# IMMUNOREGULATORY ROLE OF THE TYPE 1 CYTOKINES IN THE IMMUNE RESPONSES OF PATIENTS WITH CHRONIC RENAL FAILURE (CRF) AND A MURINE MODEL OF GRAFT-VERSUS-HOST DISEASE (GVHD)

By

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A Thesis
Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Immunology
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Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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## List of Abbreviations

| | |
|---|---|
| Ab | antibody |
| Ag | antigen |
| BSA | bovine serum albumin |
| C | complement |
| $CO_2$ | carbon dioxide |
| $^{51}Cr$ | $Na_2{}^{51}CrO_4$ |
| CPM | counts per minute |
| CRF | chronic renal failure |
| d | day |
| DPM | decay per minute |
| DNA | deoxyribonucleic acid |
| ELISA | enzyme linked immunosorbant assay |
| E:T | effector to target ratio |
| FCS | fetal calf serum |
| Fig. | figure |
| g | gram |
| GVH | graft-versus-host |
| GVHD | graft-versus-host disease |
| GVHR | graft-versus-host reaction |
| h | hour |
| $^{3}H$ | tritiated |

| | |
|---|---|
| HBSS | Hank's balanced salt solution |
| HD | hemodialysis |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| LGL | large granular lymphocyte |
| LPS | lipoplysaccharide |
| mAb | monoclonal antibody |
| mg | milligram |
| MHC | major histocompatibility complex |
| min. | minute |
| ml | milliliter |
| MLC | mixed lymphocyte culture |
| MLR | mixed lymphocyte reaction |
| mM | millimolar |
| mRNA | messenger RNA |
| ND | not detectable |
| ng | nanogram |
| NK | natural killer |
| NO | nitric oxide |

| | |
|---|---|
| OD | optical density |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| PHA | phytohemagglutinin |
| pg | picogram |
| ploy I:C | polyinositic polycytidylic acid |
| rIL | recombinant interleukin |
| RNA | ribonucleic acid |
| SEM | standard error of the mean |
| Th | T helper |
| TNF | tumor necrosis factor |
| TNFR | tumor necrosis factor receptor |
| TNFsR | tumor necrosis factor soluble receptor |
| U | unit |
| μCi | microcurie |
| μg | microgram |
| vs. | versus |

To God and To the Memories of My Father

# ABSTRACT

The outcome of *in vivo* and *in vitro* immune responses to immunogenic stimulations is profoundly influenced by the type of cytokine networks generated during the development of the response. The activation of T cells is an essential component of both the humoral and cellular immunity. Type 1 cytokines are important to elicit the cellular responses at the T cell level as well as antigen presenting cell level. IL-12 and more recently found IL-18 are known for their effects on the induction of type 1 cytokines such as IFN-$\gamma$ and TNF-$\alpha$. Patients with chronic renal failure (CRF) are known to have suppressed immune responses, whereas recipients of bone marrow transplants may develop acute graft-versus-host disease (GVHD), which is due to a severe immune reaction of the donor lymphocytes against the host cells.

The aim of the present study was to elucidate the role of regulatory type 1 cytokines, particularly IL-12 and IFN-$\gamma$, in the mechanisms by which the immune responses are suppressed or augmented. The hypothesis was that in patients with CRF, impaired immunity is caused by a defective T cell activation due to the lack of Th1-like regulatory cytokines, i.e. IL-12 and IFN-$\gamma$. We further hypothesized that in bone marrow recipients, GVHD is developed due to the activation of T cells and NK cells in parallel with the overexpression

of these cytokines. Specifically, in addition to the effects of the presence of IL-12 on *in vitro* cellular responses of patients with CRF, we examined serum TNF-α levels of IL-12 *KO* and IFN-γ *KO* mice after systemic LPS treatments. We also aimed to investigate the differences between the degree of intestinal damage occurring during the GVH reaction and its relationship with the level of LPS-induced serum TNF-α.

Our results demonstrate that:

1) The presence of exogenous IL-12 resulted in increased levels of IFN-γ and IL-10 production in the peripheral blood mononuclear cell (PBMC) cultures of the patients with CRF.

2) The sole addition of IL-12 did not correct the cytokine responses of the PBMCs from patients with CRF to match those of the healthy controls.

3) IFN-γ *KO* and IL-12 *KO* mice displayed levels of LPS-induced serum TNF-α 4 times lower than those of the wild type mice.

4) Apoptotic cells were visualized in the intestinal segments of mice with acute GVHD, confirming that apoptosis is one of the mechanism of cell death during the development of the disease.

Considering this data we concluded that IL-12 has a direct positive regulatory role in triggering IFN-γ production in both human and murine systems that we studied and it displays an indirect effect on macrophage priming, in particular LPS-induced TNF-α release by macrophages.

# INTRODUCTION

# 1. Lymphocytes And Cytokine Production Patterns (Type 1/Type 2)

## 1.1 Cells Of Immune System

The two major groups of cells that participate in the development of immune responses against pathogens are non-lymphoid (accessory) and lymphoid cells (lymphocytes). Accessory cells such as macrophages, dendritic cells and other cells from the first line of defense are involved in the induction of immune responses. However, lymphocytes are responsible for the specific recognition of antigenic determinants of pathogens and shaping specific immunity (Craddock 1967 and Singhal 1967).

Bursal lymphocytes (B cells), thymic lymphocytes (T cells) and large granular lymphocytes (LGL or natural killer cells) are three major types of lymphocytes that have distinct functions and produce different proteins such as immunoglobulins (Ig), cytokines (CK) and cytotoxic molecules. The protein products of the lymphocytes are either expressed on their surface or released by them to the extracellular environment (Lydyard 1998).

Upon the binding of specific antigens to their surface immunoglobulins and receiving costimulatory signals via adhesion molecules or cytokine receptors on their surface membranes, B cells become activated and produce antibodies of various classes (Reviewed by Clark 1991). Thus, B lymphocytes are essential for humoral immunity.

On the other hand, T cells and NK cells are more involved in cell mediated immunity and carry out their functions mainly by the production of cytokines and/or cytotoxic agents. It has been shown that even though NK cells lack a mechanism of specific recognition of foreign antigens, they specifically target tumor cells or infected cells that fail to express MHC class I molecules, in both human and mouse (Yokoyama 1993). NK cells are also characterized by IFN-$\gamma$ production and their immunoregulatory role through IFN-$\gamma$ in addition to the cytotoxic effects (Itoh 1985).

Like B cells, T lymphocytes can be activated by recognition of specific antigens through antigen/major histocompatibility complex (Ag/MHC) receptors on their surface (Meuer 1983). Based on the expression of transmembrane CD4 or CD8 molecules as a part of their Ag/MHC receptor complex, T cells are divided into two classes: helper T cells (Th cells) that express CD4 and promote the activation, differentiation and proliferation of T lymphocytes as well as other cells from the immune system and cytotoxic T cells (Tc cell) that express CD8 surface molecules and are known for their cytotoxic effects on infected cells (Krensky 1983).

## 1.2. Cytokine Production Patterns

In 1986 Mosmann defined two functionally distinct murine Th cell clones, designated Th1 and Th2, that displayed different profiles of cytokine activity (Mosmann 1986). Since then, it has been shown that Th1 cells are

associated with the activation of the cellular arm of the immune responses through IFN-γ production (Stout 1989), while Th2 cells are characterized by helping antigen specific B cells (humoral arm of immunity) through IL-4 (Killar 1987) and IL-13 (Doherty 1993) production.

Meanwhile, investigations on human cytokine production profiles resulted in the identification of two functionally distinct T cell subsets (i.e. Th1 and Th2). The *In vitro* cytokine production profiles of human T cell clones were studied and compared with those in murine systems (Rotteveel 1988). *In vivo* human cytokine responses in patients infected with Mycobacterium leprae were also investigated. It has been suggested that depending on the different symptomatic forms of the disease these patients express either type 1 or type 2 cytokine profiles (Yamamura 1991).

Further investigations on the cytokine production patterns of the subsets of helper T cells led to the characterization of a network between type 1 (IFN-γ, IL-2 and TNF) and type 2 (IL-4, IL-5, IL-10 and IL-13) cytokines as well as regulatory cytokines such as IL-12 and IL-4 with different cellular origins (Paul 1994 and Reiner 1995). Reiner and Seder suggested that IL-12 and IL-4 act on precursor T cells promoting their differentiation into type 1 or type 2 cytokine producing cells respectively. It was also suggested that IL-12 has a negative regulatory effect on the differentiation of the precursor T cells to type 2 T cells, while IL-4 inhibits the development of type 1 T cells from the precursor cell (Reiner 1995).

Furthermore, results of related experiments with $CD8^+$ cytotoxic T cell populations demonstrated that these cells can also be primed in a similar manner and express distinct patterns of type 1 and type 2 cytokine production (Croft 1994 and Seder 1995).

More recent reviews on the role of IL-10 in addition to IL-12 and IL-4 in the regulation of lymphocyte responses and cytokine production patterns consider IL-10 as a more complex factor, playing an inhibitory role in cellular immunity and an activating role in humoral immunity (Muraille 1998). Furthermore, the same reviews also suggest a positive regulatory role for IL-12 in the induction of both type 1 and type 2 immune responses. The new model for the cytokine networks that determine the outcome of the immune responses has been outlined by Muraille and Leo and is shown in figure 1 (Muraille 1998).

First described as IFN-$\gamma$ inducing factor (Okamura 1995), IL-18 is a novel cytokine with immunoregulatory functions. IL-18 has been compared with IL-12 not only for its role in the induction of IFN-$\gamma$ and stimulation of NK cells (Tomura 1998), but also for its synergistic effect with IL-12 on the *in vivo* (Yoshimoto 1997) and *in vitro* (Lauwerys 1998) inhibition of the B cell Ig production. It has also been shown that IL-18 synergizes with IL-12 in the activation of macrophages (Munder 1998) and up-regulates the LPS-induced Fas-ligand and TNF-$\alpha$ production (Tsutsui 1997). All the above findings suggest that there may be room to add IL-18 into the network model

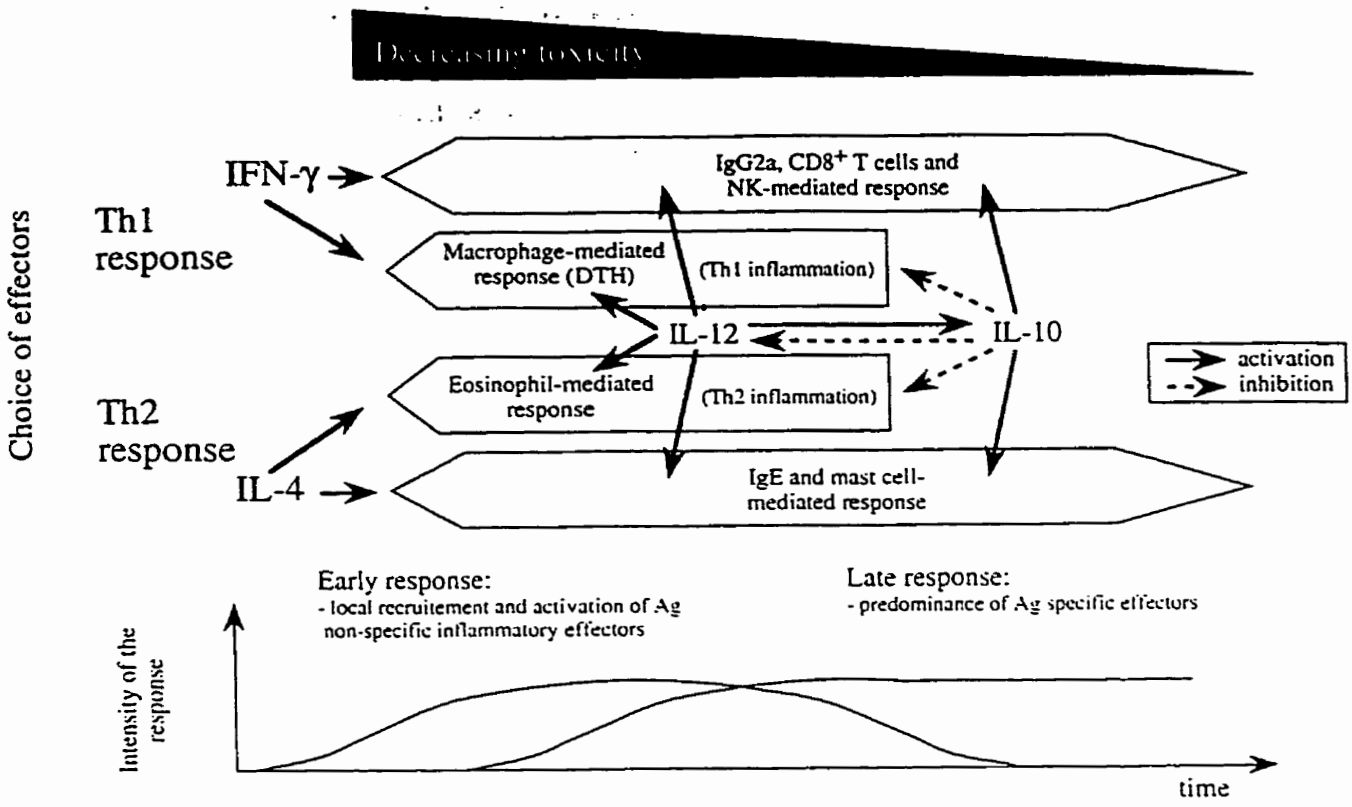


Figure 1.1: The cytokine networks involved in the regulation of the immune responses. Taken from E. Muraille & O. Leo; Revisiting the Th1/Th2 Paradigm (Muraille 1998)

suggested by Muraille and Leo shown in figure 1.1.

## 1.3. Differences Between Human and Mouse Systems

The related observations on the mechanisms involved in the development of the immune responses in humans and mice reveal some differences between the two systems. For example, production of Ig from different classes, in response to various cytokines, may vary between the two species. The expression of different transmembrane molecules such as NK1.1 and ganglio-N-tetraosylceramide (asialo-GM1; ASGM1) in murine NK cells (Rager-Zisman 1987 and Stits 1986) and NKh.1, CD 16 and CD 56 (Griffin 1983, Lanier 1986 and 1988) in human NK cells are also well known.

It has been shown that the presence of IFN-$\gamma$ in the induction of Ab production by mouse B cells results in the release of IgG2a (Mosmann 1988) and *in vivo* administration of IL-12 results in an increased IgG2a level while suppressing IgE and IgG1 levels (Rempel 1997). Humans, on the other hand, essentially lack the IgG2a isotype and express Ig molecules of IgG4 isotype instead (Kimata 1995).

Results of other studies also point to some differences between the regulatory role of cytokines such as IL-12, IL-4 and IL-10. It has been suggested that in humans, both IL-12 induced Th1 and IL-4 induced Th2 subsets of T cells produce large amounts of IL-10 suggesting that in humans IL-10 is not a typical Th2 cytokine (Sornasse 1996). Despite the differences,

most of the mechanisms involved in the regulation and development of the immune responses in humans parallel those of mice. In fact, many reviews, including the network model presented by Muraille (Figure 1.1), on the type 1/type 2 patterns of cytokine production and regulation of immune responses, include studies on both human and mouse systems.

## 2. Impaired Immunity in Patients with Chronic Renal Failure (CRF)

Pathologic renal conditions can be induced by a variety of causes and advance to renal failure. Renal failure may develop either as a primary disease or be secondary to other diseases such as infectious disease (Schwartz 1998), diabetes mellitus (Albitar 1998), enzyme deficiencies (Stokelman 1998), polycythemia vera (Shiu (1998) and other hereditary (Klemme 1998) or non-hereditary diseases. By progressive damage of the paranchimal tissue, chronic renal diseases evolve to end stage renal failure (Remuzi 1998).

Kidney transplantation and hemodialysis (HD) are among the few therapeutic choices for patients with end stage renal failure. However, neither renal transplantation nor hemodialysis is able to correct all of the consequences of chronic renal failure, in particular, the impaired immune responses associated with CRF (Goldblum 1980).

Immunodeficiency is one of the well known consequences of CRF leading to increased morbidity and mortality due to insufficient immunity to infections and a high incidence of malignant tumors. The impaired immune responses in this group of patients is also characterized by prolonged allograft survival as well as poor humoral and cellular responses (Reviewed by Descamps-Latscha 1993).

## 2.1. Defective *In vivo* Immune Responses and Abnormal Cytokine Production

CRF patients undergoing hemodialysis display an altered immunity and are highly susceptible to infectious diseases (Goldblum 1980). Tuberculosis has been reported to have a higher incidence (12 times higher) in the HD patient population in comparison with the general community (Andrew 1980). It has also been shown that in CRF patients undergoing kidney transplantation, there is a negative correlation between graft survival and *in vivo* Ab production in response to hepatitis B surface Ag (London 1977).

When the effects of the combination of vaccine treatment with recombinant human IL-2 or erythropoietin on anti-hepatitis antibody production were examined, neither recombinant human IL-2 nor erythropoietin treatment resulted in a definite positive effect on anti-hepatitis B antibody synthesis (Mauri 1998).

Other indications of impaired immune responses are the lack of antibodies to hepatitis B infection in patients with prolonged graft survival, weak Ab responses to vaccination with influenza antigens and reduced responsiveness to skin tests (Also reviewed by Descamps-Latscha 1993).

An increased plasma level of circulating cytokines and cytokine receptors such as IL-1β, TNF-α, IL-1Ra, TNF-sR 55 and TNF-sR 75 as well as T and B cell markers, i.e. CD25 and CD23 respectively, in CRF patients

has been reported (Descamps-Latscha 1995). In addition to IL-1β and TNF-α, levels of circulating IL-6 and IL-13 were examined and it was reported that the increased levels of these cytokines were significantly associated with the severity of renal failure and its relative mortality (Kimmel 1998). In contrast to the increased levels of the first group of cytokines that correlated with high mortality, the enhanced circulating IL-2, IL-4, IL-5 and IL-12 were associated with an improved survival rate (Kimmel 1998).

## 2.2. Reduced *In vitro* Proliferative Responses and Abnormal Cytokine Production

Peripheral blood mononuclear cells (PBMCs) from patients with CRF have been shown to display a decreased mitogen-induced proliferation ratio when compared to PBMCs from healthy controls. (Kurz 1986) Different studies have addressed the association of reduced *in vitro* proliferative responses with various factors involved in different stages of T cell activation. For instance, some have studied the inadequate monocyte-derived signal for IL-2 production (Meuer 1987) or the role of defective B7/CD28 costimulatory molecule interactions (Girndt 1993), while others investigated the enhancing effects of erythropoietin (Shurtz-Swirdki 1996) or macrophage-colony stimulating factor (M-CSF) (Le Meur 1996) treatments on these proliferative responses.

Despite the versatile nature of the factors that influence the *in vitro*

proliferative responses of HD patient lymphocytes, these factors mainly point investigations towards abnormal costimulatory interactions between monocytes and T cells (Meuer 1987, Girndt 1993, Shurtz-Swirdki 1996 and Le Meur 1996).

In a review article Kelly (1994) emphasizes the fact that the defective *in vitro* PBMC responses in patients with CRF is not only characterized by decreased proliferation but also by reduced inducible expression of IL-2 and IFN–$\gamma$ mRNA. Furthermore, it has been demonstrated that the level of spontaneous TNF-$\alpha$ produced by PBMCs from HD patients is significantly increased when compared with those of healthy controls (Macdonald 1993). Nevertheless, the same results could not be obtained when the levels of LPS-induced TNF-$\alpha$ of the PBMCs from the HD patients and controls were examined. In fact, it seemed as if PBMCs from patients with CRF had a lower TNF-$\alpha$ release in response to LPS than PBMCs from healthy controls (Macdonald 1993).

## 2.3. Hypothesis and Aim of the Study

Our hypothesis is based on the rapidly growing body of information on the cytokines and cytokine networks in addition to the knowledge of the immunodeficiency state of the HD patients. Since it seems that this defect is caused mainly at the T cell costimulation level, we hypothesized that the impaired immunity in patients with CRF may have resulted from an

imbalanced pattern of cytokine production due to the defective monocyte function. We hypothesized that the decreased type 1 cytokine levels (IL-2, TNF-$\alpha$ and IFN-$\gamma$) in these patients may be due to the defective IL-12 production by monocytes.

Therefore, we aimed to study the *in vitro* cytokine production and cell proliferation of the PBMCs from patients with CRF undergoing HD, in the presence and absence of the exogenous recombinant human IL-12. We specifically aimed to determine whether the presence of exogenous IL-12 corrects the *in vitro* cellular responses of HD patients to match those of healthy controls or not.

# 3. Acute Graft versus Host Disease (GVHD)

Acute GVHD is a destructive complication of allogenic bone marrow transplantation, which is life threatening to the marrow recipient. It is often associated with well-characterized damage of the epithelial cells in the skin (Gilliam 1997), lung (Madtes 1997), liver (Crawford 1997) and intestine (Mowat 1997). In addition to severe immunsuppression, cachexia, skin rashes, abdominal cramps and diarrhea are among the major symptoms of the acute GVHD (Gilliam 1997, Madtes 1997, Crawford 1997 and Mowat 1997).

## 3.1. Pathogenesis

It has been well established that the development of acute GVHD is caused by the immunological reaction of the engrafted donor lymphocytes against the immunosuppressed recipient. The principal mechanism of this reaction relies on the recognition of the host MHC molecules (foreign) by donor T lymphocytes (Korngold 1985 and Mowat 1986). The important role of the T cells in the pathogenesis of the disease is best shown by the fact that depletion of the T cells from the donor marrow prevents the development of acute GVHD (Prentice 1982).

After recognition of foreign MHC antigens, donor T cells become activated and proliferate. Depending on the regulatory cytokines or other

costimulatory factors provided by NK cells and macrophages, the donor T cells express a type 1 pattern of cytokine production (Hakim 1997).

a: Role of NK Cells

It has been demonstrated that in the process of the development of acute GVHD, in addition to T lymphocytes, NK cells also become activated (Dokhelar 1981, Borland 1983). Furthermore, it is known that NK cells can be activated in response to type 1 cytokines such as IL-2 and IFN-$\gamma$ that are released by T lymphocytes (Flamard 1996). A role for type 1 cytokines in the activation of NK cells during the development of GVHD has been well described by Krenger and Ferrara (1996).

Activated NK cells may effect the mechanism of GVHD by producing and/or releasing soluble cytokines (Gomez 1985) and other proteins (Greenberg 1986), as well as by directly participating in the cell-to-cell interactions through the expression of transmembrane molecules (Yokoyama 1993).

IFN-$\gamma$ is one of the most important cytokine products of NK cells (Trinchieri 1984), which is well known for its capacity to prime macrophages for the induction of TNF-$\alpha$ and other inflammatory cytokine release (Pase 1983, Hays 1991). IFN-$\gamma$ production by NK cells may be triggered by a variety of stimuli (Jewett 1996) including the cross-linkage of the NK1.1 surface molecules (Arase 1996).

Direct cell-to-cell interactions between the donor NK cells and the non-

self MHC class I expressing host cells seem to result in the death of the host cells through cytotoxic activity of the NK cells (Trinchieri 1997).

In previous studies, the role of NK cells in the pathogenesis of acute GVHD has been investigated by using NK cells eliminated grafts to induce GVH reaction. The results of these studies demonstrated that the lethality and severity of acute GVHD could be prevented by pre-treatment of the graft with antibodies against either $ASGM_1^+$ (Ghayur 1988) or $NK1.1^+$ (MacDonald 1992) and complement. Later, it was shown that spleen cell cultures of recipients of NK cell depleted grafts had a decreased IFN-$\gamma$ production. It was also demonstrated that these mice had a reduced serum level of LPS-induced TNF-$\alpha$ when compared to recipients of unmodified grafts (Ellison 1998).

The existence of another population of NK cells expressing the $\gamma\delta$ T cell receptor (TCR) in addition to the expression of NK1.1 membrane molecules and their involvement in the development of GVH reaction and mortality of the disease also has been reported (Ellison 1995).

Furthermore, NK cells are known to be involved in the development of the disease in the various organs and tissues of the host at different phases of GVHR (Gartner 1988 and MacDonald 1991). An essential role for these cells has been suggested in the local cellular immune responses, i.e. delayed type hypersensitivity, in the intestinal tissues of the host (Mowat 1987).

b: Role of LPS

Lipopolysaccharide (LPS) is produced by gram-negative bacteria and

is expressed as a component of their cell wall (Smith 1979). Among deleterious effects of LPS is the activation of previously primed macrophages to release large amounts of TNF-α in addition to other inflammatory cytokines (Gifford 1987 and Hays 1991).

In non-pathologic conditions, gram-negative microorganisms that are naturally present in the gut cannot pass the mucosal barrier of the intestinal tract and the small amount of the LPS produced by them can be tolerated by macrophages. However, it has been suggested that during the development of acute GVHD, the same small amount of LPS produced by these bacteria in physiological conditions can now trigger the activation of primed macrophages and consequently LPS-induced TNF-α release by them (Nestel 1992 and 1997).

This in turn causes not only intestinal damage and the passage of the gut bacteria into the host tissues, but also high levels of serum TNF-α in the systemic circulation and consequently death of the host caused by septic shock syndrome (Nestel 1997). Therefore, by acting on primed macrophages, LPS plays a central role in the development and perhaps the mortality of the disease.

C: Intestinal Damage

Epithelial tissues of the intestinal mucosa are among the targets of GVHD (Snover 1985) and the tissue injuries of the gastrointestinal tract following bone marrow transplantation have been reviewed as an important

component of the disease (Mowat 1997). An increased permeability of the mucosal barrier following bone marrow transplantation in humans, also has been well documented (Fegan 1990). It has been suggested that LPS produced by gram negative bacteria of the gut plays a central role in the development of GVHD (Nestel 1992).

The enteropathies occurring in the intestinal phase of the GVH reaction are mainly mediated by the effects of the cytokines produced by helper T lymphocytes and NK cells on the bystander enterocytes (Mowat 1986,a and 1986,b).

Two major phases of intestinal pathology during GVH reaction are crypt hyperplasia (proliferative) and villus atrophy (destructive), which have been subjected to various studies. Earlier studies had demonstrated that the GVH reaction results in an increased epithelial cell renewal and hyperplasia of the crypts (Mowat 1981 and 1982). However, more recent studies have focused on the mechanisms of cytokine-induced enteropathy and villus atrophy in the destructive phase (Reviewed by Mowat 1997).

TNF-$\alpha$ and IFN-$\gamma$ seem to be the most potent cytokines causing the death of the enterocytes in addition to the cytototoxic agents such as nitric oxide (NO) (Reviewed by Mowat 1997). Nevertheless, the mechanisms through which these cytokines deliver their functions (necrosis vs. apoptosis) need to be subjected to more studies.

## 3.2. $F_1$-hybrid Murine Model

In order to facilitate the studies on GVHD, inbred mice strains expressing different MHC haplotype commonly are being used. The major criteria for this model are that the parental strain (Type A) does not express MHC antigens foreign to the $F_1$-hybrids, i.e. immunocompetent to the $F_1$-hybrid, while the $F_1$-hybrids that are heterozygous in their MHC loci (A x B) possess MHC molecules that are foreign to the parental strain and thus not immunocompatent to the parental lymphoid cells.

Furthermore, immunocompetent parental (Type A) lymphoid and splenic cells are injected into the (A x B) $F_1$-hybrid recipients. Since the lymphocytes of the $F_1$ recipients are tolerant to parental cells, they do not respond to the engrafted cell. However, the donor cells recognize the Type B antigens, expressed by host tissues, as foreign and form an immune response against the host (Gleichmann 1976).

## 3.3. Hypothesis and aim of the study

We hypothesized that type 1 cytokines produced by alloactivated donor T cells, in particular IL-2 and IFN-$\gamma$, act on macrophages and NK cells to activate them. This in turn promotes a cascade of cytokine production by these cells.

Activated NK cells produce additional IFN-$\gamma$, which primes macrophages for the LPS-induced TNF-$\alpha$ release as well as other cytokines

such as IL-12 and/or IL-18. The latter cytokines, i.e. IL-12 and IL-18, in turn up-regulate IFN-γ production by T and NK cells.

On the other hand, a normally tolerable amount of LPS produced by intestinal gram negative bacteria, activates primed macrophages to produce large amounts of TNF-α. Binding of TNF-α to its receptors on the bystander epithelial cells results in the enteropathy, which causes the breakdown of the mucosal barrier. Following this, gram negative microorganisms can enter the intestinal tissues of the host, proliferate and produce more LPS, which leads to septic shock syndrome, which is characterized by high serum levels of TNF-α.

Therefore, we aimed to examine the effects of LPS-induced TNF-α release by exaggerating the GVH reaction during its peak days (i.e. days 8 and 15) via systemic administration of exogenous LPS to the mice with acute GVHD. We focused our studies on the level of serum TNF-α as well as the degree of intestinal damage. We also aimed to determine whether apoptosis was among the mechanisms of intestinal injury or not.

Furthermore, we aimed to study the role of NK cells in the cellular responses of GVHD in an *in vitro* model of the reaction. For this part of the studies we measured the level of IFN-γ production and cell proliferation in the mixed lymphocyte cultures (MLCs). The assays were performed with NK cell-depleted responder cell populations and the results were compared with those of cultures with non-depleted responder cells.

# MATERIALS AND METHODS

## A- *In vitro* Studies on Immune Responses of Patients with CRF

### 1. Human Subjects

The 42 human subjects in this study included 17 healthy controls and 25 patients with CRF who were on haemodialysis treatment. Sixteen of the patients had responded positively to Hepatitis B vaccination, while the other 9 patients had not developed detectable Ab to Hepatitis B antigen. Therefore, in some parts of this study, based on their antibody responses to the immunization with Hepatitis B, patients were divided into two categories of "Responders" and "Non-Responders". The patients consisted of 15 men and 10 women with a mean age of 55±13 years, ranging between 28 and 78 years. They all gave their written informed consent to participate in the study. Subjects from the control group were all university and hospital personnel who served as healthy controls, with a mean age of 35.8 ± 2.3 years.

### 2. Isolation of PBMC

15 ml of heparinized peripheral blood samples were collected from each patient and control . In the case of patients with CRF, blood was drawn

immediately prior to the haemodialysis session. PBMC were isolated from the whole blood sample by Ficoll-Histopaque-1077 (Sigma, St. Louis, MO, USA) density centrifugation (Zighelboim 1980). Briefly, whole blood was diluted to 30 ml with an equal volume of sterile saline (15 ml) at room temperature. The diluted blood was layered over 15 ml of Ficoll-Histopaque with a specific density of 1.076 g/ml and centrifuged at 400 g for 25 minutes. Following aspiration of plasma, the lymphocyte layer was collected, washed with 40-50 ml saline three times, counted with Trypan Blue exclusion and resuspended at the indicated cell concentrations, in the culture medium (RPMI 1640; Gibco, New York, USA) that was supplemented with 1% Penicillin/Streptomycin (5000 mg/ml), 10% heat inactivated fetal calf serum (FCS; Life Technologies, NY, USA) and $2X10^{-5}$ M 2-mercaptoethanol.

## 3. Cell Cultures

### 3.1. Cell Proliferation Assay:

This technique has been described by Fan (1977) and Rayat (1992). Briefly, peripheral blood mononuclear cells (PBMC) were cultured at a final density of $1X10^{5}$ cells per well in the 96-well round bottom sterile microtiter plates (Becton Dickinson, New Jersey, USA), in a final volume of 200 μl of RPMI (Gibco) supplemented with 10% heat inactivated FCS, 1% Penicillin/Streptomycin and $2X10^{-5}$ M 2-mercaptoethanol. Final concentrations of 10 μg/ml Phytohemagglutinin (PHA; Sigma), 2 ng/ml α- CD3 (OKT3; ATCC,

Rockville, MD) and 100 U/ml interleukin-2 (IL-2; Cetus) were used in order to stimulate the cell cultures polyclonally. Streptokinase (Streptase; Behringwerke AG, Marburg, Germany) at a concentration of 500 U/ml and three different strains of flu virus (B. Harbine, A. Johannesburg and A. Texas) at 1 μg/ml concentrations were used as specific antigens to stimulate the cell cultures. To purify the vaccines from stabilizers, small volumes, i.e. up to 10 ml, of the Ag preparations (i.e. Streptokinase and Flu vaccines) were dialyzed 3 times a day against 2-4 liter of PBS for 2 days, before being used in the cultures. All the above culture conditions were set in quadruplicate and similar conditions were also set in the presence of recombinant human interleukin-12 (IL-12; Hoffmann-La Roche, Nutley, NJ) at the final concentrations of 0.5 ng/ml and 2 ng/ml, in addition to the cultures without IL-12. Plates were incubated at 37° C in a humidified 5% $CO_2$ incubator for 4 days (polyclonal stimulation) or 6 days (antigenic stimulation). Approximately 16 hours prior to harvesting the cells, 0.6 μCi of $^3$H-thymidine (Amersham, USA) was added to each well. The cells were harvested onto glass-fiber filter paper (Mandel Scientific, Rockwood, ON, Canada), air-dried overnight and topped with scintillation fluid (Scytosint, Fisher Scientific). $^3$H-thymidine incorporation was assessed by liquid scintillation counting, using a β-spectrometer with a liquid scintillation system (Cambridge Technology Inc., USA) as an index of cell proliferation..

## 3.2. Cell Culture for Cytokine Production:

To generate supernatants for measuring the level of cytokines produced by PBMCs, similar culture conditions as those of the cell proliferation assay were set (i.e. cultures with polyclonal and specific antigen stimuli, in the presence and absence of 0.5 ng/ml IL-12). To obtain a large volume of supernatants, $1X10^6$ PBMCs were cultured in a final volume of 2 ml of RPMI (Gibco) supplemented with 10% FCS , 1% Penicillin/Streptomycin and $2X10^{-5}$ M 2-mercaptoethanol, in the sterile 5 ml round bottom snap-capped tubes (Falcon, Becton Dickinson, New Jersey, USA). Cultures were incubated at 37° C in a humidified $CO_2$ incubator for 4 days. The supernatants were collected after cells were centrifuged for 10 minutes at 400 g and stored at -20°c.

## 4. Cytokine Assays

The levels of different cytokines in the supernatants that were collected from cell cultures were determined either by sandwich enzyme linked immunosorbent assay (ELISA) techniques (Engvall 1971) or by a bioassay, in the case of IL-4, which is based on the biological responses of the IL-4-dependent cell line CT.h4S (Gieni 1995).

## 4.1. ELISA

a: Buffers:

The dilution buffer for the capture antibody was a 0.05 M carbonate-bicarbonate buffer with final pH of 9.6. A solution of 1% bovine serum albumin (BSA) in a 10 mM phosphate buffered saline (PBS) with a pH of 7.4 was used to block the plates for binding other proteins. Between different steps of the assay, plates were washed with a 0.05% solution of Tween 20 in 10 mM PBS at a final pH of 7.4. A 10 mM PBS buffer containing 0.05% Tween 20 and 0.5% BSA with a final pH of 7.4 was used to dilute all samples, standard proteins and developing reagents. The substrate buffer based on $MgCl_2$-6 $H_2O$ that was dissolved in diethanolamine with a final pH of 9.8, was used as a liquid phase to dissolve P-nitrophenyl phosphate (PNPP; Sigma) substrate tablets.

b: Antibodies and Standard Proteins:

b.1) Antibodies and Standard for Human IFN-γ ELISA:

Monoclonal mouse anti human IFN-γ (PharMingen, San Diego, CA, USA) at a final concentration of 2 μg/ml was the first antibody and served to coat the plates. The standard was a recombinant human IFN-γ (PharMingen) that was serially diluted 8 times in a ratio of 1:2, at a starting concentration of 2000 pg/ml. Biotin-conjugated monoclonal mouse anti-human IFN-γ

(PharMingen) at a final concentration of 1 μg/ml served as the detection reagent in combination with streptavidin-alkaline phosphatase (Bio/Can Scientific) at concentration of 1:4000 in dilution buffer. ELISAs for IFN-γ were performed by Dr. Bin Liu.

b.2) Antibodies and Standard for Human IL-10 ELISA:

The first antibody that was used to coat the plates, was a monoclonal rat anti-human IL-10 (PharMingen) diluted into a final concentration of 1 μg/ml. A recombinant human IL-10 (PharMingen) was diluted from a starting concentration of 1250 pg/ml to 4.5 pg/ml in a series of 8 dilutions in the ratio of 1:2, to serve as the standard. The second antibody was biotinylated monoclonal rat anti-human IL-10 (PharMingen), that was used at 1 μg/ml concentration, in combination with streptavidin-alkaline phosphatase (Bio/Can Scientific), diluted 1:4000 in dilution buffer as the detection reagent. All the IL-10 ELISAs were also performed by Dr. Bin Liu.

b.3) Antibodies and Standard for Human IL-5 ELISA:

The capture antibody was a purified monoclonal rat anti-human/mouse IL-5 antibody (PharMingen) used at a final concentration of 2 μg/ml. The standard for this assay was also a recombinant human IL-5 (PharMingen) used at a starting concentration of 1000 pg/ml and diluted down to 8 pg/ml. The developing antibody was a biotin-conjugated monoclonal rat anti-human

antibody used at 1 μg/ml concentration. IL-5 ELISAs were carried on in Dr. K. HayGlass' laboratory by Ms. Yan Li.

b.4) Antibodies and Standard for Human IL-13 ELISA:

The coating antibody for this assay was a biopilot purified anti-human IL-13 (JES 10-5A2/11-95; DNAX, CA, USA) used at the concentration of 5 μg/ml. Human IL-13 (PeproTech) was used as the standard at 200 pg/ml concentration and was serially diluted to 1.56 pg/ml. Biotinylated rabbit anti-human polyclonal antibody (PharMingen) at 0.5 μg/ml served as the detection antibody. As with the IL-5 ELISAs, all the IL-13 ELISAs were also performed by Ms. Yan Li in Dr. K. HayGlass' laboratory.

c: Assay Procedure:

Flat bottom 96 well microtiter plates (Corning) were coated with 50 μl/well of capture antibody and incubated at 4° C overnight. Plates were blocked with 75 μl of blocking buffer for a period of 1 hour at room temperature. Some wells were specified as blanks and did not receive any samples. However, designated wells received 50 μl of samples or standard which were diluted to indicated dilutions and incubated at 4° C overnight. A volume of 50 μl/well of Biotin-conjugated detection antibody was added to the plates and incubated at room temperature for 1 hour. The colorimetric reaction of the enzyme and substrate was completed by adding 50 μl of

substrate into each well and incubating the plates for 30 minutes at room temperature. The optical density (OD) of each well was obtained by reading the absorbance at 405 nm and subtracting background absorbance at 690 nm using a UV max kinetic microplate reader (Molecular divices, USA).

## 4.2. IL-4 Bioassay

The level of IL-4 was measured by a bioassay (Gieni 1995) based on the biological activity of the cell line CT.h4S initially provided by Dr. W. Paul. In order to maintain this cell line the culture medium (RPMI containing10% FCS) was supplemented with 8 ng/ml rIL-4. The cells were subcultured on the day prior to the assay, to achieve log phase growth at the time of application. After washing the cells twice with 10% FCS/RPMI, aliquots of 5 x $10^3$ cells in 50 µl of medium were added into the wells containing an equal volume of either standard recombinant human IL-4 (Immunex Manufacturing Corp., Seattle, WA) at 40 pg/ml starting concentration or supernatant samples that were serially diluted in twofold dilutions. Wells designated as blank and negative controls also were included. To ensure that the cell response was not due to the presence of IL-2, some control wells were cultured in the presence of neutralizing anti-IL-2 antibody. The plates were incubated at 37° C for 40 hours before they received 50 µl/well of (3-carboxymethoxyphenyl)-2-(4-sulfuphenyl)-2H-tetrazolium (MTS) solution. This solution contained 2/3 5% RPMI with PSF (penicillin G sodium, streptomycin sulfate, amphotericin B)

and 1/3 MTS at 1 mg/ml and 0.008% phenazine Methosulfate (PMS) at 0.92 mg/ml. After a further incubation at 37° C for 24 hours in the dark due to the sensitivity of the MTS, the adsorbance was assessed at 492 nm subtracting background absorbance at 690 nm. The lower limit of the detection of the assay was routinely between 1-2.5 pg/ml. All the IL-4 bioassays were performed by Ms. Yan Li at Dr. K. HayGlass' laboratory.

### 5. Statistics

The Wilcoxon Test was used to examine the significant differences in the indicated parameters between matched pairs, and the Mann-Whitney Two Sample Test served to evaluate the significant differences between non-matched pairs.

## B- Murine Model of GVHD

### 1. Mice

Female C57BL/6J (H-$2^b$) mice were purchased from Jackson Laboratory, Bar Harbor, ME. Female (C57BL/6 x DBA/2) $F_1$-hybrids (H-$2^{b/d}$) were obtained from Charles River, Wilmington, MA. C57BL/6J-*Ifg*$^{um1Ts}$ (IFN-$\gamma$ *KO*) and C57BL/6 IL-12 p40 *KO* (IL-12 *KO*) breeding mice were obtained from Jackson Laboratory and bred in the animal care facility of the University of Manitoba.

## 2. Cell Lines

The NK-sensitive, Moloney murine virus-induced T cell lymphoma YAC-1 (H-$2^a$), obtained from the American Type Culture Collection (ATCC; Rockville, MD) served as a target cell in the NK cytotoxicity assay. The murine hybridoma, PK136 was also obtained from the ATCC and used as a source of anti-NK1.1 antibody. Both cell lines were maintained in RPMI 1640 (Life Technologies, Grand Island, NY, USA) medium that was supplemented with 10% FCS (Life Technologies), 1% Penicillin/Streptomycin (5000 mg/ml), Sodium pyruvate (100 mM; Gibco) and glutamine (200 mM; Gibco).

## 3. Induction of GVHR

Acute graft-versus-host reactions (GVHR) were induced by injecting 60 x $10^6$ parental C57BL/6 (H-$2^b$) cells into $BDF_1$-hybrid (H-$2^{b/d}$) mice (Gleichmann 1976 and Van Elven 1981). Briefly; sex and age matched C57BL/6J donor mice were killed in a $CO_2$ chamber, spleen and lymph nodes (inguinal, cervical and axillary) were taken and placed into a petri dish containing fresh Hank's buffered salt solution (HBSS) at room temperature. In order to homogenize the graft, these tissues were pressed into #60 stainless steel mesh screens, and washed in HBSS (Ghayur 1987). To remove any clumps of cells or connective and fat tissue, the cell suspension was passed through a double layer of gauze and washed again. The number of viable

cells was determined by trypan blue exclusion, and the cells were resuspended in RPMI at a final concentration of $200x10^6$ cells/ml. Using a 26 gauge catheter and an insulin syringe (1 ml), a unit of 0.3 ml of the donor cell suspension ($60x10^6$ cells) was injected to each recipient mouse, via their tail vein.

## 4. Tissue Processing

Mice at days 8 and 15 post-induction, and in some experiments healthy mice, received an i.v. dose of 10 or 20 μg lypopolysaccharide (LPS; Sigma, St. Louis, MO). 18 hours prior to the LPS administration some groups of the healthy mice were treated with an i.p. injection of 100 μg Poly I:C. Control groups were either treated with Poly I:C alone or not subjected to any treatment. Mice were euthanized in $CO_2$ chamber and various tissues were collected and processed as follows:

### a: Blood

Prior to sacrificing, a volume of 0.5-1 ml of peripheral blood was drown from each mouse using their tail vein. In order to collect the sera from blood samples after an overnight incubation at 4°C, blood samples were centrifuged at 300 g for 10 minutes. Using pasture pipettes sera were harvested and stored in 1 ml eppendorf tubes at -70° C.

b: Intestinal Mucosa

Immediately after the mouse was killed, a 1-2 cm long segment of jejunum (almost 10 cm after pylorus) was taken and placed in HBSS. Using a pair of blunt end 12.5 cm surgical scissors, the intestinal segment was longitudinally opened, washed thoroughly with HBSS, and pinned over a small piece (1.5 x 1.5 cm) of styrofoam in a way that the luminal side would face up. To fix the intestinal tissues, segments were incubated in 10% phosphate-buffered neutral (pH 7.2) formalin for 24 hours at room temperature, by placing the styrofoams in the formaline containers in an upside-down position so that the intestinal sections were facing the surface of the formline. Using a sharp blade, damaged edges of the tissue were cut and each intestinal tissue sample was longitudinally divided into two pieces and placed in labeled cassettes. The fixation procedure was continued by two 15-minute incubations in fresh 95% ethanol, three 20-minute incubations in fresh absolute ethanol, and two 15-minute incubations in fresh xylol. After fixation steps were completed, using heat and cold blocks the intestinal sections were carefully embedded in paraffin, in a straight position. Using microtome 4 μm sections of each specimen were cut and placed on the sialinized slides.

c: Spleen

Spleens were also taken immediately after the mice were killed, and placed in the cold sterile RPMI. Using a 7 ml glass tissue homogenizer splenic

cells were brought into a suspension phase. Cells were washed in 15 ml of cold RPMI and then passed through a sterile layer of HC3-125 Nytex mesh (B&H Thompson & Co. LTD, ON, Canada) to remove any clumps of cells or other tissue elements. Then they were washed two more times with cold RPMI and resuspended in RPMI supplemented with 10% FCS and 1% Penicillin/Streptomycin at the identified concentrations. Red blood cells were eliminated by Lympholyte-M density centrifugation. Briefly; 4 ml of cell suspension (with a concentration less than 20 $x10^6$ cells/ml) was layered over 4 ml of Lympholyte-m, in a 15 ml conical centrifuge tube. The cells were centrifuged at 400 g for 20 minutes. Using a pasture pipette the lymphocyte layer was transferred to another tube and washed two more times with 15 ml of HBSS. All the above procedures took place under sterile conditions and between the steps cells were either kept on ice or left at 4° C.

In experiments involving mice with acute GVHR, immediately after mice were killed spleens were extracted and weighed. Prior to any dissection mice were also weighed and the spleen index (S.I.) was calculated as follows:

$$SI = \left(\frac{\text{Spleen weight (expt.)}}{\text{Body weight (expt.)}}\right) \div \left(\frac{\text{Spleen weight (control)}}{\text{Body weight (control)}}\right)$$

## 5. Histopathology

In addition to hematoxylin and eosin (H&E) staining of the intestinal sections for the routine histopathological analysis (Longnedker 1966), in order to visualize and evaluate the number of the apoptotic cells on each tissue

section, an in situ apoptosis detection kit (ApopTag; oncor, Gaithersburg, MD, USA) was used following the manufacturer instructions. The mechanism of the detection was based on the chemical incorporation of digoxigenin conjugated nucleotides (digoxigenin-11-dUTP), to the nick ends of the DNA fragments of the apoptotic nuclei, in the presence of terminal deoxynucleotidyl transferase (TdT) enzyme that was provided by the kit. Prior to the in situ staining of the apoptotic nuclei that was instructed by manufacturer, slides with paraffin-embedded tissues were deparaffinized by washing the specimens in two changes of xylene for 5 minutes each, followed by two 5 minutes washes of absolute ethanol, two 3 minutes washes in 95% and 70% ethanols and a final wash in PBS for 5 minutes. As a protease, proteinase K (Sigma) was applied to the specimens at a final concentration of 20 μg/ml for 15 minutes. This step was followed by four 2 minutes washes in distilled water. The slides were subjected to the steps instructed by the protocol of the kit. Briefly; to block the endogenous peroxidase, slides were treated with 2% hydrogen peroxide and rinsed with PBS. After a quick treatment with the equilibration buffer, the TdT enzyme was applied and the slides were incubated at 37° C for 1 hour before the reaction was stopped by applying the stop/wash buffer. The sections were treated with peroxidase conjugated anti-digoxigenin as the detection antibody and to complete the detection procedure, the tissue sections were covered with diaminobenzidine (DAB; Polysciences, Inc., Warrington, Pa) and hydrogen peroxide in substrate solution. Staining with methyl green as a counterstain was the final step of

the procedure before the specimens were mounted under a glass coverslip. Then the tissue sections were examined by light microscopy for the number of apoptotic nuclei, as an index of histological damage to the intestinal mucosa.

## 6. NK Cell Depletion

Some of the C57BL/6 mice received an intraperitoneal injection of 100 μg of Poly I:C (Sigma) 18 hours prior to the time they were killed. Splenic cells of these mice were subjected to the NK cell depletion procedure, using either anti-ASGM1 rabit antiserum (Wako Chemicals, Dallas, TX) or anti-NK1.1 (PK136; ATCC) antibodies (MacDonald 1991). Briefly; cells were resuspended in cytotoxicity medium (Cedarlane, Hornby, ON, Canada), containing either PK136 ascites at 1:20 dilution or α-ASGM1 at 1:100 dilution (Kasai 1980), and a final concentration of 20 x $10^6$ cells/ml. After 1 hour of incubation on ice, cells were washed with HBSS, resuspended in the cytotoxicity medium containing a 1:8 dilution of Low-Tox rabbit complement (Cedarlane) and incubated at 37° C for 1 hour. After another washing with HBSS, the cells were counted by Trypan blue exclusion and resuspended to the indicated concentrations. In addition to these groups, there were control groups consisting of cells that were either treated with medium or antibody or complement alone.

## 7. NK Cell Cytotoxicity Assay

C57BL/6 splenic cell suspensions that were treated with different antibodies with or without C, C alone or received no treatment were adjusted to a concentration of $10^7$ cells/ml in RPMI containing 5% FCS and used as effector cells (Kasai 1979 and MacDonald 1991). The cell suspensions were serially diluted four times in a two fold volume of medium to provide the effector:target cell (E:T) ratio range of 100:1 to 12.5:1. A volume of 100 μl of each dilution was added into the wells of a 96 well v-bottom microtiter plates (Corning) in triplicates.

Using trypan blue staining, the concentration of viable YAC-1 tumor target cells in the culture was determined. A volume of the culture containing 1-2 x $10^6$ cells was centrifuged at 300 g for 10 minutes and the cell pellet was resuspended by gently tapping the bottom of the 15 ml conical centrifuge tube. A volume of 100 μl of $Na_2{}^{51}CrO_4$ (Amersham, Oakville, ON, Canada) containing an activity of 100 μCi was directly added to the pellet before the cells were gently resuspended by tapping the bottom of the tube, and incubated in a shaking water bath at 37° C for 1 hour. Then the labeled cells were washed with RPMI containing 5% FCS three times and resuspended in a final concentration of $10^5$ cells/ml. 100 μl of this cell suspension was added to each well of the plates containing the effector cells. In addition, 100 μl of the labeled target cell suspension was added into the 6 wells without effector

cells three of which were designated to measure the level of minimal and three for maximal spontaneous release of $^{51}Cr$.

The plates were incubated at 37° C in a 5% $CO_2$ humidified incubator for a period of 4 hours after which the plates were centrifuged at 300 g for 10 minutes to pellet the cells. The pellets in the wells specified for maximal release were resuspended and 100 μl of the cell suspension was transferred into disposable borosilicate glass tubes 6 x 50 mm (Kimble, VWR Scientific, San Francisco, CA). A volume of 100 μl of the supernatants of the wells designated for minimal release as well as 100 μl of the supernatants from other wells of the plates were also harvested and transferred to the same size disposable borosilicate glass tubes to be sampled for the amount of $^{51}Cr$ release. The percent lysis of the target cells in each well was calculated by an LKB γ-counter (LKB, Rockville, MD) as follows:

$$\text{Percent Lysis} = [(CPM_{Expt.} - CPM_{Spontaneous}) \div (CPM_{Max.} - CPM_{Spontaneous})] \times 100$$

The mean percent lysis for each dilution was also calculated by LKB γ-counter by averaging the percent lysis of the triplicate samples for each dilution, and dose response curves were drawn by using percent lysis data over the 4 E:T ratios.

## 8. Mixed-Lymphocyte Reaction

Prior to being used in the culture, some portions of C57BL/6 splenic responder cells were subjected to the NK cell depletion procedure. The

protocol for mixed-lymphocyte culture has been well described (Meo 1973 and Festenstein 1973), but minor modifications have been made in our laboratory. Briefly, 4 x $10^5$ responder cells were cultured with or without 4 x $10^5$ splenic stimulator cells from $BDF_1$ mice, in triplicate wells of the 96-well round bottom microtiter plates (Falcon, Becton Dickinson, NJ), in a final volume of 200 μl of RPMI (Gibco) that was supplemented with 10% FCS and 1% Penicillin/Streptomycin (5000 mg/ml). The cell cultures were incubated at 37° C in a humidified 5% $CO_2$ atmosphere for four days before they were harvested. On day three the cells were pulsed with 0.5 μCi of $^3$H-thymidine (Amersham) per well and incubated overnight in the same conditions. Using a cell harvester (Skatron Inc., Sterling, VA.) cells were harvested onto the glass-fiber filter paper (Mandel Scientific, Rockwood, ON, Can.), air-dried overnight and topped with 2 ml scintillation fluid (ScintiVerse II, Fisher Scientific). As an index of cell proliferation, the degree of $^3$H-thymidine incorporation was assessed by measuring the mean decay per minute (DPM) of triplicate samples, by using a β-spectrometer with a liquid scintillation system (Beckman Instrument, Inc., Irvine, CA).

Identical culture conditions were also set to generate supernatants for the cytokine (IFN-γ) production assay. At different times after setting the cultures, plates were centrifuged at 300 g for 10 minutes and 175 μl of the supernatants were collected. The supernatants were stored at -20° C.

## 9. ELISA

Sandwich ELISA techniques were employed to measure the level of TNF-α in the mouse sera or IFN-γ in the supernatants that were harvested from murine splenic cell cultures.

a: TNF-α ELISA:

In most experiments, a monoclonal rat anti-mouse TNF-α ( clone MP6-XT22; PharMingen, San Diego, CA) was used as coating antibody and a polyclonal rabbit anti-mouse/rat TNF-α (PharMingen) served as the detection antibody. Later, TNF-α ELISAs were performed using a monoclonal anti-mouse TNF-α (clone G281-2626; PharMingen) to coat the plates, and a biotinylated monoclonal rat anti-mouse TNF-α (PharMingen) to develop the assay.

ELISA techniques for the detection of murine TNF-α are well described (Schreiber 1985 and Lucas 1990). In our laboratory, 100 μl of the primary antibody at the concentration of 4 μg/ml, in a 0.1 M $NaCO_3$ buffer with a pH of 8.2, was added to each well of the flat-bottom 96 well, high binding microtiter plates (Costar). After an overnight incubation at 4° C, the plates were washed and then blocked with 200 μl of PBS containing 3% BSA at room temperature for 2 hours. Plates were washed again and either 100 μl of recombinant mouse TNF-α (R&D Systems Inc., Minneapolis, MN) with a starting

concentration of 4 ng/ml, or 100 μl of samples were added into the wells containing an equal volume of dilution buffer (PBS/3% BSA) and serially diluted down in doubling dilutions. Plates were sealed and incubated overnight at 4° C. After washing the plates with PBS/Tween 20, 100 μl/well of the biotinylated secondary antibody was added at a concentration of 4 μg/ml. The plates were sealed and incubated at room temperature for 45 minutes. The next wash was followed by a further incubation step with 100 μl of avidin-peroxidase (Sigma) at a final concentration of 1 μg/ml at room temperature for 30 minutes. The colorimetric changes were developed after washing the plates, by adding 100 μl/well of substrate which was prepared by dissolving 2,2'-Azino-bis (3-Ethylbenz-Thiazoline-6-Sulfonic Acid) (ABTS; Sigma) in 0.1 M citric acid and immediately before using $H_2O_2$ was added at a final concentration of 2.7%,. After 30 minutes of further incubation at room temperature the optical density of the wells were read at a wave length of 405 nm using a Microtiter reader (Bio-TEK instrument, Inc.). The detection limit of the assay was 60 pg/ml. All the TNF-α ELISAs were performed by Ms. Jacquie Fisher.

b: IFN-γ ELISA

b.1) Preparation of Standard for IFN-γ ELISA

Splenic cells from a C57BL/6 mouse were isolated and adjusted to 7.5 x $10^6$ cell/ml in RPMI containing 10% FCS and 1% Penicillin/Streptomycin.

Cells were cultured with 5 μg/ml of concanavallin A (Con A; Sigma) at 37° C in a humidified 5% $CO_2$ incubator for 2 days. The supernatant was harvested and using the same ELISA technique, calibrated against world health organization (WHO-NIAID) international reference reagent Gg02-901-533 (originally provided by Dr. C. Laughlin, national institute of health; NIH-NIAID). The supernatant was aliquoted into small volumes (1 ml) and stored at -70° C (Yang 1993).

b.2) Antibodies, Reagents and Assay Procedure

This ELISA technique has been developed and described by Yang in 1993. The rat anti-mouse IFN-γ monoclonal antibody, XMG 1.2 (ATCC), was diluted in a 0.05 M carbonate-bicarbonate buffer with a pH of 9.6 at a final concentration of 3 μg/ml. A volume of 50 μl of this antibody was added to each well of a flat-bottom 96 well microtiter plate (Corning, Fisher Scientific) and incubated at 4° C overnight. The coating solution was aspirated and each well was incubated with 75 μl of a 0.17% BSA/PBS blocking buffer with a pH of 7.4, at 37° C for 45 minutes. Before proceeding to the next step and between all other steps of the assay, plates were washed four times with a 0.05% Tween 20/PBS solution (final pH of 7.4). A volume of 50 μl of the standard at 100 U/ml concentration and supernatants from MLR experiments were added into the designated wells and were serially diluted by equal volumes of PBS with 0.085% BSA and 0.05% Tween 20 at pH 7.4. The plates

were incubated at 37° C for 3 hours. To develop the assay, 50 μl/well of biotinylated rat anti-mouse IFN-γ antibody (R4-6A2; ATCC) at 1:2500 dilution was added and the plates were incubated at 4° C overnight. The developing procedure was continued by adding 50 μl/well stereptavidin-alkaline phosphatase (Bio/Can Scientific) at a concentration of 1:2500 and incubating at 37° C for 45 minutes, followed by a further incubation at room temperature for 60, 90 or 120 minutes, with 50 μl/well of substrate P-nitrophenyl phosphate (PNPP; Sigma) dissolved in substrate buffer prepared by dissolving $MgCl_2$-6 $H_2O$ in diethanolamine with pH 9.8 (1 PNPP tablet/5 ml substrate buffer). The level of IFN-γ was measured by the optical density (OD) of each well obtained by reading the absorbance at 405 nm and subtracting background absorbance at 690 nm. The lower limit of the detection of this assay was 0.8 U/ml IFN-γ.

# RESULTS

## A- *In vitro* Cellular Responses of HD Patients to Specific Antigens and Polyclonal Stimuli

To determine the effects of IL-12 on *in vitro* cellular responses of patients with CRF, we examined the proliferative responses and the level of various cytokines in the supernatants of the PBMC cultures from hemodialysis patients and healthy controls, in the presence and absence of 0.5 ng/ml recombinant human IL-12. In order to stimulate the PBMCs, two different sets of activators were used. In the first approach we used polyclonal activators of T cells such as PHA (10 μg/ml), α-CD3 (2 ng/ml) and IL-2 (100 U/ml), and in the second approach, specific antigens such as various strains of the flu virus (attenuated or inactivated, 1 μg/ml) and streptokinase (500 U/ml) were used. Optimal concentrations for either of the stimuli as well as that of rIL-12 were determined in a series of dose response experiments (data not shown). Kinetic experiments were also carried out and days 4 and 6 were chosen as optimal time points for both cytokine production and cell proliferation assays for polyclonal activation and antigen stimulation respectively (data not shown).

### 1. Cytokine Production

The level of IFN-γ in the supernatants obtained from cell cultures was assayed using sandwich ELISA techniques. Our results showed that both mitogen-induced (Fig. 1) and antigen specific (Fig. 2) IFN-γ production by

PBMCs from patients with CRF were lower than those of the healthy controls. Specifically, the antigen specific responses of the patient group to the flu antigens were significantly ($p<0.05$) lower than those of the controls (Fig. 2). The presence of IL-12 enhanced the level of IFN-$\gamma$ and this effect of IL-12 was significantly ($p<0.05$) pronounced in the patient group. (Fig. 1 and 2) Addition of IL-12 alone did not increase the level of the IFN-$\gamma$ production of the patient group to match that of the controls in response to specific antigens (Fig. 2). However, it resulted in the higher levels of IFN-$\gamma$ production, by the patient group, in response to polyclonal stimuli with the exception of OKT3 stimulation (Fig. 1).

IL-10 production was also measured using sandwich ELISA techniques. In general, in response to both type of stimuli, PBMCs from patients produced lower amounts of IL-10 in comparison with those of the controls (Fig. 3 and 4), and in particular antigen specific IL-10 production by PBMCs from HD patients was significantly ($p<0.05$) lower than that of the healthy controls (Fig. 4). The presence of IL-12 did not seem to have a significant effect on the level of IL-10 production in either the patient or control groups, whereas the presence of IL-12 in addition to IL-2 resulted in a significant ($p<0.05$) increase in the IL-10 production in both groups (Fig. 4).

Furthermore, we examined the level of various type 2 cytokines produced by PBMCs of the HD patients in comparison with the healthy controls, to determine whether the impaired cellular responses of the HD

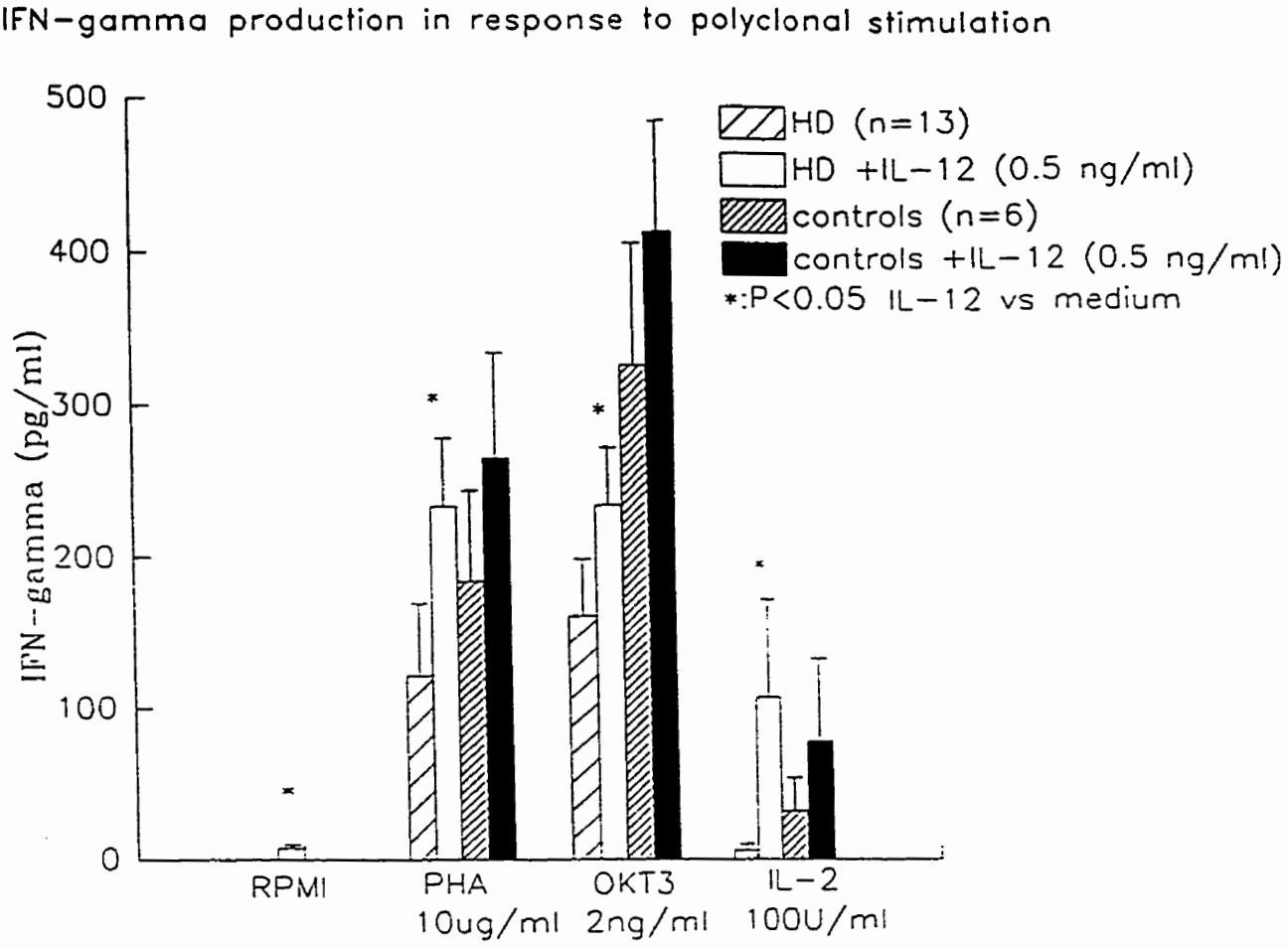


Figure 1: 1 x $10^6$ PBMCs from each subject were cultured with either no treatment or PHA or OKT3 or IL-2 at the indicated concentrations in the presence or absence of 0.5 ng/ml rIL-12 in a final volume of 2 ml of RPMI for 4 days. The level of IFN-γ in the supernatants was measured by sandwich ELISA technique as described in "Materials and Methods" and is expressed as pg/ml.

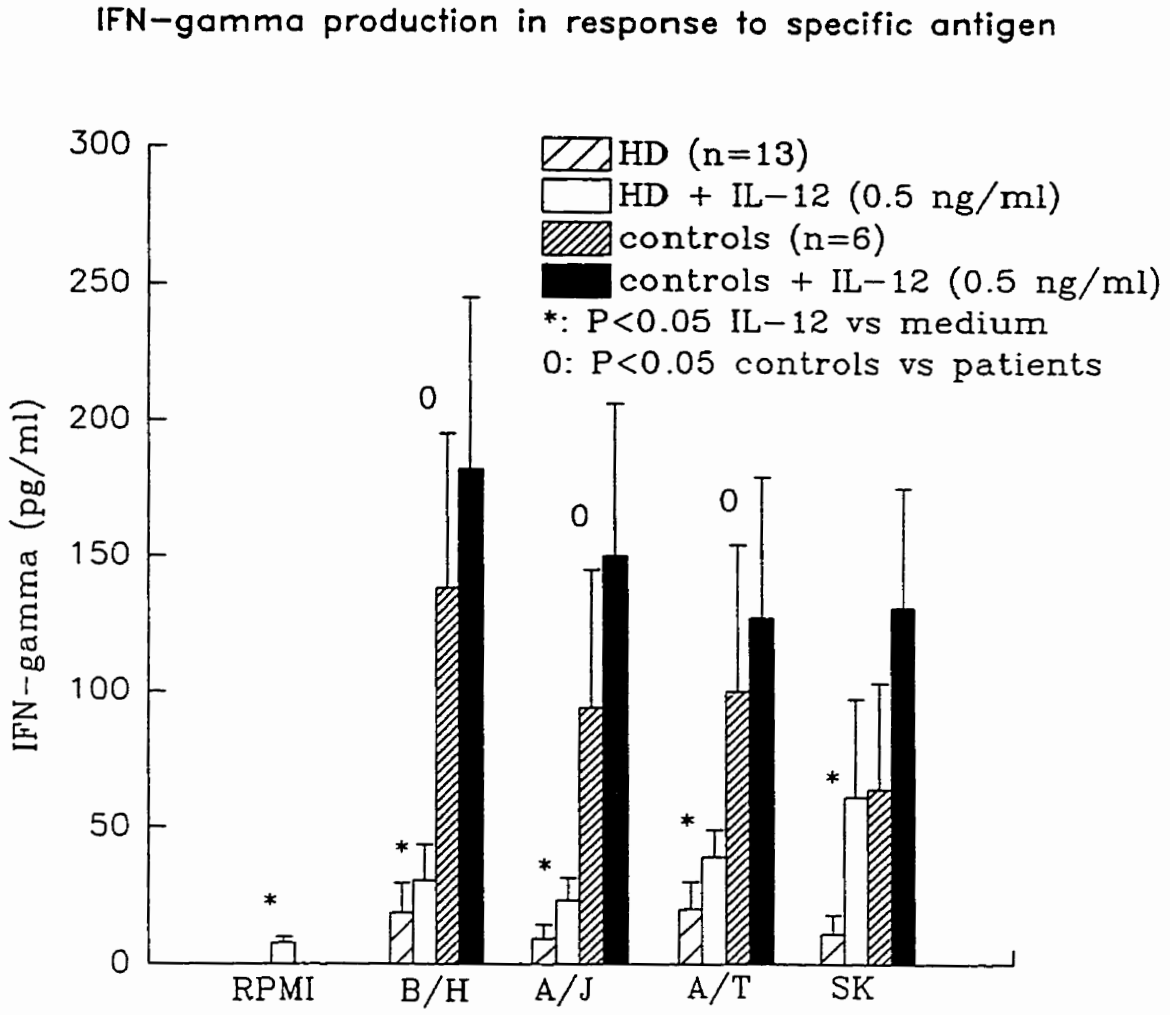


Figure 2: IFN-γ (pg/ml) production in the supernatants of the PBMC cultures from each subject was measured by sandwich ELISAs. Supernatants were collected after 1 x $10^6$ cells were incubated for 6 days with either no treatment or 1 μg/ml of flu antigens (i.e. B. Harbine, A. Johannesburg and A. Texas) or 500 U/ml stereptokinase (SK) in the presence or absence of 0.5 ng/ml rIL-12 in a final volume of 2 ml.

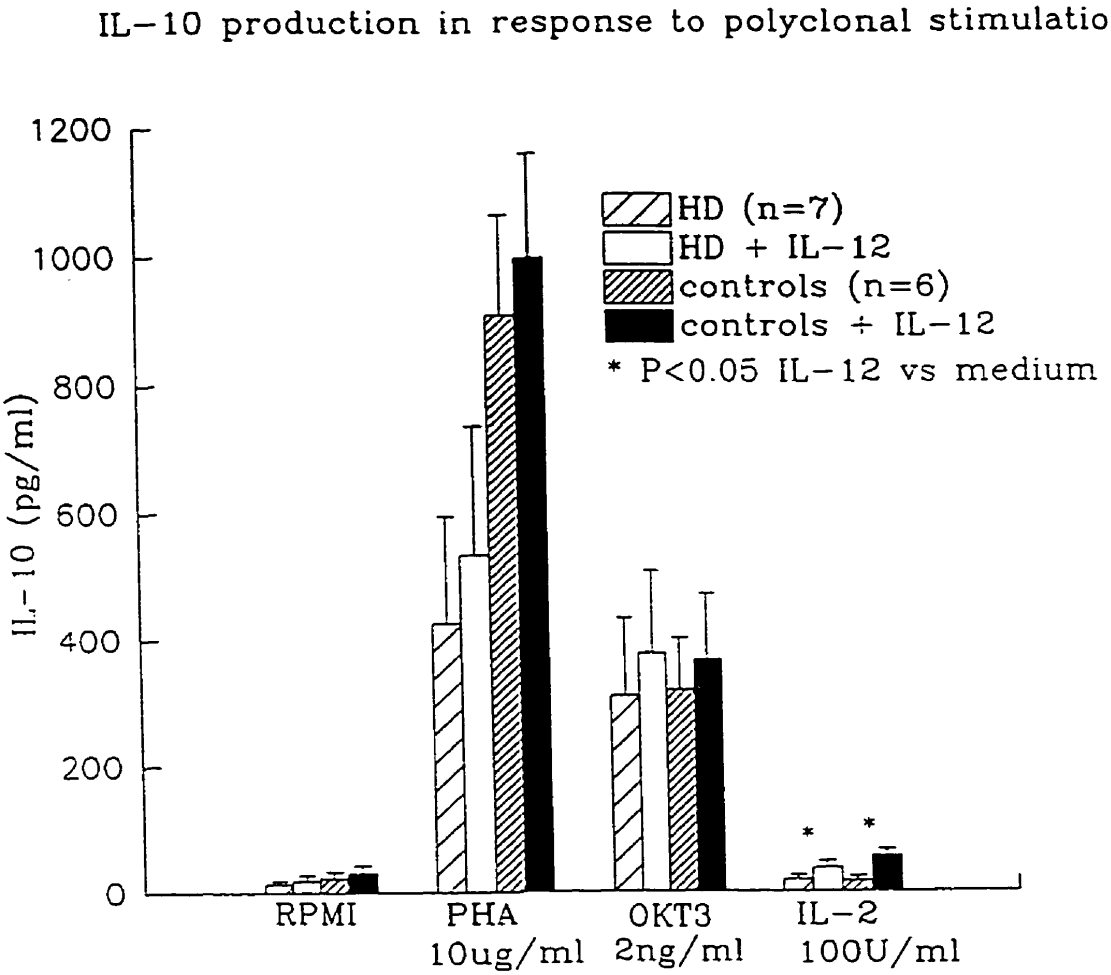


Figure 3: IL-10 production (pg/ml) by PBMCs from each subject in response to no treatment, PHA (10 μg/ml), OKT3 (2 ng/ml) and IL-2 (100 U/ml) in the presence or absence of 0.5 ng/ml rIL-12 was measured by ELISA. The cultures were incubated for a period of 4 days at a final concentration of 5 x $10^5$ cells/ml before the supernatants were collected to be assayed for their IL-10 levels.

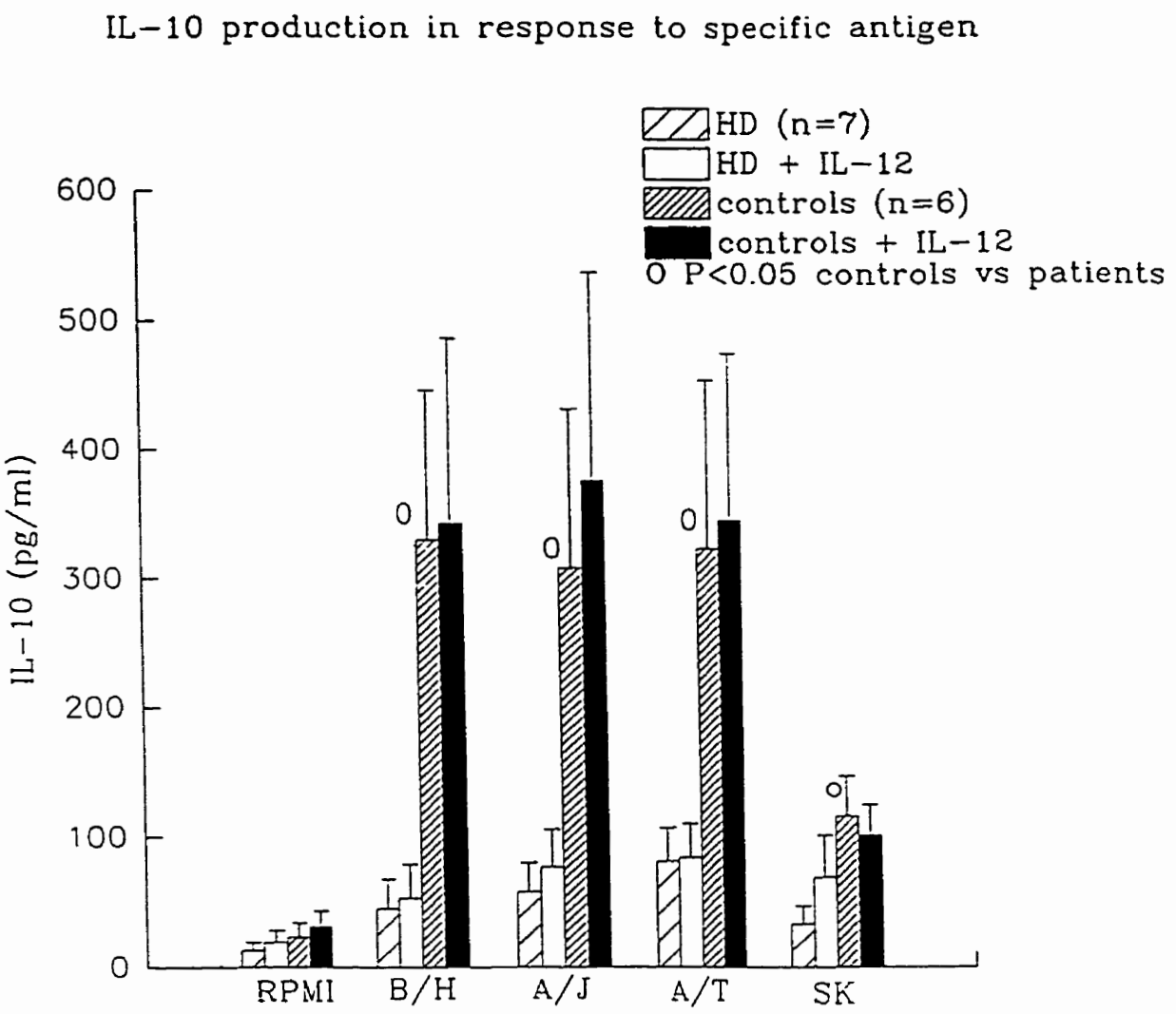


Figure 4: 1 x $10^6$ PBMCs from each subject were cultured with either no treatment or 1 μg/ml flu antigens (B. Harbine, A. Johannesburg or A. Texas) or 500 U/ml stereptokinase (SK), in the presence or absence of 0.5 ng/ml rIL-12 in a final volume of 2 ml of RPMI for 6 days. IL-10 production in the supernatants was measured by sandwich ELISA and is expressed by pg/ml.

patients was due to a switch of the immune responses towards a type 2 pattern. Using a bioassay in the case of IL-4 and sandwich ELISAs for IL-5 and IL-13, we also studied the level of these cytokines in the same supernatants from HD patients and controls (Table 1). In most cases patients had lower cytokine responses to various stimuli. However, the level of IL-5 and IL-13 in both responder and non-responder groups, i.e. responders and non-responders to hepatitis B vaccination, was higher than that of the control groups in response to PHA, whereas non-responder patients produced significantly ($p<0.05$) higher levels of IL-4 than the responder group when stimulated with PHA.

## 2. Cell Proliferation

Cell proliferation was another criteria of the *in vitro* study of immune responses of HD patients that was examined. The results of these studies are summarized in figures 5 and 6. In general, it seemed that the PBMCs from the patients had lower proliferative responses to either groups of stimuli when compared with the results of the controls with the exception of the results of B. Harbine stimulation (Fig. 5 and 6). Our results suggest that the presence of exogenous IL-12 did not increase the level of cell proliferation of the PBMC cultures(Fig. 5 and 6).

## Comparison of type 2 cytokine responses of HD patients with those of healthy controls (mean ± SE)

| Variable | Controls | Total Patients | P vs Cont. | Responder -HD | P vs Cont. | Non-Responder | P vs Cont. |
|---|---|---|---|---|---|---|---|
| **IL-4** | | | | | | | |
| RPMI | 2.6±0.007 | 2.5±0 | | 2.5±0 | | 2.5±0 | |
| PHA | 12.8±2.6 | 11.6±3.2 | | **5.9±1.4** | 0.05 | **20.4±7.1** | 0.05* vs Res. |
| α-CD3 | 16.4±13.5 | 2.8±0.15 | | 2.8±0.2 | | 2.7±0.2 | |
| A. Texas | 2.5±0 | 2.7±0.17 | | 2.5±0 | 0.05 | 3.1±0.04 | |
| SK | 20±6.7 | 13.4±5.3 | | 15.2±7.1 | | 8.9±1.4 | |
| **IL-5** | | | | | | | |
| RPMI | 32.6±5.8 | 16.9±2 | 0.02 | 18.2±3.2 | 0.05 | 15±0 | 0.05 |
| PHA | 172±43 | 203±70 | | 193±101 | | 217±93 | |
| α-CD3 | 186±43 | 149±39 | | 129±53 | | 183±56 | |
| A. Texas | 35±6.7 | 22±4.5 | 0.04 | 17.3±1.6 | 0.03 | 28±11.2 | |
| SK | 49±10.7 | 17.2±1.5 | 0.003 | 16.4±1.4 | 0.008 | 19±4 | 0.05 |
| **IL-13** | | | | | | | |
| RPMI | 262±75 | 48±14 | 0.002 | 44±8.4 | 0.02 | 54±35 | 0.001 |
| PHA | 1960±431 | 2761±669 | | 2063±770 | | 3846±1185 | |
| α-CD3 | 719±141 | 850±204 | | 640±211 | | 1218±409 | |
| A. Texas | 279±67 | 41±6.2 | 0.0026 | 48±7.6 | 0.029 | 29±10.1 | 0.005 |
| SK | 330±86 | 79±25 | 0.04 | 54±10.5 | 0.05 | 140±85 | |

Table 1: PBMCs from each subject were cultured in RPMI at a final concentration of 5 x $10^5$ cells/ml, with either no treatment or PHA (10 mg/ml, 4 days), OKT3 (2 ng/ml, 4 days), flu antigen (A. Texas; 1 mg/ml, 6 days) and streptokinase (SK; 500 U/ml, 6 days) for indicated periods of time. IL-4 (pg/ml) production was measured by CT.h4S bioassay while the levels of IL-5 (pg/ml) and IL-13 (pg/ml) were assessed by sandwich ELISAs. p values were calculated by the Non-parametric Mann Whitney-U test.

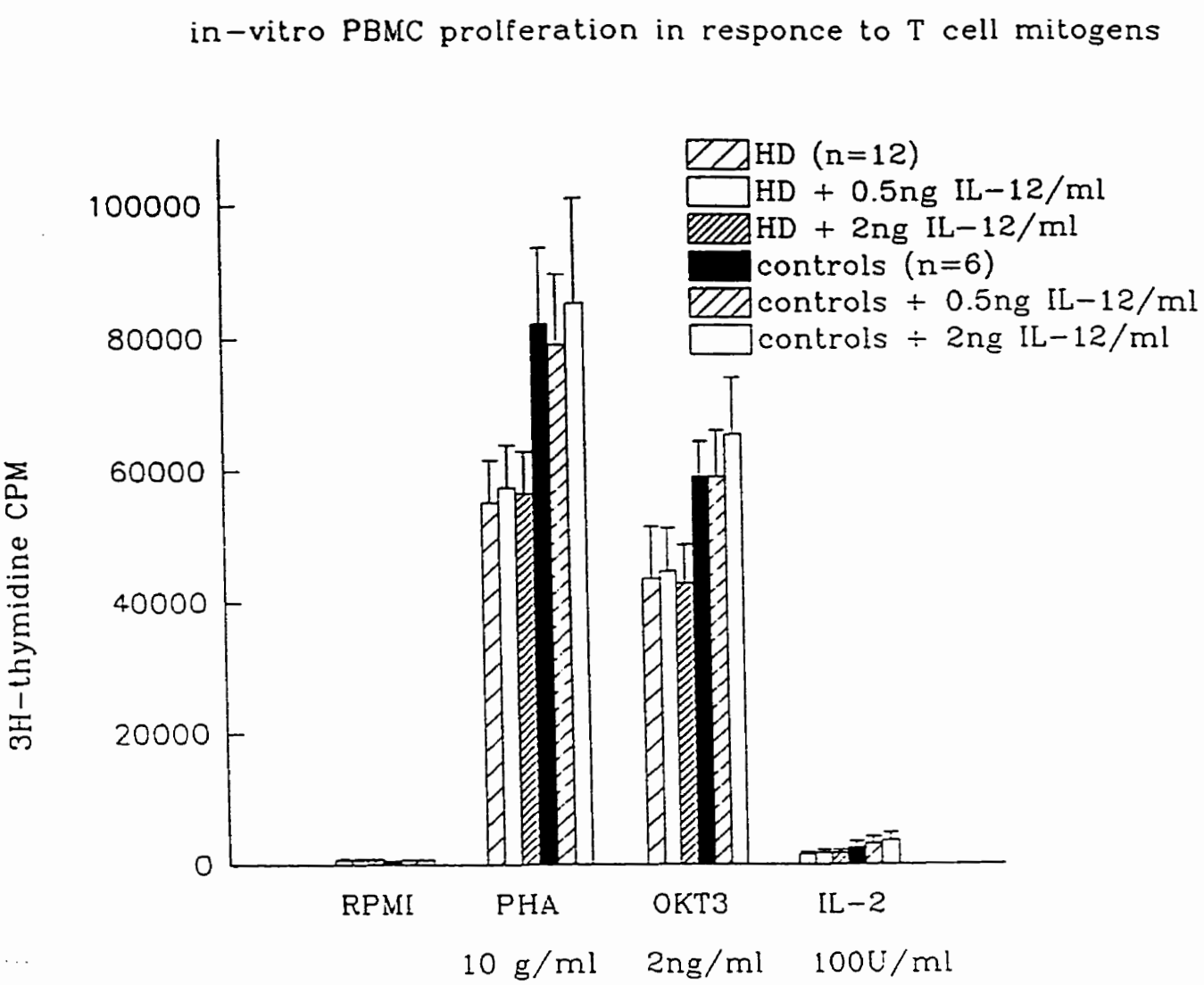


Figure 5: Proliferative responses of each subject was assessed by culturing 1 x $10^5$ PBMCs with no treatment, PHA (10 μg/ml), OKT3 (2 ng/ml) or IL-2 (100 U/ml), in the presence or absence of 0.5 or 2 ng/ml rIL-12 in final volume of 200 μl for 4 days. Cells were pulsed with 0.6 μCi $^3$H-thymidine 16 hours prior to harvesting. The degree of $^3$H-thymidine uptake was measured and expressed by CPM as an index of cell proliferation.

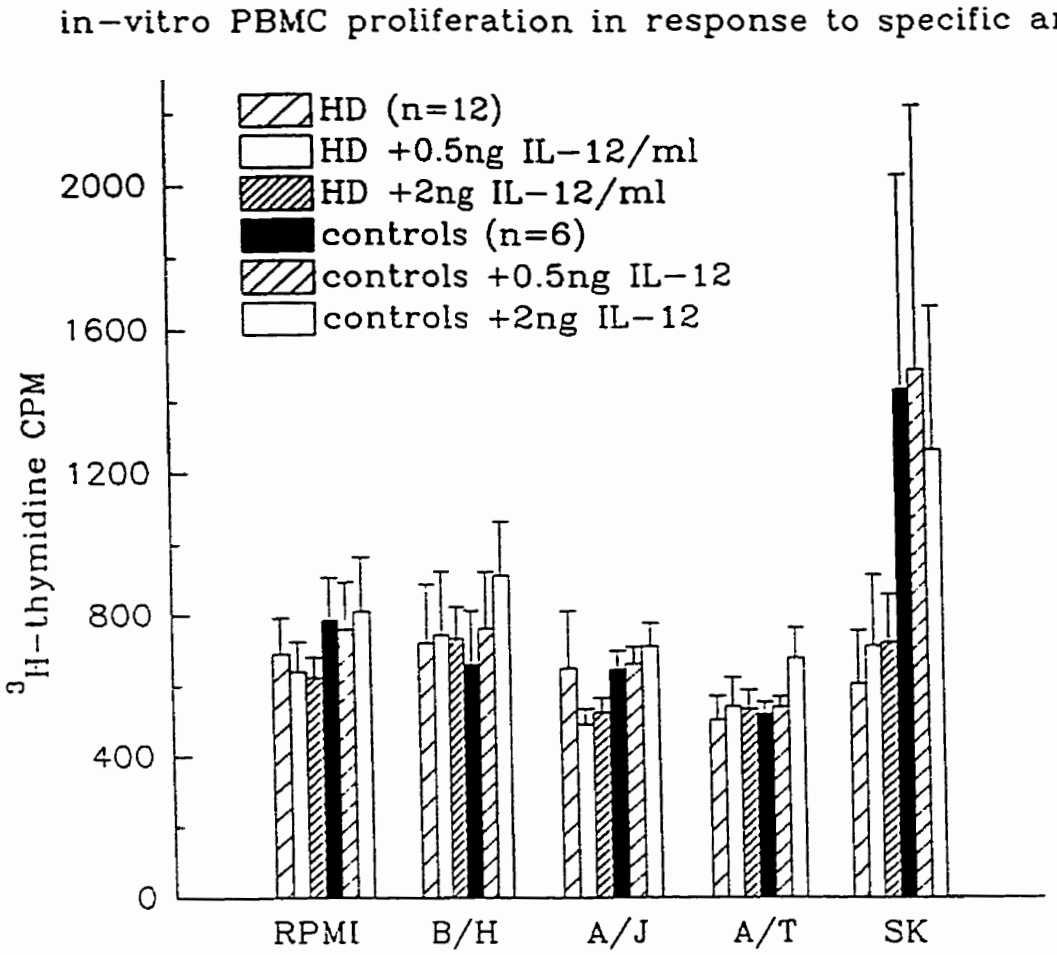


Figure 6: Proliferative responses to specific antigens were measured by culturing 1 x $10^5$ PBMCs from each subject in a final volume of 200 µl with either no treatment or 1 µg/ml flu antigens (i.e. B. Harbine, A. Johannesburg, A. Texas) or 500 U/ml stereptokinase (SK), in the presence or absence of rIL-12 at the indicated concentrations, for 6 days. Sixteen hours prior to harvesting, 0.6 µCi of $^3$H-thymidine was added to each well. $^3$H-thymidine incorporation was evaluated and expressed as CPM to assess the level of cell proliferation.

## B- *In vivo* and *In vitro* Studies on Murine Model of GVHR, Cytokine and Proliferative Responses

### 1. *In vivo* Studies

The serum levels of TNF-α and the degree of the pathological changes in the intestinal mucosa (i.e. the level of apoptosis in the epithelial tissue of the intestine) were measured as *in vivo* indices of GVHR. Firstly, a series of kinetic experiments were carried out to determine the time points at which LPS-induced TNF-α release and apoptosis of the epithelial tissue of the intestine were at their highest levels. A number of healthy mice received an i.p. dose of 100 μg Poly I:C 18 hours prior to a systemic administration of 20 μg LPS. At different time intervals after the LPS treatment mice were killed and their sera and intestinal tissues were subjected to ELISA and histopathological analysis respectively as is discussed in "Materials and Methods". The results of these experiments suggested that the level of serum TNF-α was at its peak at one hour after the LPS treatment (Fig. 7) and the number of apoptotic nuclei in a cluster of 10 vili/crypts was at its peak at 6 hours after the systemic LPS administration. (Fig. 8)

The effect of Poly I:C and LPS alone on the induction of the TNF-α release were also studied (Table 2). Our results suggested that Poly I:C alone did not induce a detectable response, while administration of LPS alone resulted in the release of up to 35 ng/ml of TNF-α in the mouse sera.

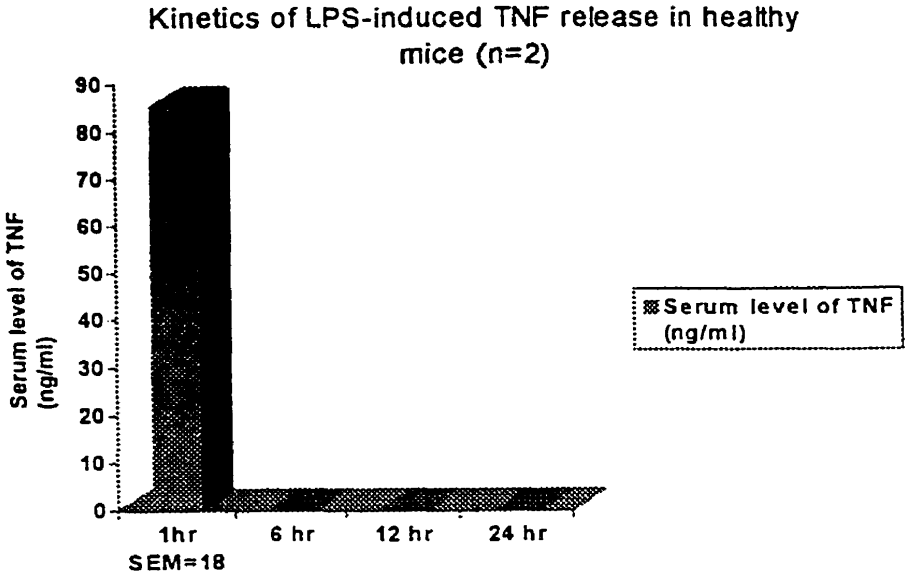


Figure 7: Eight age-matched female C57BL/6 mice received an i.p. dose of 100 μg Poly I:C/mouse which was 18 hours later followed with an i.v. injection of 20 μg LPS in 0.2 ml of RPMI. Blood samples were drawn at 1, 6, 12 and 24 hour intervals after the LPS administration and left to clot at 4° C overnight. Sera were collected and the serum level of TNF-α (ng/ml) was measured by ELISA as described in "Materials and Methods".

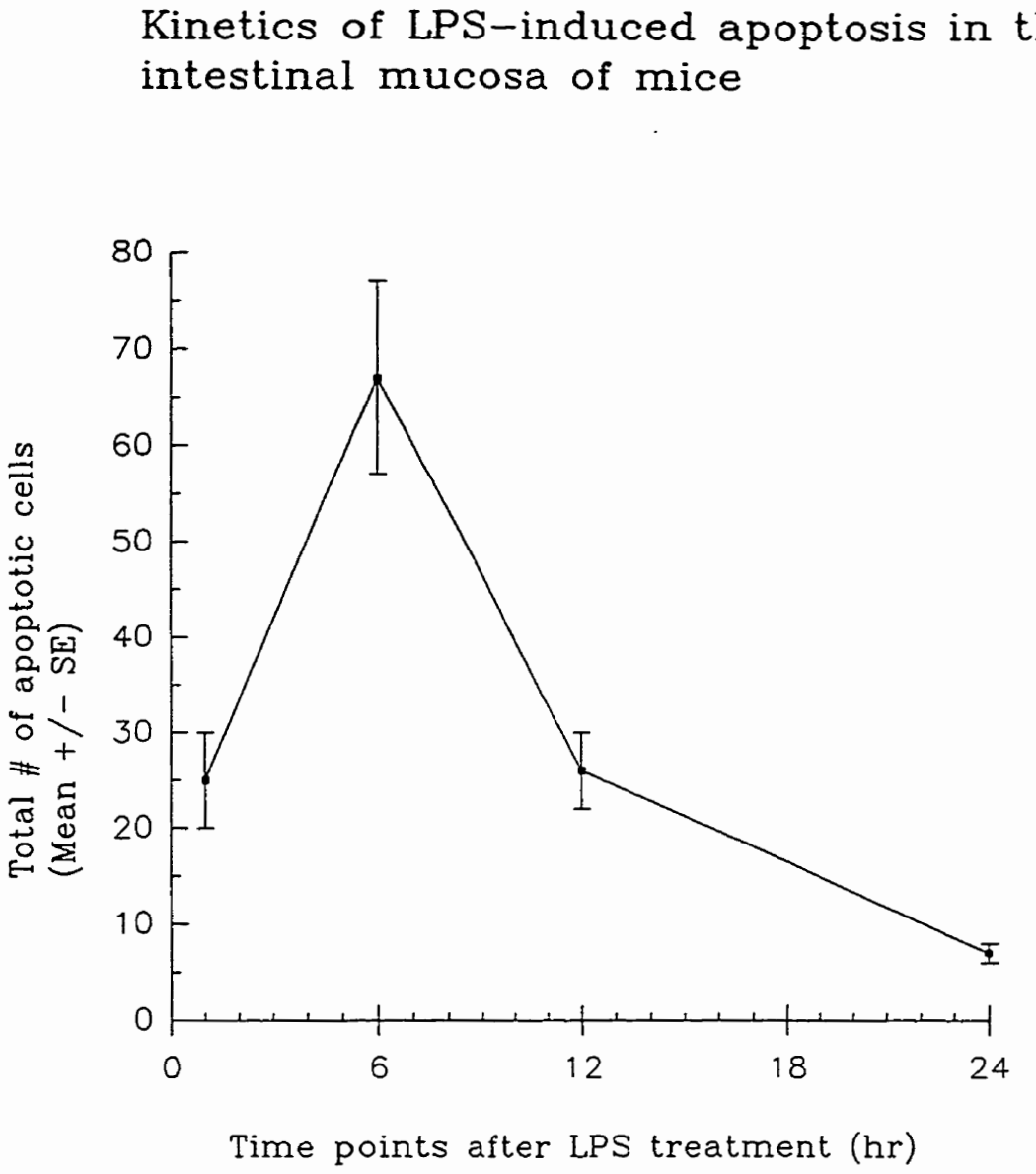


Figure 8: Mice that were pretreated with an i.p. dose of 100 μg Poly I:C received an i.v. injection of 20 μg LPS in 0.2 ml RPMI. Mice were sacrificed at the indicated intervals and 1-2 cm long intestinal segments from jejunum were taken and fixed in a 10% neutral formalin. Slides with intestinal sections were prepared and stained with the ApopTag kit as described in "Materials and Methods". The total number of apoptotic nuclei in a cluster of 10 vili/crypts on each slide was determined by counting brown stained nuclei under light microscopy.

## Serum TNF-α levels at different time points after various treatment

| Treatment groups | Time of bleeding | Serum TNF-α Mean±SE (ng/ml) |
|---|---|---|
| Control (n=3) | 0 | ND |
| Poly I:C (n=2) | 18 hrs after treatm. | ND |
| LPS (n=2) | 6 hrs after treatm. | ND |
| LPS (n=2) | 1 hr after treatm. | 35.1±3.0 |

Table 2: 15-week old female C57BL/6 mice were either injected with 100 μg Poly I:C or 20 μg of LPS and were bled at the indicated times after the injections. The control mice received no treatments. Blood samples were left to clot overnight at 4° C. Sera were collected and the level of serum TNF-α was for each sample measured by ELISA techniques. ND means not detectable.

1.1. Intestinal damage and LPS-induced TNF-α release in mice with acute GVHD

Next we induced an acute GVH reaction as is described in "Materials and Methods", and examined the level of apoptotic cells in the intestinal tissue of mice with acute GVHR as well as the level of serum TNF-α in the same mice with or without LPS treatment at days 8 and 15 post-induction. A set of 3 slides containing tissue sections from the same intestinal segments were stained either with hematoxylin and eosin (H&E) or with the ApopTag kit in the presence or absence of the TdT enzyme, the later for negative control. Figure 9 shows an H&E stained intestinal section from a GVH mouse without LPS administration. In the slides that were stained with the Apoptag kit we were able to detect apoptotic nuclei in the sections from different treatment groups, including LPS recipients and GVH mice without LPS administration, at days 8 and 15 post-induction. (Fig. 10 a, b, c) However, these results were not consistent either on the same tissue section or on the sections from various mice. The level of LPS-induced TNF-α in the sera of mice was also assessed and compared with those of control mice that did not receive the systemic injection of LPS (Table 3). The systemic administration of LPS resulted in high serum TNF-α levels at both days 8 and 15 post-induction while mice that did not receive LPS treatment did not display a detectable level of serum TNF-α. The level of LPS-induced TNF-α was higher in the sera

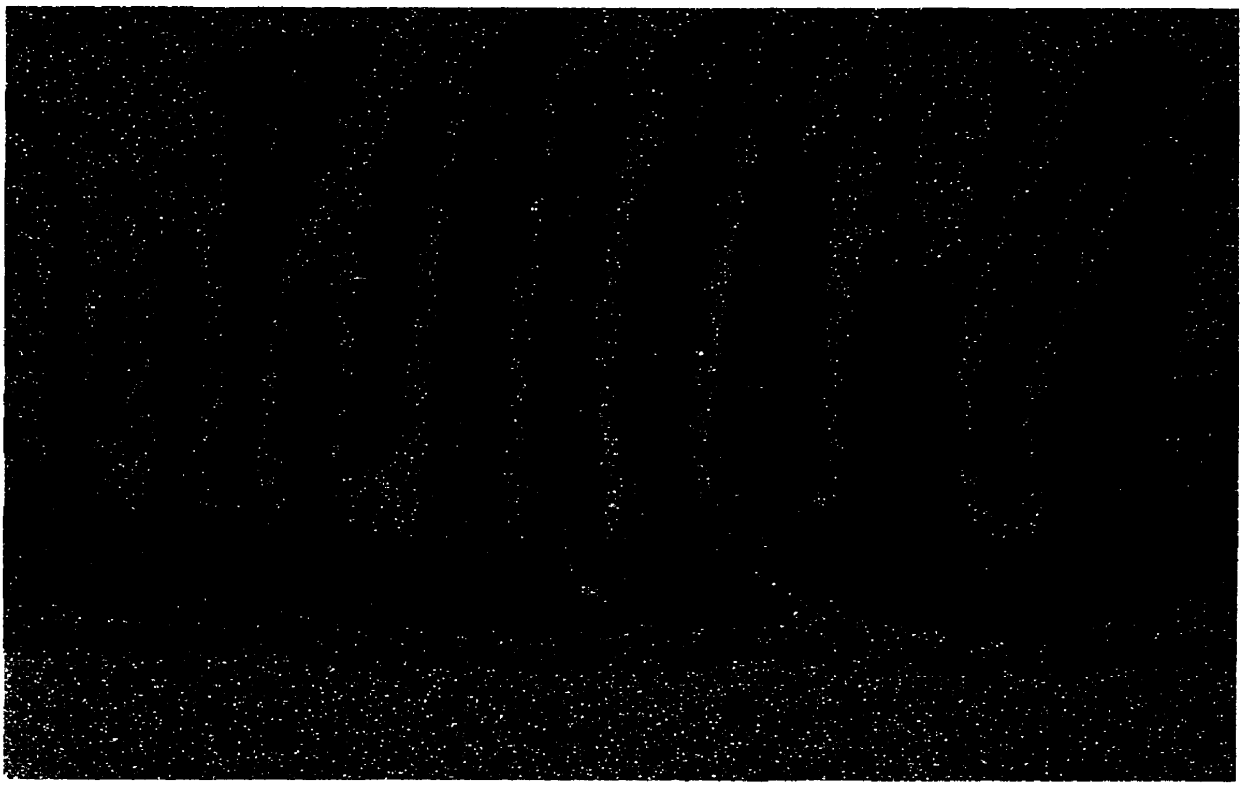

Figure 9: Immediately after the mice were killed, intestinal segments were extracted, fixed in neutral formalin and embedded in paraffin. A set of three 5 μm sections were cut and placed on three sialinized slides. Sections were deparaffinized and one slide of each set was stained with hematoxylin and eosin for the routine histopathological analysis of the intestinal segment.

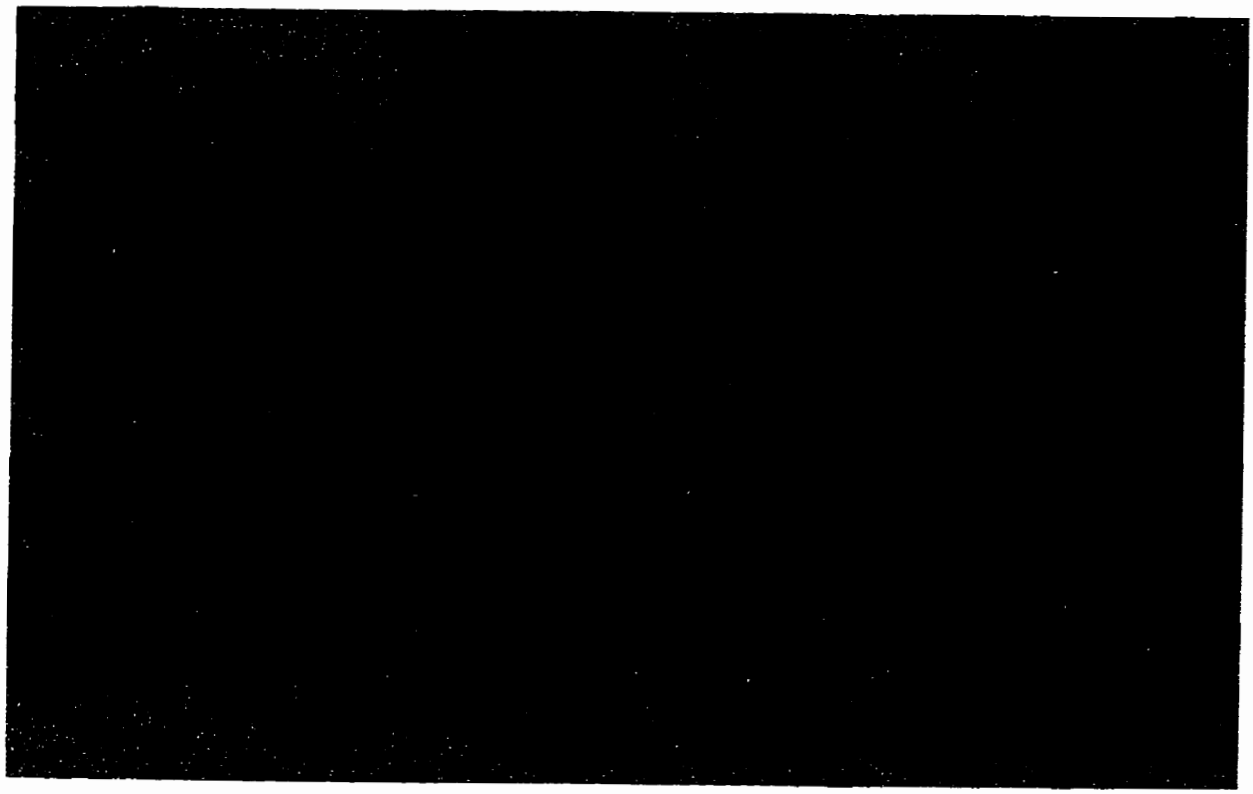

**Figure 10 (a)**

Figure 10: (a) day 8, control. (b) day 8, LPS treated. (c) day 15, LPS treated. GVHR was induced by the injection of 6 x $10^7$ C57BL/6 parental cells to each BDF1-hybrid mouse. At days 8 (a and b) and 15 (c) groups of mice received a systemic dose of LPS (b and c) while control mice did not. 90 minutes later mice were killed and intestinal segments were immediately collected, fixed and placed in the paraffin blocks. Tissue sections were prepared and stained with the in-situe apoptotic nuclei detection kit as explained in "Materials and Methods". Apoptotic cells stained with brown color as well as normal cells stained with methyl green were visualized by light microscopy and documented by photographic techniques.

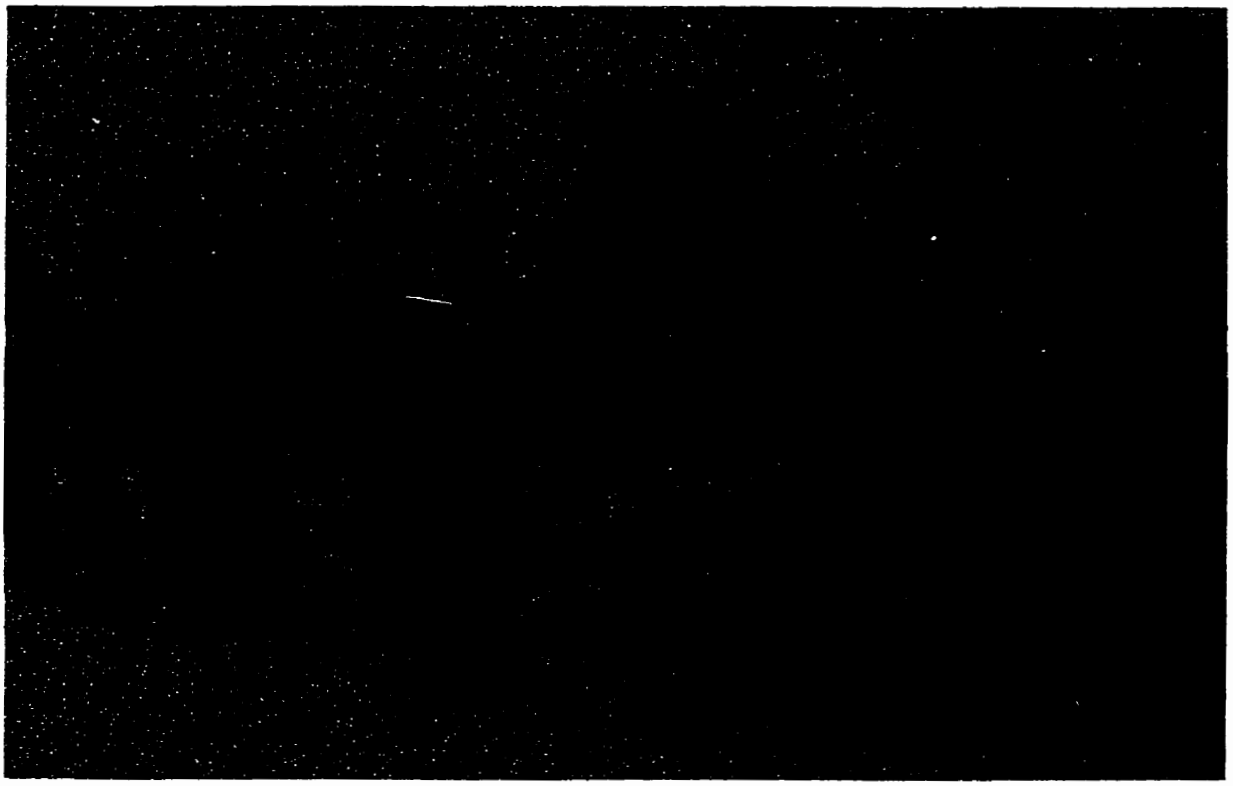

Figure 10 (b)

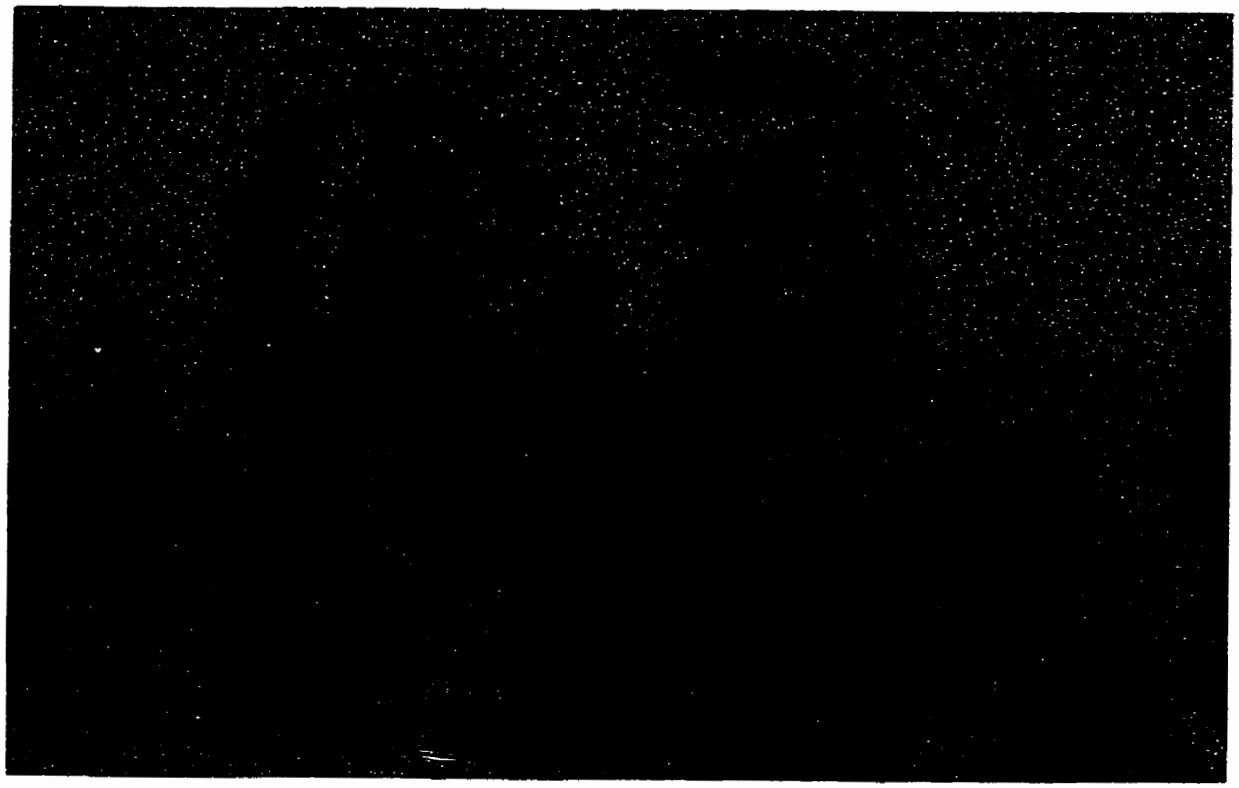

Figure 10 (c)

## LPS-induced TNF-α release in mice with acute GVHR

| Days after induction | S.I. | n | LPS | TNF-α (ng/ml) Mean ± SE |
|---|---|---|---|---|
| 8 | 3.70±0.00 | 3 | + | 343±220 |
| | | 3 | – | ND |
| 15 | 1.96±0.02 | 3 | + | 472±83 |
| | | 3 | – | ND |

Table 3: GVHR was induced by injection of 6 x $10^7$ C57BL/6 parental splenic and LN cells to each BDF1-hybrid mouse. Groups of three mice were administered with a dose of 10 μg LPS 90 minutes prior to bleeding at the days 8 and 15 post-induction. Control mice with GVHR were also bled without being administered with LPS. To measure the level of TNF-α, sera were collected and subjected to TNF-α ELISA. Immediately after bleeding, the mice were killed and weighed. The spleens were also extracted and weighed in order to calculate the splenic index (S.I.). ND means not detectable.

of mice on day 15 post-induction when compared to those of the mice on day 8 after induction (Table 3).

1.2. LPS-induced TNF-α release in IFN-γ *KO* and IL-12 *KO* mice

In order to further the dissection of the mechanism involving the LPS-induced TNF-α release and the role of Poly I:C in this cascade we carried out a set of similar experiments using IFN-γ *KO* mice with a similar genetic background to those of the mice routinely used in our murine model of GVHR (i.e. C57BL/6). As it is shown in Table 4, mice that were treated with Poly I:C and LPS had up to 48 ng/ml serum TNF-α, whereas mice that received LPS alone did not have detectable levels of TNF-α in their sera (Table 4). However, in a similar experiment that was performed later where the level of TNF-α was measured with a different ELISA system, 4 ng/ml TNF-α was detected in the sera of IFN-γ *KO* mice in response to LPS alone (Table 5). Considering the age of the IFN-γ *KO* mice used in this experiment that were up to 8 months old, this may be related to the redundant mechanisms that in time have taken over the priming role of the IFN-γ on macrophages. In the first experiment with the IFN-γ *KO* mice, a group of mice were treated with $10^3$ U recombinant IFN-γ per mouse 18 hours prior to the LPS injection. The dose of the rIFN-γ was based on the studies of the resistance of a primary monolayer intestinal cell culture to different concentrations of IFN-γ (Madara, 1989). Even though it was shown later that mice would tolerate higher *in vivo* doses of IFN-γ (i.e. 25 x $10^3$ U/ml), in our experiment an i.p. administration of $10^3$ U

IFN-γ prior to the LPS injection resulted in the release of up to 2 ng/ml of serum TNF-α while the administration of LPS alone did not induce detectable serum TNF-α (Table 4).

In another experiment we added a group of IL-12 p40 *KO* mice to the previous experimental groups (i.e. IFN-γ *KO* mice), to study the role of IL-12 in the mechanisms of LPS-induced TNF-α responses. After receiving an i.v. injection of LPS that was preceded by a Poly I:C injection or LPS alone, the level of TNF-α in the sera of both IFN-γ *KO* and IL-12 p40 *KO* mice was measured and compared to each other as well as those of wild type mice (Table 5). The wild type mice had the highest level of LPS-induced serum TNF-α while the IFN-γ *KO* mice expressed the lowest level and IL-12 p40 *KO* ranged in the middle of the other two groups (Table 5). The i.p. injection of 100μg poly I:C prior to the LPS administration resulted in higher serum levels of TNF-α in all three different mice groups.

## 2. *In vitro* Studies

In order to initiate an *in vitro* model of murine GVH reaction parental splenic cells from C57BL/6 mice that were treated with an i.p. injection of 100 μg Poly I:C were isolated and used as responder cells. The stimulator splenic cells from $BDF_1$-hybrid mice were also isolated and co-cultured with responder cells at different concentrations for variable periods of time (data

## LPS induced TNF-α release in IFN-γ ko mice

| Treatment groups | n | TNF-α (ng/ml) Mean ± SE |
|---|---|---|
| LPS only | 3 | ND |
| Poly I:C + LPS | 3 | 48.0 ± 13.0 |
| rIFN-γ + LPS | 3 | 1.8 ± 0.4 |

Table 4: Eighteen hours prior to the LPS injection 13-week old male IFN-γ ko mice received an i.p. administration of either 0.1 mg Poly I:C or 1 x $10^3$ U of rIFN-γ which was followed by an i.v. injection of 20 μg LPS. A group of 3 mice did not receive any treatment prior to the LPS administration. Blood samples were collected 90 minutes after the LPS treatment and were left to clot overnight at 4° C. Sera were collected and the level of serum TNF-α was assayed by ELISA as described earlier. ND means not detectable.

## LPS-induced TNF-α release in various mice groups

| Mice | Treatment groups | n | TNF-α (ng/ml) mean ± SE |
|---|---|---|---|
| wild type | LPS only | 3 | 35.1 ± 3.0 |
| | Poly I:C + LPS | 2 | 85.5 ± 18.0 |
| IFN-γ ko | LPS only | 2 | 3.8 |
| | Poly I:C + LPS | 3 | 20.9 ± 2.9 |
| IL-12 p40-ko | LPS only | 2 | 4.5 |
| | Poly I:C + LPS | 3 | 26.2 ± 7.1 |

Table 5: A group of 3 retired male IFN-γ ko mice in addition to 3 retired male IL-12 p40 ko mice received an i.p. dose of 0.1 mg Poly I:C, while 2 more retired male IFN-γ ko and 2 retired female IL-12 p40 ko did not receive the treatment. 18 hours after the Poly I:C all of the mice were injected with an i.v. dose of 10 μg LPS. 90 minutes later blood was drawn and after an overnight incubation at 4° C sera were collected. Serum TNF-α levels were measured by ELISA techniques as described in "Materials and Methods" and are expressed in ng/ml.

not shown), and the optimum assay conditions were determined by comparing the results of these pilot experiments.

2.1. NK Cell Depletion

In the next experiments, responder cell populations from C57BL/6 mice were subjected to various Ab and C treatments as described in "Materials and Methods". The degree of NK cell depletion in each treatment group was assessed by an NK cell cytotoxicity assay using the YAC-1 target cell.(Fig. 11) Both α-ASGM1 and PK136 antibody treatments were able to remove up to 85% of the NK cell lysis activity of the parental splenic cell population, when combined with a subsequent complement treatment. (Fig. 11) Then, equal numbers of the parental C57BL/6 responder cells, from various treatment groups and $BDF_1$-hybrid stimulator splenic cells were cultured together as described in "Materials and Methods".

2.2. Proliferative Responses in MLR

As an index of cell proliferation, the degree of $^3$H-thymidine incorporation for each treatment group was assessed and compared with each other as well as to those of the responder cells alone (Fig. 12). The level of cell proliferation was the highest in the Ab and C treated groups, and both of the α-ASGM1 and PK136 antibody treatments resulted into the comparable enhancement of proliferative activities in the cultures when

## Percent Lysis of the YAC−1 cells by C57 splenic cell populations.

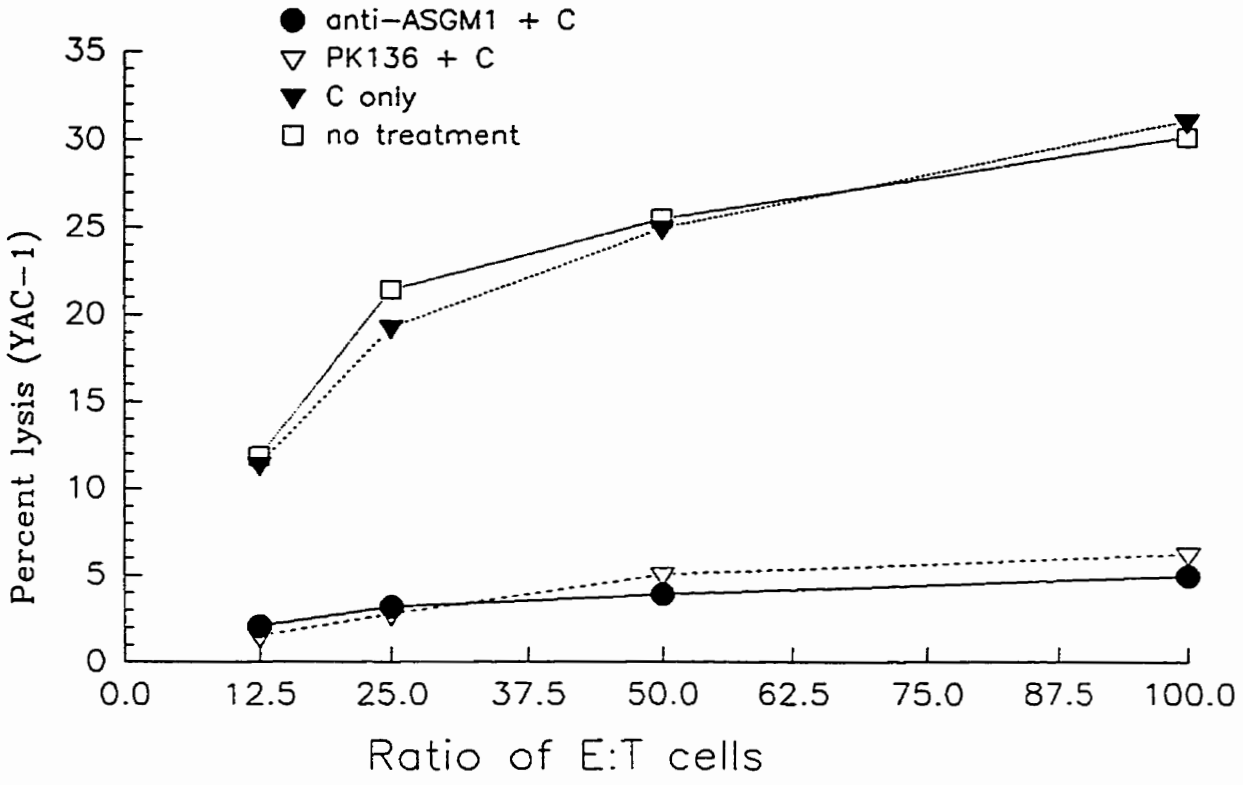


Figure 11: Eighteen hours prior to being sacrificed, two 15 weeks old female C57BL/6 mice were injected with an i.p. dose of 100 μg Poly I:C. Splenic cells were isolated and pooled together. The cells were subjected to the NK depletion procedure as described earlier and portions of each treatment group were used in the cytotoxicity assay as effector cells. A number of $10^4$ $^{51}$Cr-labeled YAC-1 cells were added to each well of the plates as target cells. To achieve the indicated E:T ratios a number of $10^6$, $5 \times 10^5$, $2.5 \times 10^5$ or $1.25 \times 10^5$ effector cells were also added into the wells in triplicates. After 4 hours of incubation the degree of $^{51}$Cr release in the supernatants was assessed and expressed as an index of percent lysis.

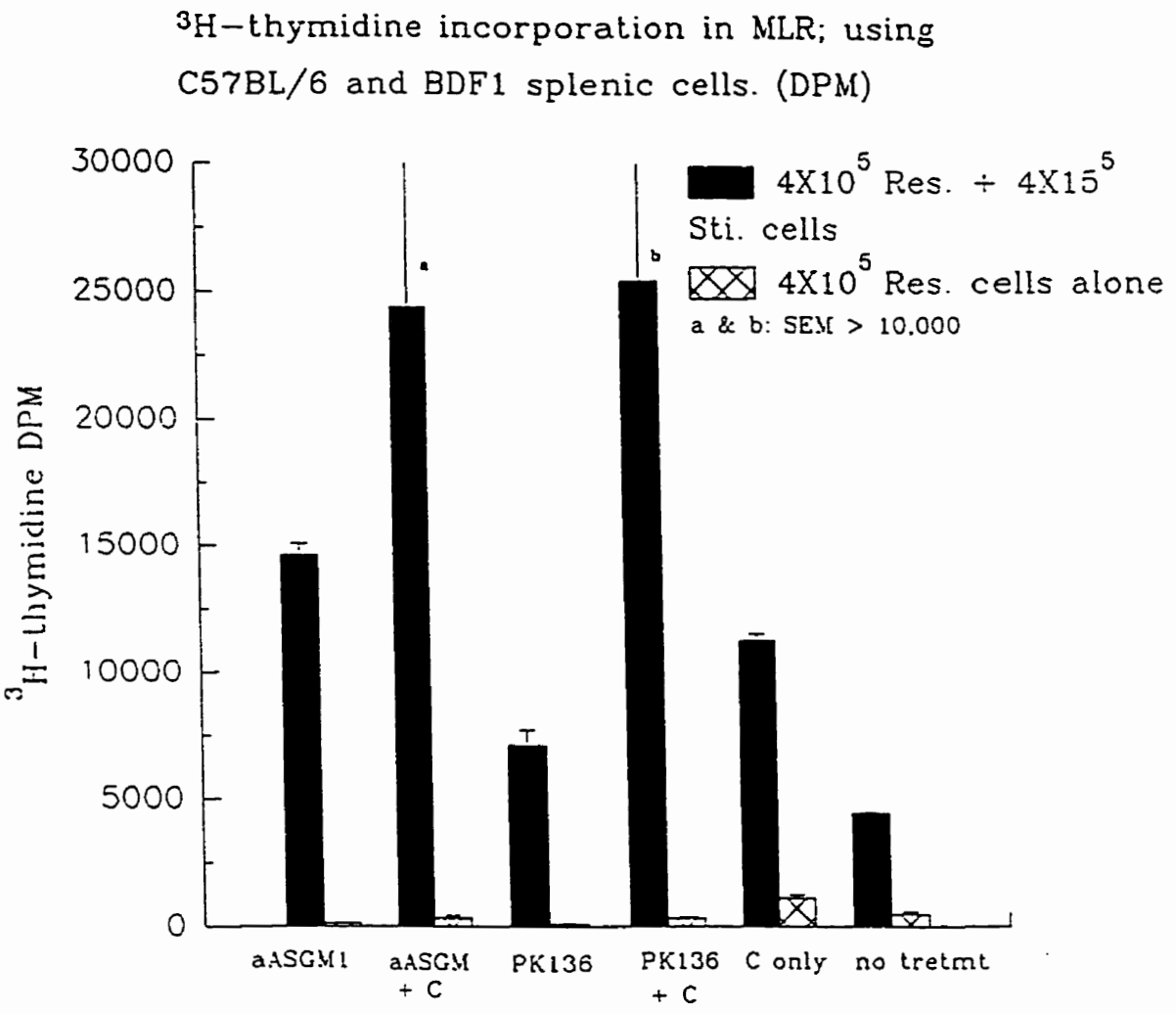


Figure 12: Responder splenic cells from Poly I:C treated H-2$^b$ C57BL/6 mice were treated with indicated treatments and 4 x $10^5$ cells from each treatment group were cultured in triplicates in the presence or absence of 4 x $10^5$ stimulator splenic cells from $BDF_1$-hybrid H-2$^{b/d}$ mouse. 16 hours prior to harvesting, cells were pulsed with 0.5 μCi $^3$H-thymidine. The level of cell proliferation was measured by $^3$H-thymidine incorporation and expressed as DPM.

combined with C treatment (Fig. 12). Responder cells that were treated solely with either of the two antibodies also displayed higher proliferative responses than those of cells from no treatment group. However, this increasing effect of the antibodies on the proliferative activities of the cells was more pronounced in the case of α-ASGM1 than that of PK136 (Fig. 12). A treatment with C alone also resulted in enhanced proliferative responses of the mixed lymphocyte culture (Fig. 12). The levels of spontaneous proliferation in the absence of stimulator cells was also measured Fig. 12).

2.3. IFNγ Production in MLR

The level of IFN-γ in the mixed lymphocyte cultures was another index of the *in vitro* GVH reaction that was studied. The culture conditions were similar to those that were set for cell proliferation studies and are described in "Materials and Methods". Supernatants obtained from these cultures were subjected to sandwich ELISA for IFN-γ as explained earlier and the results are summarized in Table 6. Supernatants from cultures with Ab treated responder cells, i.e. Ab only, had the highest levels of IFN-γ and in fact supernatants from the PK136 treatment group displayed the highest level of this cytokine, which was 37 U/ml followed by those of α-ASGM1 up to 21 U/ml. However, the degree of the IFN-γ production of the cultures was much lower when responder cells were treated with complement following the treatment with either of the antibodies (Table 6). A treatment of C alone

resulted in more than twice the IFN-γ production, i.e. 12 U/ml, when compared with no treatment group, which produced up to 5 U/ml IFN-γ.

IFN-γ production in MLR (U/ml, Mean ± SE)

| Treatment groups | αASGM1 | αASGM1 + C | PK136 | PK136 + C | C only | no tremt. | Sti.Cells Alone |
|---|---|---|---|---|---|---|---|
| Res. Cells +Sti. Cells | 21.0 ± 4.3 | 8.0 ± 0.3 | 37.0 ± 3.5 | 9.0 ± 1.7 | 12.0 ± 0.5 | 5.0 ± 0.7 | ND |
| Res. Cells Alone | ND | ND | ND | ND | ND | ND | |

Table 6: A number of $4X10^5$ Poly I:C treated responder C57BL/6 H-$2^b$ mice splenic cells were treated with various treatments and cultured in triplicates with or without equal number of splenic cells from stimulator $BDF_1$-hybrid H-$2^{b/d}$ mice for 4 days. The supernatants were harvested and the level of IFN-γ for each treatment group was measured by ELISA techniques as explained in "Materials and Methods". ND means not detectable.

# DISCUSSION

## 1. Studies on Immune Responses of HD Patients

IL-12, a monocyte-derived cytokine, was first known as NK cell stimulatory factor (NKSF) (Kobayashi 1989) and studied for its role in the differentiation of precursor T cells to Th1-like cells (Trinchieri 1993). Earlier studies focused mainly on the effects of IL-12 on the development of IFN-γ producing $CD4^+$ T cells, i.e. Th1-like cells. (Heish 1993 and Seder 1993) However, more recent evidence for an enhancing effect of IL-12 on IL-10 production (Morris 1994), as well as further evidence for its indirect role in eliciting Th1-like cells, through IFN-γ (Bruselle 1997) resulted in the suggestion that IL-12 may have a positive regulatory effect not only on the type 1 responses, but also on type 2 immune responses (Muraille 1997). In fact, our results from the experiments with PBMCs from patients with CRF suggested that the presence of IL-12 not only significantly ($p<0.05$) increased the level of IFN-γ production in response to both type of stimuli, but also enhanced the level of IL-10 production in response to polyclonal stimuli. These results were more pronounced when the PBMCs were stimulated by specific antigens in comparison with the results of the PBMCs responding to polyclonal stimuli. Although not certain, this may be due to either a lack or decrease in memory T cells caused by a chronic immunosupression or a defective antigen presentation that may have occurred at the monocyte or T cell level.

Moreover, IL-18 is another novel cytokine that has been reported to exhibit similar and synergistic effects with IL-12 in the induction of IFN-γ (Okumara 1995), stimulation of NK cells (Tomura 1998) and activation of macrophages (Munder 1998). Our results suggest that the sole exogenous IL-12 treatment did not enhance the level of cytokines produced by PBMCs from patients to match those of the healthy controls. Considering the recent data on IL-18, perhaps investigating the role of IL-18 alone and together with IL-12 will lead to a more clear understanding about the regulatory function of these cytokines.

Furthermore, it has been suggested that various polyclonal activators may elicit qualitatively different cytokine production patterns. In particular, it has been shown that stimulation with the anti-CD3 monoclonal antibody induces a Th1-dominant response, while PHA evokes a Th2-dominant type of response. (Imada 1995) Our data from the *in vitro* cytokine production by PBMCs from patients with CRF, in response to polyclonal stimulation, was also parallel to these results with the exception of the PHA-induced IL-4 level that was almost 10 times lower in our samples. If the lower level of PHA-induced IL-4 production was restricted to the HD patients, it could be related to the impaired immunity of these patients. However, the results of the experiments with the PBMCs from healthy controls also followed the same pattern and expressed almost 10 times lower levels of IL-4 in response to PHA stimulation when compared to the results published by Imada (1995), suggesting that this was likely caused by an interassay variation.

One of the indications of the impaired immune responses in HD patients is the lack of detectable serum Ab levels in response to vaccinations with various antigens. (Reviewed by Descamps-Latscha 1993) HD subjects in our study also were examined for their serum Ab levels after the hepatitis B vaccination and based on this criteria were divided into Responder and Non-responder groups. Interestingly, the level of PHA-induced IL-4 production by PBMCs from Non-responder subjects was significantly ($p<0.05$) higher, i.e. almost 4 times than those of PBMCs from responders. However, this pattern was not observed with either of other stimuli, i.e. anti-CD3, flu Ag and streptokinase, or other type 2 cytokines that were examined, i.e. IL-5 and IL-13. Taking together, these results suggest that in HD patients, production of IL-4, IL-5 and IL-13 may be via independent regulatory mechanisms.

Another aspect of the impaired immunity of patients with CRF is the suppressed *in vitro* T cell proliferation (Reviewed by Kelly). The results of our experiments confirm a decrease in cell proliferation in response to T cell mitogens and demonstrate that this holds true for specific antigens. However, simultaneous IL-12 treatment in addition to either of the stimuli did not have an enhancing effect on the *in vitro* proliferative responses of PBMCs. Hence, we conclude that the defective *in vitro* cell proliferation of HD patients may be either due to primary T cell abnormalities or secondary to the abscence of accessory signals for T cell activation.

## 2. Studies on Murine Immune Responses and GVHD

### 2.1. LPS-Induced TNF-α Release and Intestinal GVHD

The two different mechanisms of cell death that have been identified and compared with each other are necrosis and apoptosis. Necrosis is one of the mechanisms of cell death caused by non-physiological disturbances including Ab and complement as well as cytotoxic molecules such as granzymes, perforin (Kagi 1994) and nitric oxide (NO) (MacMicking 1997). Apoptosis, on the other hand, is a more recently described mechanism of cell death induced by physiological stimuli and characterized by non-random oligonuclesomal length fragmentation of DNA molecules (Thompson 1992). Moreover, the presence of a death domain on the cytoplasmic tail of both FAS and TNF-α receptor molecules (Smith 1994 and Hsu 1995) has been reported. More recent literature has suggested that both the upregulated expression of FAS ligand on activated T cells (Kagi 1994) and the increased TNF-α release by activated macrophages (Ruckdeschel 1998 and Penning 1998) play a role in the induction of apoptotic signals. Furthermore, In addition to the enhancing effect of IFN-γ on TNF-α production, it has been shown that IFN-γ enhances the expression of the FAS antigen (Sayama 1994).

In humans, acute graft-versus-host disease is one of the major causes of intestinal injury accompanied with the diffuse ulceration of the intestines following bone marrow transplantation (Spencer 1986). On the other hand it

has been shown that the anti-TNF treatment of the mice with acute GVHD, almost completely prevented the isolated enterocyte necrosis as well as other characteristic signs of intestinal pathology (Piguet 1987). Furthermore, the relation between local GVH reaction in the gastrointestinal tract and the LPS-induced TNF-α release has been well documented and different aspects of it reviewed separately by different investigators (Mowat 1997 and Nestel 1997). Our data also provides evidence for a strong relation between LPS and TNF-α release in mice with acute GVHD.

In our experiments, in addition to the relation between LPS and serum TNF-α, we were able to observe a relationship between the time intervals after the LPS treatment and the number of the apoptotic cells in the intestinal tissue of normal mice. However, in the experiments using mice with GVHD we could not distinguish this difference among intestinal segments from mice on day 8 or day 15 post-induction that did or did not receive systemic LPS treatment. Therefore, while there is clarity in our results visualizing the apoptotic cells in the intestinal tissues of mice with acute GVHD, there remains uncertainty regarding the inconsistent relationship between the LPS treatment and the observation of the apoptotic cells. However, the fact that apoptotic cells were detected in the intestinal segments of GVH mice that did not receive LPS treatment as well as those from LPS administered mice suggest that apoptosis in this group of mice may be induced by local cellular responses such as TNF-α production. In addition to this, inconsistency of the number of the apoptotic cells on the same tissue section may also be related

to the local GVH reaction expressed by macrophages and lymphocytes in the intestinal tissues. In some cases, we were not able to detect apoptosis in sectioins from same experiment in following Apoptag assays suggesting interassay variations caused by technical difficulties of the assay procedure.

### 2.2. LPS-Induced TNF-α Release Using IFN-γ or IL-12 *KO* Mice

A central role for IFN-γ in cell mediated immunity has been suggested by a number of immunologists and in particular, its characteristic role in the augmentation of macrophage activity has been reviewed and revealed in various studies (Nathan 1983 and Adams 1984). The results of our studies using IFN-γ *KO* mice to investigate the level of LPS-induced TNF-α in these mice, which was much lower than those of the wild type, argues for the priming role of this cytokine on macrophages to elicit TNF-α release upon LPS stimulation in this system.

On the other hand, the role of IL-12 in the activation of NK cells and thereby synthesis of IFN-γ that was discussed above, was also indirectly demonstrated in our experiments using IL-12 *KO* mice. The data from these experiments revealed a decreased level of LPS-induced TNF-α release by IL-12 *KO* mice that matched those of IFN-γ *KO* mice in comparison with the results of experiments with control mice. Collectively, these results suggest an indirect positive regulatory function for IL-12, i.e. probably through induction of IFN-γ, in the priming of macrophages to release TNF-α upon LPS stimulation.

2.3. IFN-γ Production and Proliferative Responses of *In Vitro* Model of GVHD

As the results of pervious *in vivo* experiments suggested splenic cells from NK cell depleted graft recipient $F_1$-hybrid mice produced lower levels of IFN-γ (Ellison 1998). Large granular lymphocytes known as NK cells express various membrane bound molecules such as ASGM1 and NK1.1 on their surface. Considering their cytokine production abilities, NK cells have been defined as powerful producers of IFN-γ (Trinchieri 1997). IFN-γ production by NK cells may be triggered by the cross-linkage of the NK1.1 surface molecules (Arase 1996). The data from our experiments on the *in vitro* model of GVH reaction, using NK cell-depleted responder cell populations, is somewhat parallel to these findings as well as previous MLR experiments(MacDonald 1993) as well as mitogen responsiveness of the spleen cell cultures from recipients of NK cell-depleted grafts (Ellison 1998) performed in our laboratory. Nevertheless, some parts of our results that can not be interpreted at present time may need further investigation. For instance, the proliferative responses of the NK cell depleted responder cells and one of the control responder cell population that was treated with complement alone matched those that were previously reported (MacDonald 1993). However, the two other control cell populations that were treated with different antibodies alone also had higher proliferative responses than the no-

treatment control group. Even though this may be attributed to a stimulatory effect of the Ab treatments on the cells, further experiments may give more secure interpretations for this observation. The results of *in vitro* IFN-γ production assays were also parallel to the proliferative responses and the same discussion applies for them as well,with the exception of the high level of IFN-γ in the cultures with anti-NK1.1 treated responder cells. Based on the suggestion made by Arase in 1996 that IFN-γ production by NK cells may be augmented upon the cross-linking of NK1.1 molecules, we may be able to relate the high level of IFN-γ in the anti-NK1.1 treatment culture to the cross-linkage of these molecules by the antibody. In fact these results However, the results obtained from the anti-ASGM1 alone or complement alone treatment groups can not be interpreted at this time.

## 3. Conclusion

Taking together, our results confirm a direct positive regulatory role for IL-12 in the augmentation of IFN-γ production in both human and murine systems as well as an indirect enhancing role in macrophage priming and in particular LPS-induced TNF-α release by macrophages.

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