

**THE IMPACT OF NITRIC OXIDE AND RECOMBINANT HUMAN KERATINOCYTE
GROWTH FACTOR ON THE PATHOGENESIS OF ACUTE GRAFT-VERSUS-HOST
DISEASE**

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

University of Manitoba

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ABSTRACT

When BDF1 mice are injected with lymphoid grafts from wild-type C57Bl/6 donor mice, acute, lethal graft-versus-host disease develops. Although GVHD injures many organs, the intestine is of particular interest since it is directly involved in the lethality of the disease. Apoptotic epithelial crypt cells form during the later "destructive" phase of intestinal GVHD, permitting endotoxin to enter the circulation. Studying the spontaneous development of the apoptotic intestinal lesions is difficult, firstly, because they appear shortly before death and, secondly, because the time at which individual mice succumb to the disease is variable. We have developed a means of studying the mechanism underlying intestinal lesion formation by injecting GVH mice with a small dose of LPS. Administration of as little as 10 μ g of endotoxin was lethal in BDF1 recipients of wild-type C57Bl/6 grafts. Such an injection of LPS induced apoptosis of the intestinal epithelial crypt cells and was responsible for the production of a burst of NO in the intestine. L-NAME and L-NIL, inhibitors of NO synthesis, blocked NO release and abrogated LPS-induced intestinal injury thus implicating NO as the principal mediator of the intestinal injury. Using IFN- γ gene knockout (gko) mice we were able to investigate the role of IFN- γ in the pathogenesis of intestinal GVHD. BDF1 recipients of IFN- γ gko grafts, in contrast to their wild-type graft recipient counterparts, did not die when injected with LPS. Neither LPS-induced NO production nor intestinal epithelial cell apoptosis was seen in recipients of IFN- γ gko grafts suggesting that donor-derived IFN- γ is necessary for the development of intestinal GVHD.

The effect of rHuKGF on the GVH reaction was studied in order to determine whether or not the growth factor could protect against lethal, acute GVHD in a Parent

into F₁-hybrid mouse model. Of particular interest was the ability for rHuKGF to prevent the intestinal injury that is seen in the GVH reaction. Mortality and weight loss were ameliorated in the rHuKGF-treated mice. These mice had elevated levels of NO and TNF- α in the intestine but intestinal lesions could not be detected. LPS levels remained statistically similar to those seen in control mice that were not suffering from acute GVHD. Despite augmenting NO and TNF- α levels, LPS administration failed to induce the formation of the apoptotic intestinal lesions that are normally seen in LPS-treated mice with aGVHD. This indicates that rHuKGF can protect the intestine from the NO-mediated damage that occurs when LPS is present. IFN- γ may be responsible for suppressing KGFR expression in the intestine of mice suffering from aGVHD as receptor levels are reduced on day 8 when IFN- γ cytokine levels are at their highest. This reduction in KGFR expression was not seen in recipients of IFN- γ gko where IFN- γ is virtually absent from the GVH reaction. Interestingly, treatment with rHuKGF redirected the cytokine response seen in mice with acute GVHD from a Th1 response to a mixed Th1 and Th2 pattern. IFN- γ levels were reduced in mice that received KGF treatments when compared to their untreated, GVH counterparts. Histological findings suggested that rHuKGF-treated GVH mice were beginning to suffer from chronic GVHD indicating that rHuKGF may play an immunoregulatory role in acute GVHD.

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ABBREVIATIONS

AGVHD; acute graft-versus-host disease

BDF₁; (C57BL/6 x DBA/2)F₁

BMT; bone marrow transplantation

BSA; bovine serum albumin

CGVHD; chronic graft-versus-host disease

CNOS; constitutive nitric oxide synthase

CPM; counts per minute

CTL; cytotoxic T lymphocyte

DETC; diethyldithiocarbamate

DNA; deoxyribonucleic acid

ELISA; enzyme linked immunosorbent assay

ENOS; endothelial nitric oxide synthase

EPR; electron paramagnetic resonance

FasL; Fas ligand

FCS; fetal calf serum

FGF; fibroblast growth factor

G; gauss

GI; gastrointestinal

GKO; gene knockout

GVH; graft-versus-host

GVHD; graft-versus-host disease

GVL; graft-versus-leukemia

HBSS; Hank's balanced salt solution

HLA; human leukocyte antigen

IBMTR; international bone marrow transplant registry

IEL; intraepithelial lymphocytes

IFN interferon

IL; interleukin

INOS; inducible nitric oxide synthase

KGF; keratinocyte growth factor

KGFR; keratinocyte growth factor receptor

L-NAME; N^oNitro L-arginine methyl ester

L-NIL; N⁶-(1-Iminoethyl)-L-lysine

LAL; limulus amebocyte lysate

LBP; LPS-binding protein

LPS; lipopolysaccharide

MHC; major histocompatibility complex

MLR; mixed lymphocyte reaction

ND; not determined

NK; natural killer

NMMA; N^G-mono-methyl-L-arginine

NNOS; neuronal nitric oxide synthase

NO; nitric oxide

NOS; nitric oxide synthase

PBS; phosphate buffered saline

pNA; p=nitroaniline

RHuKGF; recombinant human keratinocyte growth factor

RPA; ribonuclease protection assay

TCR; T cell receptor

TLR; toll-like receptor

TNF; tumor necrosis factor

TNFR; tumor necrosis factor receptor

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CHAPTER 1
LITERATURE REVIEW

1.1 Introduction:

Many years have passed since the first pioneers in the field offered proof that it would be possible to reconstitute diseased bone marrow with a marrow transplant. Since then, allogeneic bone marrow transplantation (BMT) has become a common clinical approach for treating hematological disorders. Today, BMT is frequently used to treat patients with malignant diseases such as leukemia, lymphoma, myeloma, and some solid tumors. Non-malignant diseases such as aplastic anemia, thalassemia major, immune deficiencies and autoimmunity, and a variety of inherited disorders also require BMT as part of their treatment protocols.^{1,2}

The International Bone Marrow Transplant Registry (IBMTR) estimated that 40,000 to 50,000 hematopoietic stem cell transplants were performed in 2001. Despite the therapeutic potential for BMT, a variety of limitations, such as GVHD, still hinder overall transplant success.

According to the IBMTR, the 100-day mortality rate after transplant ranged from 10% to 40% when human leukocyte antigen (HLA)-identical siblings were used as donors. Approximately 20% of these deaths were due to GVHD.¹ Unfortunately, only 30% of transplant recipients have an HLA-identical sibling necessitating the use of partially-matched, related or completely unrelated donors for transplantation. Associated with the use of these types of donors, however, is a higher incidence of mortality post-BMT. The incidence of GVHD in such cases can reach as high as 90% depending on the number of risk factors involved.¹⁻³ Transplant related mortality and GVHD also remain a serious risk to a large number of patients aged 50 or older and to patients for whom a matched donor cannot be found. Although vast improvements in transplant outcome have

been seen in recent years, GVHD remains as a formidable opponent, impeding the cure for hematological disease.

1.2 Historical overview of GVHD

James B. Murphy was the first to record his observations of the GVH reaction, although he failed to correctly interpret the immunological significance of his findings. Murphy made his unexpected discovery while working on a control experiment for his cancer research. In his experiment, he placed fragments of rat tumor and pieces of normal adult chicken organ on the chorioallantoic membrane of seven-day old chick embryos. As a control, he used the adult chicken organ pieces alone. When the embryos were examined 11 days later, Murphy noticed several changes in the control eggs. He described small nodules with white centres scattered throughout the various membranes of the embryo as well as on the spleen, on the skin and in the subcutaneous tissue. The spleens in these engrafted embryos were also found to be greatly enlarged. Murphy noted that only certain tissues were capable of inducing these changes. Notably, spleen and bone marrow induced these effects while liver and kidney produced minor changes. Bone, muscle and cartilage however had no effect on the chick embryos.

Murphy erroneously concluded that his findings were the result of a stimulation factor present in the transplanted adult tissues that could induce growth in the immature embryo. That he was actually observing a GVH reaction evaded his thoughts.⁴

1.2.1 The discovery of the GVH reaction:

Morten Simonsen was the first to present the idea of the GVH reaction.⁵ This was based on histological evidence that arose from his work with allografted kidneys in dogs. Examination of the allogeneic kidney transplants, stained with Unna-Papenheim stain revealed the presence of many pyroninophilic cells classified as “reticulo-endothelial” cells in the interstitial tissue. Simonsen believed these pyroninophilic cells to be of local origin and represented the response of the transplanted kidney to recipient-specific antigen.

Dempster, a contemporary of Simonsen’s, also became convinced of this graft-versus-host concept based on the data from his own experimentation.⁶

Convincing evidence of the GVH reaction came a little later as a result of work based on tolerance induction. Billingham, Brent and Medawar, who were studying the induction of tolerance at the time, found that they could induce tolerance to future skin grafting in rodents and chickens by intravenously injecting them as embryos with cells from an unrelated adult donor. This procedure however was fraught with difficulty as many of the embryos died shortly after birth.⁷⁻¹⁰

Simonsen’s work had become highly influenced by that of Billingham, Brent and Medawar and, after studying this form of immunological tolerance for several years himself, Simonsen realized that he now had a means with which to test his hypothesis that grafted cells could indeed react against the host.¹¹ By 1957 Simonsen published his findings and showed that chick embryos, injected with adult chicken cells from either spleen or peripheral blood, showed splenomegaly and usually ended in the death of the

newborn chicks a few weeks after hatching. He obtained similar results using mice provided that an allogeneic donor strain was used.⁹

Billingham and Brent followed Simonsen's publication shortly thereafter with their description of a syndrome they called runt disease. This syndrome developed in immunologically immature neonatal mice injected with genetically different adult cells. Billingham had observed this syndrome while continuing his work on tolerance. He discovered that not all strain combinations resulted in the induction of healthy, tolerant newborn mice. Billingham noted that while certain strain combinations resulted in mortality rates of 100%, other combinations showed less severe signs of disease.¹² He and his colleague Brent went on to study and describe this disease in more detail.¹³

Through his experiments, Simonsen was the first to describe the necessary conditions for the development of a GVH reaction and he noted that the disease would only arise if these conditions were met.⁹ Billingham later presented these findings in his 1966 Harvey Lecture.

- 1) The graft must contain immunocompetent cells.
- 2) The host must possess antigens that appear foreign to the donor.
- 3) The host must be incapable of reacting immunologically against the graft.¹¹

Another key finding of Simonsen's 1957 paper was the observation that an injection consisting of only the leukocyte fraction of peripheral blood could cause the same reaction that he had seen previously when using spleen cells. Further investigation

into identifying the cell responsible for the GVH reaction established that it was indeed the lymphocyte that was responsible for the GVH effect.¹³⁻¹⁷

1.3 Experimental models to study GVHD

1.3.1 Runt Disease:

While attempting to elucidate the mechanisms of tolerance, Billingham and Brent stumbled upon a lethal disease in newborn mice. They injected suspensions of spleen cells from adult mice into genetically different strains of newborn mice. The expectation was that the newborns would be rendered tolerant to future skin grafts from the original donor strain of mice. The results, however, were unanticipated as certain strain combinations developed a disease that culminated in the death of the neonates. This observation was named Runt disease; a descriptive term based on the retarded rate of growth seen in the affected newborn mice. They determined that the injected spleen cells reacted against the immunologically immature neonates and caused the wasting and death of the animals. Other characteristics associated with this Runt disease were abnormalities in the skin, splenomegaly, thymic involution, anemia, and lymphocytic infiltration into the portal spaces of the liver.¹³

1.3.2 Homologous Disease:

Homologous disease, also known as secondary disease, is used to describe the GVH reaction that results from a post-irradiation transplant of bone marrow or spleen cells. Such treatment results in protection from otherwise lethal doses of irradiation as the graft re-populates the host's hematopoietic tissue.¹⁸ The obvious flaw in this type of

treatment is that mortality can follow the initial protective period due to the development of GVHD. It was Trentin who suggested that the irradiation itself was essential to the progression of the disease because it rendered the recipient tolerant to the donor cells.¹⁹ The donor cells would then be free to react against host tissue antigens, unhindered by any host immune response since this had been wiped out by the irradiation treatment. Trentin also identified the symptoms of this secondary disease with those seen by Billingham in neonatally injected mice.^{18,19}

Use of radiation treatment on mice prior to bone marrow transplantation as a model for studying GVHD does closely resemble clinical GVHD, however any experimental results must be carefully interpreted in order to discern the effects of the GVH reaction from the antecedent irradiation.

1.3.3 F₁-hybrid Disease:

The breeding of two different strains of inbred mice will result in an F₁-hybrid. F₁-hybrid disease results from an injection of parental strain cells into an unirradiated F₁-hybrid. The basis for F₁-hybrid disease rests in the genetics of the major histocompatibility complex (MHC) in which the alleles inherited from both parents are co-dominantly expressed. Because, an F₁-hybrid mouse, is a cross between two inbred parental mice, it will express the MHC antigens from both parents. Due to the presence of a common antigen, the F₁-hybrid will be unable to mount any response against cells from either parental strain. The same is not true however when cells from either parent are injected into the F₁-hybrid, provided that the parents each express a different haplotype.

The donor lymphocytes from one parent will recognize this MHC antigen from the opposite parent as foreign and react against it.

The F₁-hybrid model holds several advantages over other murine models when studying the GVH reaction. Firstly, it is a clean model allowing for the direct interpretation of the results. This contrasts with homologous disease where the effects of the GVH reaction must be differentiated from the radiation and immunosuppressive pre-conditioning regimens. In addition, the F₁-hybrids can be irradiated in subsequent experiments to more closely resemble clinical GVHD. Another advantage of this model is that it allows for a study of the different MHC disparities. The use of various Parent into F₁-hybrid strain combinations can be used to determine how the GVH reaction changes with respect to different MHC incompatibilities.

1.3.4 Parabiosis Intoxication:

Parabiosis intoxication experiments require the surgical union of the circulatory systems of two different animals. Depending on the histocompatibility between the two animals, a GVH reaction may result, with one of the partners developing a lethal, wasting syndrome.²⁰ The most commonly used pair in this type of experiment is the joining of an F₁-hybrid with one of its parents. This ensures that the reaction can only proceed in one direction. The use of such a technique to study GVHD however is uncommon due to the complex nature of the procedure.

1.4 The role of the MHC in the GVH reaction

The existence of the MHC (known as H-2 in mice) and its importance to transplantation can be traced back to the 1930s when work by both Gorer and Snell led to the demonstration that tumor immunity and skin graft rejection are associated with certain genetic loci.²¹ As their importance to tissue rejection became clear, these loci began to be referred to as histocompatibility genes. Today, they are collectively known as the MHC.

The MHC is essential to the functioning of the immune system and T cell activation. The MHC is involved in antigen presentation and aids the immune system in the proper identification and destruction of foreign invaders and in distinguishing pathogens from normal host tissue. Class I MHC antigens can be found on the surface of all nucleated cells in the body and they are responsible for the typical host-versus-graft transplantation reaction. Rejection occurs when the recipient's immune system recognizes the histocompatibility antigens on the donated organ as foreign and initiates an immune response against them. When immunologically competent cells are present in the graft, the immune response can occur in the opposite direction and may potentially result in GVHD. The severity of the GVH reaction varies with the degree of histoincompatibility between donor and host. Lethal GVHD can result from both class I and class II differences. It has, however, been noted that fewer cells seem to be necessary to establish GVHD in mouse strains differing at the class II region.²²

1.4.1 Responses to class I and class II differences:

It is well known that CD8⁺ T cells recognize MHC class I antigens and that CD4⁺ T cells recognize class II antigens. Korngold and Sprent analyzed these two subsets of T cells for their ability to cause GVHD. They found that lethal GVHD occurred in MHC class I mismatched strains only when unseparated or Lyt-2⁺ (CD8⁺) killer T cells were used. In class II disparate strains, unseparated or L3T4⁺ (CD4⁺) helper T cells lead to fatal GVHD. Finally, in the case of a full mismatch, either set of T cells could cause lethal GVHD.²³

CD4⁺ and CD8⁺ T cells possess distinct functional properties suggesting that the different subsets may be responsible for causing different forms of GVHD. Rolink *et al.* worked to ascertain the mechanism behind the development of an acute or of a chronic GVH response believing that the different subsets of T cells were responsible for causing the different forms of GVHD. They first looked at whether GVHD resulting from either class I, class II, or both class I and class II differences would selectively activate different subsets of T cells; thus promoting either acute or chronic GVHD. Their experiments demonstrated that when class II disparate strain combinations were used, the resulting reaction developed into a chronic GVHD. Class I differences alone could not clearly cause either form of GVHD and both class I and class II differences were necessary for the development of acute GVHD²⁴ However, in another series of experiments, using a different class I and class II disparate model, these same investigators showed that depletion of the Lyt2⁺ cells from the graft could result in chronic GVHD but both T cell subsets needed to be present for acute GVHD to develop. The presence of Lyt2⁺ T cells alone in the graft could not induce either form of GVHD.²⁵ This seems to indicate that

class I differences and Lyt2⁺ cells are necessary for the development of acute GVHD provided that Lyt1⁺/Lyt2⁻ T cells are present to provide initial help. These Lyt1⁺/Lyt2⁻ T helper cells also appear to be capable of reacting to class II differences all by themselves resulting in chronic GVHD.

These findings contrast somewhat with the work done by Korngold and Sprent who demonstrated that Lyt2⁺ T cells alone could cause lethal GVHD in certain strain combinations. Since their model used irradiated recipients, they were careful to point out that distinct patterns of GVHD may develop when host hematopoietic cells remain present in the recipient, as is the case for Rolink's work. Korngold and Sprent also emphasized that functional overlap exists between these two subsets of T cells with each showing signs of both cytolytic activity and lymphokine production.²⁶

1.4.2 The Importance of Minor H-2 Antigens:

The importance of HLA-matching, to the bone marrow transplant recipient, is now clear. However, despite efforts to achieve such donor/recipient compatibility, a high incidence of GVHD still occurs. This type of GVHD is directed against non-HLA differences, or minor histocompatibility antigen disparities, between the donor and the recipient. These minor histocompatibility antigens are thought to consist of small peptides encoded by genes scattered throughout the genome. They are MHC-restricted and only T cells have been shown to respond to them.²⁷⁻³¹

Using an irradiated H-2 compatible mouse model that differed at multiple minor histocompatibility antigen loci, Korngold and Sprent assessed the ability for different subsets of T cells to mediate a GVH reaction to these minor antigens. They demonstrated

that the cells that initiate GVHD to minor histocompatibility antigens have a class I restricted phenotype. Elimination of the cytotoxic T cells from the graft resulted in a failure to cause GVHD. On the other hand, removal of the T helper cell population had little effect as lethal GVHD still developed. These results suggested to the authors that the T-helper cell population does not contribute to GVHD against minor histocompatibility antigens.²⁸ However, in later experiments, these authors reported that L3T4⁺ T cells could participate in lethal GVHD to minor histocompatibility antigens when a different strain combination was used.³² Hamilton also showed in three different strain combinations that L3T4⁺ cells either participated in or were the dominant population of cells that initiated GVHD to minor histocompatibility antigens.³³

While multiple minor histocompatibility antigen differences can result in lethal GVHD, Rappaport demonstrated that chronic GVHD might also develop. Injection of B10.D2 cells into (DBA/2/B10.D2) F₁-hybrids resulted in widespread tissue injury, lymphocytic infiltration, scleroderma and glomerulonephritis, all features of chronic GVHD.³⁴ It therefore seems that, much like GVHD to major H-2 antigens, minor H-2 differences can elicit a variety of GVH responses depending on the strain combination used. One possible explanation for this occurrence is the theory of immunodominance. This theory stems from the observation that despite there being a large number of minor histocompatibility antigen genes, only a limited number are recognized by T cells. Those minor histocompatibility antigen capable of stimulating a response are referred to as immunodominant.^{30,35,36} Why certain minor histocompatibility antigens are dominant over others has not yet been elucidated. Several theories, such as, peptide binding

affinity, T cell repertoire and tissue distribution have been put forth in an attempt to explain this phenomenon.³⁶

1.5 Clinical bone marrow transplantation and GVHD

1.5.1 Preparation of the bone marrow graft:

One of the first human bone marrow transplants was performed by Mathé when he attempted to rescue six victims of an irradiation accident.³⁷ Such early trials were fraught with difficulties as most recipients of marrow grafts were terminally ill and did not live long enough to have proper graft evaluation. Those few who were successfully engrafted succumbed to the lethal effects of GVHD.³⁸ Today, BMT is used to treat a variety of malignant and non-malignant hematological diseases. Based on animal experimentation indicating that MHC mismatching often resulted in lethal GVHD, HLA matching in humans became an important selection criterion for choosing the proper donor. The ideal candidate for marrow transplantation would be an identical twin who would match at all genetic loci. Since most patients requiring BMT do not have such a syngeneic sibling, HLA-typing has become an important test to determine the degree of compatibility between donor and recipient. The most critical loci to match seem to be the HLA-A, HLA-B and HLA-D genes.^{30,39-43} Serotyping is used to determine the identity of the HLA-A and HLA-B haplotypes in both the donor and the recipient. HLA-D matching is ascertained by mixed leukocyte culture where a lack of reactivity is indicative of a match.^{41,44} Matching with a sibling or other family member is preferable to an unrelated donor since the minor histocompatibility antigens are also more likely to match.^{40,45,46}

DNA based methods are sometimes used as a more sensitive approach to typing and have been correlated with improved transplant outcome.^{41,47,48}

Once a suitable donor has been chosen, the marrow is usually harvested from the iliac crest of the pelvis or from the sternum by multiple sterile aspirations.^{44,49}

Unless the graft is completely syngeneic, the recipient must undergo some form of immunosuppressive treatment in order to avoid rejection.^{40,50,51} Patients who have malignant disorders become immunosuppressed as a side-effect of their treatment regimen that is designed to kill the cancerous cells. Often, recipients undergo total-body irradiation and receive a mixture of chemotherapeutic drugs to ensure for maximum cytoreduction of the cancerous cells as well as proper graft acceptance.^{38,44} Recipients of marrow grafts for non-malignant conditions receive similar types of treatments prior to the bone marrow transplant that may vary slightly depending on the hematological disorder. High-dose cyclophosphamide is commonly used as a conditioning regimen with the administration of additional drugs and radiation therapy if the recipient has been previously sensitized to the donor. Such prior sensitization often results from blood transfusions and pregnancy.^{52,53} The recipient can now be intravenously infused with the collected marrow cells.⁴⁴

As the pre-conditioning regimen ensures for proper engraftment, GVHD is the next major obstacle to the success of allogeneic BMT.

1.5.2 Attempts to prevent GVHD:

Since the initiation of GVHD is mediated primarily by donor T cells⁵⁴⁻⁵⁷, investigators sought to eliminate them from the graft. This met with some success in

murine models.^{58,59} Results from human trials revealed a reduced incidence of GVHD, but new complications developed as the marrow failed to engraft⁶⁰⁻⁶³ and leukemic relapse increased.⁶⁴⁻⁶⁶

One known benefit of GVHD is the graft-versus-leukemia effect (GVL). In GVL, the donor cells appear to eradicate any residual cancerous cells that survived the initial chemoradiotherapy, thus achieving complete remission of the malignant insult. With the discovery of immunodominant minor histocompatibility antigens, investigations have begun adoptive immunotherapy experiments that target the minor histocompatibility antigen present on the cancerous cells. Such treatments transfer T cells specific for certain minor histocompatibility antigens to the patient in an attempt to eradicate any remaining cancer cells. The obvious risk associated with such a treatment is GVHD. In order to avoid this, immunodominant minor histocompatibility antigens would have to be identified and found distributed in such a way that their recognition would result in GVL and not GVHD. It has been suggested that such difficulties may be overcome by targeting the minor histocompatibility antigens on hematopoietic cells since the main target for GVHD are epithelial cells.⁶⁷ More importantly, minor histocompatibility antigens have been detected on leukemic cells of both myeloid and lymphoid origin indicating a potential treatment for both types of leukemia.³⁶ Supporting this is a study of such an effect in mice that observed T cells, targeted to such antigens with wide tissue distributions, causing GVL and not GVHD. They proposed that single minor histocompatibility antigen differences rarely cause GVHD as multiple incompatibilities are necessary for GVHD to occur.⁶⁸ Despite its far-reaching therapeutic potential as a

cancer treatment, investigators have so far been unable to fully separate the GVL effect from GVHD.^{69,70}

Since a little GVHD appears to be a good thing, current treatments attempt to moderate the severity and prevent its lethal outcome without completely eliminating the reaction. A number of methods are presently used that modulate the immune response to limit the GVH reaction. The administration of many of these immunosuppressive therapies must begin before any symptoms of GVHD become apparent.^{52,71} Several of these prophylactic agents include cyclosporine and methotrexate for the prevention of GVHD and corticosteroids to treat the disease. Although individually, each has been shown to be effective in modulating GVHD, various combinations are employed in an attempt to improve their efficacy.⁷²⁻⁷⁶ Many other agents, such as FK 506, are also being used clinically since the effectiveness of methotrexate and cyclosporine is limited in recipients of grafts from unrelated donors.⁷⁷ As is the case with many drug therapies, use of these agents have been commonly associated with the development of hypertension, liver and renal toxicity.⁷⁸⁻⁸⁰

Treatment with these immunosuppressive agents, while allowing engraftment and preventing lethal GVHD, leaves the patient susceptible to opportunistic infections by viral, fungal and bacterial organisms.^{53 44} Even though the attempts to control GVHD are being met with some success, there are still a large number of obstacles that require further investigation.

1.5.3 Acute GVHD

Clinically, GVHD is classified as being acute when it develops within the first one hundred days following an allogeneic bone marrow transplant. Acute GVHD (aGVHD) arises when donor T cells, present in the graft, recognize foreign histocompatibility antigens on host tissues and become activated. These T cells are then either directly or indirectly involved in producing the symptoms associated with aGVHD.

Many studies have been done to ascertain the parameters that would potentially increase the risk of developing aGVHD. As mentioned earlier, the most important parameter to control is the HLA compatibility between the donor and the recipient. However, both age and sex have been associated with the development of aGVHD. Young patients, age twenty or less, have a low incidence of severe GVHD. As the age of the patient increases, the frequency of lethal GVHD also rises to a point where allogeneic BMT is not recommended in elderly patients.^{76,81-83} Gender matching of the donor and the recipient has also been associated with decreasing the potential risk of serious aGVHD.⁸²⁻⁸⁴ In particular, it has been shown that the male Y chromosome encodes a powerful minor histocompatibility antigen. This increases the risk for male recipients of female grafts.⁸⁵ A history of transfusions and/or pregnancy for the donor also increases the potential for developing aGVHD due to the allosensitization that may occur during such events.^{85,86}

The three major targeted organs in aGVHD are the skin, the gastrointestinal tract and the liver. Coincidentally, symptoms of aGVHD include skin rash, jaundice, wasting and diarrhea. Clinically, these three organs are assessed for signs of disease and are assigned a representative grade from zero (no GVHD) to four (life-threatening GVHD).

The grade increases as the severity of the disease and organ involvement increases and as the outlook for the patient becomes less favourable.^{71,87}

1.5.3.1 The Skin:

GVHD first manifests itself in the skin as a maculopapular rash that must be verified by histology in order to distinguish it from rashes that are drug related or virally induced. "Satellite cell dyskeratosis" is a characteristic feature seen histologically and consists of an apoptotic keratinocyte surrounded by several lymphocytes. The rash itself is distributed throughout the body, initially on the palms, soles and ears but later also on the trunk and face. Hair follicles may also be targeted. In severe cases, bullae will form and develop into epidermal necrolysis over most of the body. The onset of such events usually fall within the first month after transplantation. However, in hyperacute cases, desquamation of the skin may develop as early as seven days post-transplant.^{40,88-90}

1.5.3.2 Liver:

Following the involvement of the skin, the liver is often the second organ to show signs of GVHD. The liver disease is associated with a rise in both serum bilirubin levels and in other liver enzymes such as alkaline phosphatase and transaminase. Jaundice is evident in severe cases. This kind of liver dysfunction is non-specific and may be caused by other common disorders associated with BMT such as venoocclusive disease, infection and drug-induced hepatitis. As is the case with the skin, histology must be used to confirm the presence of GVHD. Donor lymphocytes can be seen in the small bile ducts in close proximity to the ductal epithelial cells. These infiltrates are responsible for the

exfoliation of necrotic epithelial cells into the duct lumen. Those epithelial cells that remain appear “squamous-like” giving the ductal epithelium a withered look. Hepatocytes may also be attacked resulting in hepatitis-like necrosis of the liver. Such extensive bile duct injury and loss is highly suggestive of GVHD.^{40,88,90,91}

1.5.3.3 Gastrointestinal Tract:

One of the most characteristic signs of GVHD is diarrhea. Such diarrhea is initially watery and voluminous with blood appearing as the damage to the gut becomes more extensive. This may be accompanied by abdominal pain, nausea and vomiting as well as paralytic ileus and anorexia. Since diarrhea can also be a complication of chemotherapy and infections, histological confirmation of GVHD in the gastrointestinal tract is also needed. Lesions can be seen throughout the gut and are characterized by crypt epithelial cell apoptosis as these cells are the primary targets of GVHD in the intestines.^{40,92}

According to Mowat, the injury that occurs in the intestine is associated with three distinct phases. The first is known as the “proliferative” phase whereby increased epithelial cell turnover leads to crypt hypertrophy as the rate at which the enterocytes migrate from the crypts to the tips of the villi increases. Lymphocytes can also be seen infiltrating into the epithelium.

In more severe cases of GVHD, the enteropathy may progress into a second “destructive” phase. Here, extensive villus damage is seen as crypt cell hyperplasia and crypt stem cell necrosis and apoptosis promote intestinal destruction. The infiltrating lymphocytes are lost and inflammatory cells can now be seen in the lesion.

Under certain conditions the third “atrophic” phase may develop. Here, the lesion consists of a thinning and shortening of the crypts and villi as well as a complete absence of all lymphoid cells.⁹²

Such alterations in the crypts can be responsible for the diarrhea seen in GVHD. The more rapid migration of cells from the crypts results in functionally immature epithelial cells at the villus tips. These cells lack the microvilli and functional enzymes needed for proper water absorption in the intestine. Villus atrophy results in further water loss as the absorptive surface area becomes reduced. Poor sugar absorption by the damaged intestine may also contribute to the diarrhea by osmotic water loss.

Damage to the gastrointestinal tract plays a major amplificatory role in the pathogenesis of aGVHD. Such amplification occurs when LPS, a normal constituent of the gut flora, passes through the injured intestinal tract to the circulation. There, it is responsible for triggering a variety of cellular and inflammatory effector mechanisms that further damage the gut and ultimately lead to septic shock and death.

1.5.4 Chronic GVHD

Chronic GVHD (cGVHD) presents later than aGVHD and has a clinical benchmark of developing one hundred days or more post-transplant. Clinically, cGVHD has occurred after partial or complete resolution of aGVHD and may also develop when there is no prior evidence of aGVHD. As is the case for the acute disease, T cells are responsible for inducing cGVHD. These T cells, however, produce IL-4 and IL-10 with little IFN- γ being detected. For this reason, cGVHD is associated with having a Th-2-like cytokine profile.⁹³

Autoreactive T cells are responsible for much of the pathology that occurs in cGVHD. This may, in part, be due to thymic dysregulation brought about by the GVH reaction or the involution that results with aging. Certain risk factors have been associated with the development of cGVHD. Most importantly is a history of aGVHD. Grafts from female donors given to male recipients as well as increasing age of the recipient also put patients at a higher risk for cGVHD.^{94,95}

Chronic GVHD manifests itself as a multi-organ disease with many autoimmune-like features. The most commonly affected organ in cGVHD is the skin. This injury is characterized by patchy dyspigmentation and Lichen planus-like papules. Fibrosis develops and the skin becomes indurated and attaches to the underlying fascia. In the end, fibrosis causes the skin to thicken and harden and joint contractures limit movement, much like what is seen in scleroderma.⁹⁶ Often, severe dryness of the eyes and mouth is described as lymphocytes infiltrate the salivary gland producing a syndrome similar to Sjogren's.⁹⁷ Injuries characteristic of primary biliary cirrhosis also occur as the bile ducts become damaged by lymphocytes causing fibrosis of the liver.⁹⁸ B cell dysregulation results in the production of autoantibodies and, in particular, antibodies to nuclear antigen and anti-double stranded DNA antibodies. These autoantibodies are generally of the IgG class which deposit on the basement membrane and lead to immunocomplex glomerulonephritis. Proteinuria and ascites develop. These symptoms greatly resemble what is seen in systemic lupus erythematosus.⁹⁹ The gastrointestinal tract and the lungs are also targeted in cGVHD with fibrosis commonly developing in these organs.

Diagnosis of cGVHD follows from histological evidence using skin and oral biopsies. Other organs can also be examined and generally a score is assigned related to

the severity of the disease. Like aGVHD, many prophylactic and immunosuppressive treatments have been used in an attempt to limit the severity of cGVHD and the mortality associated with it. A risk of infection is also high in these patients.^{87,96}

1.6 Pathogenesis of acute GVHD

1.6.1 Initiation and Propagation:

Donor T cells initiate GVHD in response to alloantigen where either recipient MHC-bound peptides or the recipient MHC itself appears foreign.^{30,40,54-57} These T cells undergo maximal proliferation and secrete large amounts of IL-2 within the first few days post-induction.¹⁰⁰ This production of IL-2 is necessary for the expansion and maturation of donor T cells.^{30,40,100,101} These early T cells also produce the Th2 cytokines IL-4 and IL-10 during the initial stages of the GVH reaction and B cells become activated. At this stage, acute and chronic GVHD cannot be distinguished from one another. The presence of donor CD8⁺ T cells and the ability for CD4⁺ and CD8⁺ T cells to produce IFN- γ determine whether or not the GVH reaction will progress into an acute or a chronic form.¹⁰² If IFN- γ is not produced, the GVH reaction will continue on a Th2 cytokine-dominated course resulting in chronic GVHD.^{93,103,104} The exact mechanism by which the switch to a Th1 dominated response occurs is not known. However, when acute GVHD does develop, IFN- γ can be detected early in the response and is seen to peak around day 8.^{30,105,106} Our lab proposed that natural killer (NK) cells are instrumental to the development of Th1-mediated acute GVHD.¹⁰⁵

The importance of IFN- γ to the progression of an acute GVH reaction is demonstrated by a study where neutralizing antibodies to this cytokine were

administered. In this investigation, mice that received anti-IFN- γ antibody treatments did not develop the severe enteropathy normally associated with the disease. These mice were also protected from the weight loss and mortality normally seen in the model.¹⁰⁷ In our own studies, we have similarly found that removal of donor-derived IFN- γ from a mouse model of aGVHD results in a disease that closely resembles cGVHD.^{103,104} The activated T cells secrete IFN- γ which primes and activates macrophages.^{30,102,108} It has been suggested that some of the pathology seen in aGVHD is a direct result of the ability for IFN- γ to prime macrophages. This priming results in the release of a large amount of TNF- α upon stimulation with LPS.¹⁰⁹ IFN- γ may also increase the susceptibility of the tissues to attack as this cytokine has been shown to have the ability to up-regulate the expression of MHC class I and class II molecules as well as orchestrate the trafficking of immune cells to the site of inflammation.^{30,110-112}

Macrophages are key to the GVH reaction for several reasons. As the GVH reaction progresses, T and B cells become immunosuppressed whereas macrophages remain activated.¹¹³ These activated macrophages produce IL-12 which maintains the Th1 immune response by sustaining IFN- γ production by NK cells.^{114,115} If IL-12 is removed from the reaction using IL-12 p40 gene knockout mice or antibodies, the severity of the GVH reaction lessens.¹¹⁶ NK cells respond to the IL-12 by producing IFN- γ creating a positive feedback loop with the activated macrophages and enhancing their primed state. Removal of the NK cells from the graft protects against the mortality associated with acute GVHD and also prevents macrophages from becoming primed.¹⁰⁵

As all of these interactions are occurring, the permeability of the intestine increases¹¹⁷ and LPS begins to leak slowly from the lumen of the intestine.¹¹⁸

1.6.2 Endotoxin and GVHD:

Endotoxin is a component of gram-negative bacteria and is found in the bacterial cell wall. It is composed of protein, lipid, known as lipid A, and a polysaccharide moiety. LPS consists of the lipid A and the polysaccharide components of endotoxin and is responsible for a variety of effects in mammals. These include non-specific activation of the immune system, activation of the complement cascade and blood clotting and septic shock.¹¹⁹⁻¹²⁴

Endotoxin had long been known to be an important component in the pathogenesis of GVHD. Germfree mice housed in pathogen-free conditions are generally unaffected by the effects of secondary disease when irradiated and given bone marrow grafts.¹²⁵ Furthermore, the use of a competitive inhibitor to LPS for six days following allogeneic BMT results in a decrease in the intestinal pathology and improves survival in a mouse model of aGVHD.¹²⁶ Studies of this nature have provided a foundation for clinical trial work where intestinal bacteria are eliminated from the allogeneic BMT recipient patient by an anti-microbial treatment. These clinical trials have also demonstrated that intestinal decontamination results in a significant reduction in the severity of aGVHD.^{127,128}

The ability for LPS to behave as an adjuvant has been known for many years. Its mode of action is thought to be mainly through macrophage activation and polyclonal B cell stimulation.^{111,119,129} T cell responsiveness in MLR can also be enhanced by LPS. Although there have been a few instances where LPS has been shown to directly stimulate various subsets of T cells,¹³⁰⁻¹³² it is generally thought that LPS affects T cells in an indirect manner. This mechanism is believed to proceed through LPS-stimulated

macrophages that are induced to release cytokines that can affect T cell activation and proliferation.^{119,133} LPS is recognized by macrophages with their pattern recognition receptors. These receptors are found on the surface of cells of the immune system and are important for the detection of LPS as they recognize conserved molecular motifs on pathogens. CD14, LPS-binding protein (LBP) and Toll-like receptor 4 (TLR4) are three such molecules that interact to signal the presence of LPS. LBP has binding specificity for the lipid A moiety of LPS. Once formed, the LBP-LPS complex is recognized by CD14 on the surface of macrophage and LPS gets transferred to the CD14 receptor.^{123,134} It has been suggested that the binding of LPS to CD14 allows for further associations within the plasma membrane. CD14 interacts with TLR4 and initiates a signal transduction pathway with the help of adaptor protein MD-2.^{123,135} Ultimately, this results in the release of NF- κ B from its inhibitor where it is now free to induce transcription of a variety of genes important to the inflammatory and immune responses. These macrophages will now secrete cytokines that further affect macrophage function or that can regulate lymphocytes through activation and suppression. In this way, LPS is a potent stimulator of macrophage and the immune system.^{122-124,136}

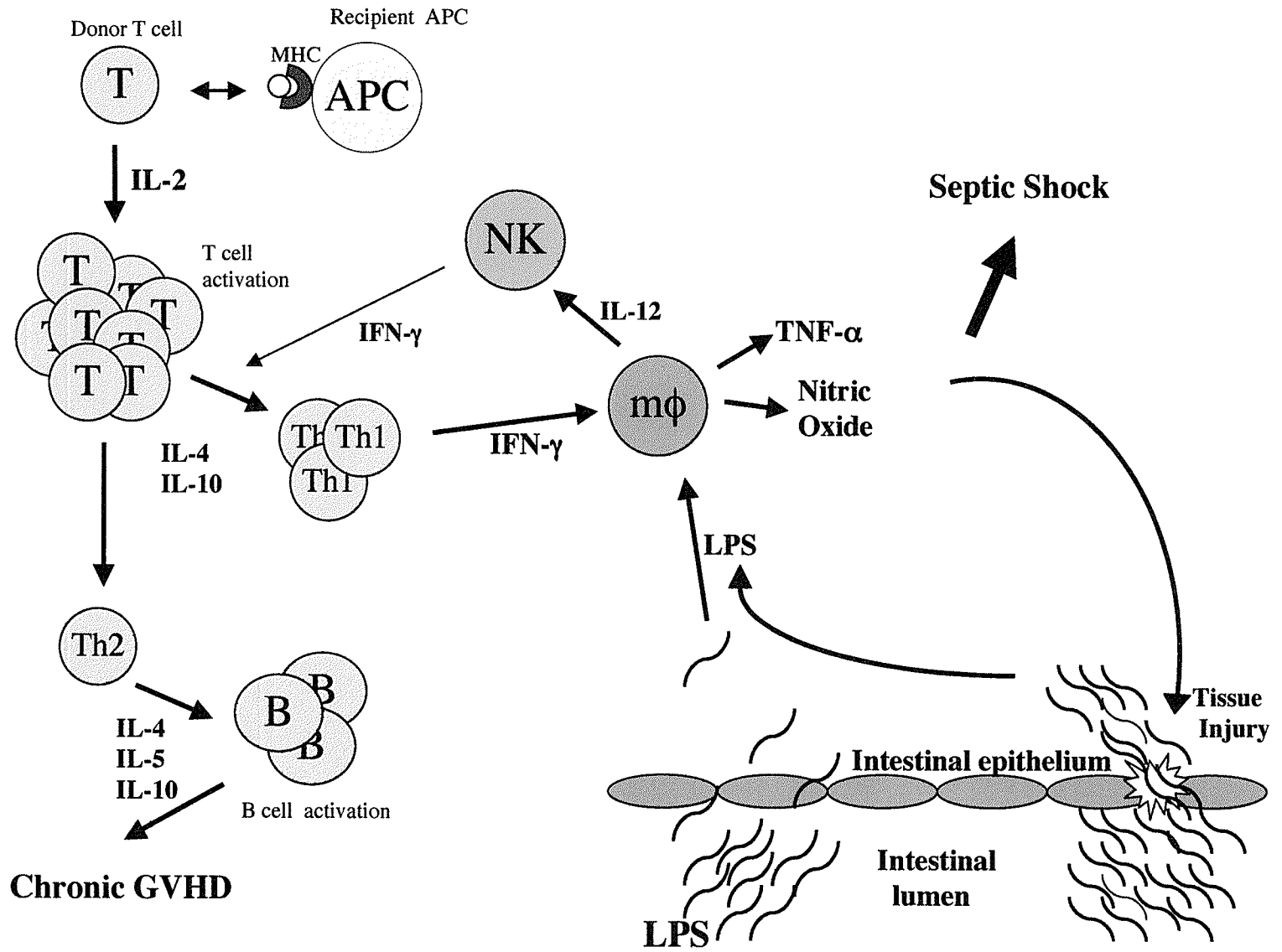
The cytokines released by LPS-stimulated macrophages are an important component in the pathogenesis of acute GVHD. Despite these cytokine-producing abilities, macrophages also play another important role in the GVH response to endotoxin. This is because macrophages become primed for TNF- α production when they are exposed to IFN- γ .^{30,124,137,138} Nestel *et al* were the first to explain the significance of this priming in the GVH reaction. They demonstrated that the administration of a normally non-lethal dose of LPS to a mouse suffering from acute GVHD results in a

rapid onset of sepsis and in the death of the animal. This outcome was thought to be due to the macrophage priming that had occurred earlier in the GVH reaction. When primed, a small dose of LPS was shown to trigger the release of large quantities of TNF- α from macrophage. TNF- α , however, could not be detected in the serum of GVH mice that were not given LPS and could not be detected in LPS-treated control mice. Coincident with the onset of mortality, Nestel and colleagues could detect LPS in the serum of the GVH mice demonstrating that the second signal needed to trigger primed macrophage is indeed present during the later stages of the GVH reaction.¹⁰⁹ Despite this late appearance, LPS is present earlier in the reaction but it first accumulates in the liver and the spleen before appearing in the systemic circulation.¹¹⁸ LPS, by leaking out of the injured gut and triggering macrophages, promotes and amplifies the inflammatory response seen in aGVHD. This essentially results in a cycle of positive feedback. LPS triggers the release of more inflammatory cytokines, which contribute to more extensive intestinal injury thereby further promoting LPS leakage and the cycle, begins anew (Figure 1.1).

1.6.3 TNF- α and GVHD:

TNF- α only appears late in the course of the GVH reaction when extensive tissue injury begins to occur and the onset of death is near.¹⁰⁶ TNF- α is known for its ability to contribute to the cardiovascular collapse seen in septic shock. LPS triggers the systemic release of the large quantities of TNF- α associated with the fatal outcome of sepsis.¹⁰⁹ Several investigators have also implicated TNF- α as a mediator of tissue injury and cell death in GVHD. One study, using an irradiated parent-into F₁-hybrid murine model of

Figure 1.1 Diagram illustrating the cells, cytokines and other molecules that may be involved in the pathogenesis of aGVHD.



acute GVHD demonstrated that administration of anti-TNF- α antibodies prevents most of the skin and gut injury associated with the disease. An increase in survival of these mice was also seen.¹³⁹ Another group demonstrated that TNF- α mediates intestinal crypt cell apoptosis since blockage of the cytokine with antibody or pentoxifylline appeared to inhibit intestinal epithelial cell apoptosis.¹⁴⁰

Clinical studies have correlated the appearance and the level of TNF- α in the serum with the severity of the GVH reaction and with the period of survival. Consequently, anti-TNF- α antibodies have been administered to patients suffering from GVHD or at risk for the disease and have met with some success.^{30,141,142}

1.6.4 Nitric Oxide:

Up until the late 1980s nitric oxide (NO) was known only as a toxic molecule and as such, the discovery of its importance in biology came as quite a surprise.¹⁴³ NO is a small, gaseous molecule that acts as a biological messenger in the body. It is a free radical consisting of an unpaired valence electron that makes it highly reactive and gives it a relatively short half-life. NO relies on nitric oxide synthase (NOS) enzymes for its synthesis from the oxidation of the amino acid L-arginine. The reaction is catalyzed by NOS, forming one molecule of NO and one molecule of citrulline. Nitric oxide synthases are found in, but not limited to neuronal cells, skeletal muscle, vascular endothelium, platelets, macrophages, vascular smooth muscle cells, hepatocytes and cardiac myocytes.¹⁴³⁻¹⁴⁷ There are three types of NOS which are classified as being either constitutive (cNOS) or inducible (iNOS). The constitutive enzymes are always available to provide brief bursts of NO that are essential for physiological maintenance. Both

endothelial NOS (eNOS) and neuronal NOS (nNOS) are in this category. These constitutive isoforms of NOS require elevated intracellular Ca^{2+} levels that enable calmodulin to bind to the NOS and activate the enzyme.^{144,147-150} Physiological levels of NO are important for such processes as the maintenance of vascular tone, blood flow and adequate perfusion, neurotransmission, digestion, modulation of platelet aggregation and adhesion to the vessel wall. Vascular endothelial cells release NO which migrates to nearby smooth muscle cells causing them to relax. This results in vasodilation and a lowering of blood pressure. Further evidence for this stems from evidence that overproduction of NO causes the hypotension associated with septic shock.^{145,151} NO has similar effects on the smooth muscle cells surrounding the intestines. Here, NO relaxes smooth muscle cells after a wave of peristalsis allowing food to move through the gut. NO has also been shown to work as a messenger molecule in neurotransmission.^{143,144}

On the other hand, inducible NOS (iNOS) is not constitutively expressed and requires the activation of protein synthesis in order for it to be created. One exception to this is in the intestine where small amounts of the enzyme have been detected under normal conditions. The presence of a constitutive iNOS here is not surprising considering that the intestinal mucosal surface is continuously exposed to foreign antigen.¹⁵² iNOS also does not depend on high intracellular Ca^{2+} levels for calmodulin binding. Instead, this NOS has a high affinity for calmodulin such that levels of Ca^{2+} in resting cells are sufficient for calmodulin binding and enzyme activation.¹⁴⁴

Synergism between various cytokines and microbial by-products has been shown to be the most effective means of stimulating NO production via iNOS. Although IFN- γ alone has been demonstrated to be capable of stimulating iNOS expression, the largest

increases in iNOS induction are seen when IFN- γ and LPS work together.¹⁴⁷ TNF- α has also been shown to act synergistically with IFN- γ and LPS to increase iNOS.^{145,147}

The induced iNOS can produce high levels of NO for a period of days, which is important in host defense and for tumoricidal activity.¹⁵³ Furthermore, NOS inhibitors have been shown to worsen the course of disease caused by virus, bacteria, fungi, helminths or protozoa. The survival of iNOS -/- mice is also diminished upon infection with these organisms.^{145,146,153} As a defense mechanism, NO works in both a cytotoxic^{145,147,154} and cytostatic^{153,155} manner. As NO can freely diffuse through the plasma membrane, it can interact with many intracellular proteins and inhibit cell growth and cell division. Often, NO reacts with iron groups that are essential for enzymes involved in DNA synthesis and cellular respiration. NO also readily reacts with oxygen to form radicals and other reactive intermediates that may cause cell death. This allows NO to be a primary defense pathway against intracellular microorganisms and pathogens that are too large to phagocytose such as fungi and helminths. High output NO production from iNOS does have a downside and can be responsible for adverse side effects to both those cells producing the NO and to any adjacent cells. This can result in local and systemic tissue damage to the host.^{144,153} Macrophages are a major source of high output NO production. They express large amounts of iNOS in response to LPS and these levels are further increased through the synergistic actions of IFN- γ when it is also present.^{30,146,156-158}

1.6.4.1 Nitric oxide and GVHD:

Aside from its normal physiological and immunological functions, NO has been associated with various pathological disorders such as GVHD. Elevated serum levels of NO_2^- and NO_3^- , two byproducts of NO, have been observed in several animal models and clinical studies of GVHD.¹⁵⁹⁻¹⁶³ Macrophages, responding to IFN- γ and LPS, are thought to be the major producers of NO in the GVH reaction with iNOS as the enzyme responsible for this NO production.^{159,164} NO has been shown to be responsible for the immunosuppression seen in GVHD as well as playing a role in the destruction of host tissues.

Using macrophages obtained from animals suffering from acute GVHD, Nestel and colleagues provided evidence for NO-induced cytostasis. These macrophages were primed by IFN- γ during the GVH reaction and released large amounts of NO in culture when stimulated by LPS. This NO release resulted in complete inhibition of target cell proliferation and was only reversed by treatment with a NOS inhibitor, N^G-mono-methyl-L-arginine (NMMA).¹⁶⁵ Another group looked specifically at the impaired B cell proliferation seen in aGVHD. Again, NO was found to be responsible for the inability of these B cells to respond to stimulation with mitogen.¹⁶⁶ Other studies agree that NO acts in an immunosuppressive manner¹⁵⁹ but also demonstrate that NO can be a mediator of tissue injury as well.¹⁶⁷

Mowat first demonstrated a specific role for NO as a mediator of intestinal tissue injury. Using a CBA into (CBA x BALB/c) F_1 mouse model for GVHD, Mowat studied the effects of inhibiting NO production during the GVH reaction. He observed that the

treatment of these GVH mice with the NOS inhibitor L-NMMA prevented the intestinal pathology normally associated with this proliferative form of intestinal GVHD.¹⁶⁸

Addition of a specific iNOS inhibitor, aminoguanidine, also appeared to prolong survival in a model of GVHD resulting from small bowel transplantation.^{164,169} NO_2^- / NO_3^- levels and bacterial translocation were also reduced by treatment with this iNOS inhibitor.¹⁶⁴ Together, these studies indicate that NO, produced in the GVH reaction, may play a role in the development of an impaired mucosal barrier making it an important contributor to the pathogenesis of aGVHD.

1.7 Keratinocyte Growth Factor:

Keratinocyte growth factor (KGF) was first identified by Rubin *et al* who recognized that a great number of human malignancies were derived from epithelial tissues. Using this knowledge, they searched for growth factors specific to epithelial cells thinking that such factors may have a role in the transformation process. A 28 kDa protein was identified from a human embryonic fibroblast stromal cell line. This protein stimulated DNA synthesis in epithelial cells. Keratinocytes, in particular, were extra sensitive to these mitogenic effects, hence the name KGF. It was observed that fibroblasts and endothelial cells did not show any enhanced proliferation when treated with KGF. This led to the conclusion that KGF was a growth factor specific to epithelial cells.¹⁷⁰ This activity of KGF was later confirmed in a subsequent paper where KGF transcript was found to be widely expressed in stromal cell lines derived from embryonic, neonatal and adult epithelial tissues. The cDNA sequence of KGF demonstrated that it was a member of the fibroblast growth factor (FGF) family and is otherwise known as FGF-

7.¹⁷¹ Since then, KGF has been found to be secreted from a variety of cultured stromal cells including cells of the lung, skin, mammary gland, stomach, bladder and prostate.¹⁷²

The receptor for KGF (KGFR), is a high affinity receptor that also binds acidic FGF with high affinity and basic FGF with low affinity. KGFR is expressed in a wide array of epithelial tissues. The receptor is almost identical to BEK/FGFR-2 but diverges from it in one section of an immunoglobulin loop. In this particular section, there is only 47% homology. These differences between the two receptors were found to be a result of alternative splicing of the exons.¹⁷²

KGF has been found to be important in the process of wound healing. High levels of this mitogen are expressed in the fibroblasts below the wound and at the wound edge within 24 hours of injury. This induction of KGF immediately precedes the onset of epithelial cell proliferation. KGF will also persist for the first seven days after injury during which epithelial cells are proliferating and migrating as part of the healing process. The KGFR is also the predominant splice variant of the FGFR-2 found in the epidermis of the wound providing further evidence for a paracrine mechanism of stimulation.¹⁷³ In addition, it has been shown that local application of recombinant KGF (rKGF) to deep partial-thickness wounds accelerates re-epithelialization and increased the number of immature keratinocytes present in the regenerating epidermis.^{174,175} Hyperproliferation of the epithelium was also apparent in transgenic mice engineered to express KGF by keratinocytes and have it act in an autocrine fashion.¹⁷⁶

Interestingly, KGF knockout mice demonstrated no abnormalities in epithelial growth and wound healing as only the coat appeared to be affected.¹⁷⁷ However, different observations were made using a transgenic mouse developed to express a truncated form

of KGFR in the basal keratinocytes. This truncated receptor lacked two tyrosine kinase domains and resulted in epidermal atrophy, morphological abnormalities in the hair follicles and a delay in re-epithelialization after wounding.¹⁷⁸ This suggests that other growth factors may bind KGFR in the absence of KGF to trigger proliferation of keratinocytes. When the receptor itself is knocked-out however, no compensatory mechanism seems to exist.

KGF can also be produced by lung fibroblasts to stimulate DNA synthesis and proliferation in alveolar type II cells.¹⁷⁹ Expression of both KGF and the KGFR has also been detected in various tissues of the mouse throughout development.¹⁷²

1.7.1 KGF and the gastrointestinal tract:

The ability for the gastrointestinal tract to respond to KGF was demonstrated by Housley and colleagues who detected KGF and KGFR transcript expression throughout normal rat gut. Interperitoneal administration of rHuKGF to animals was found to increase the proliferation of epithelial cells along the intestine. Aside from stimulating elongation of the crypts, rHuKGF treatments also resulted in an increase in mucin-producing cells and goblet cells.¹⁸⁰

With these results in mind, groups began to investigate the potential for KGF to reduce irradiation-induced toxicity in the GI tract. Indeed, treatment of mice with KGF for two consecutive days prior to and a third dose a few hours after whole-body irradiation enhanced the survival of intestinal crypt cells.¹⁸¹ Another study has demonstrated that KGF protects against the mucositis induced by chemotherapy and radiation treatments. Mortality, weight loss and subsequent weight gain during the

recovery were all seen to significantly improve with KGF administration. Crypt survival and proliferation were also ameliorated following KGF pre-treatment. This pre-treatment with KGF, before chemotherapy and radiation therapy were administered, was crucial to a successful outcome. Post-treatment with KGF had no beneficial effect. Interestingly, KGF was not seen to alter the growth rate or alter the ability for chemotherapy to eradicate tumor cells.¹⁸² This protection was found to be due in part to an increase in stem cell numbers at the base of the crypts.¹⁸³ Protection by pre-treatment with KGF, before cytoablative or hyperoxia-induced injuries, was also seen in a variety of other tissues such as the lung, bladder, oral mucosa and thymus.¹⁸⁴⁻¹⁹⁰

One possible endogenous source of KGF in the intestine is intraepithelial $\gamma\delta$ T cells. In particular, a unique subset of $\gamma\delta$ T cells expressing a V γ 3V δ 1 T cell receptor (TCR) reside exclusively throughout the various epithelia, including the intestine. This TCR has a limited antigen recognition capability when compared with non-epithelial $\alpha\beta$ and $\gamma\delta$ T cells but can recognize stressed or damaged keratinocytes. Once activated, these T cells secrete KGF suggesting that a subset of $\gamma\delta$ T cells surveys epithelial tissues and, if damaged, aid in their repair.¹⁹¹

Recent evidence supports this hypothesis as it has been demonstrated in mice that $\gamma\delta$ T cells localize to sites of intestinal epithelial cell injury and express KGF. KGF -/- and TCR δ -/- mice also sustain more serious intestinal injuries and have a significant delay in tissue regeneration.¹⁹² $\gamma\delta$ intraepithelial lymphocytes (IEL) have also been shown to play a similar role in the response to skin injuries.¹⁹³

1.7.2 KGF and GVHD:

Since intense pre-conditioning regimens often precede bone marrow transplantation, and since GVHD is a major complication that develops post-BMT, Panoskaltis-Mortari *et al* began to investigate the potential therapeutic value of KGF in preventing GVHD. Using lethally irradiated B10.BR mice transplanted with C57Bl/6 bone marrow and spleen cells, and a three-day pre-BMT KGF treatment regimen, this group observed significant ameliorations to the GVHD that developed. KGF treatment increased the survival rate and limited the weight loss normally seen in this model. KGF also ameliorated tissue damage, particularly in the lungs and in the skin.¹⁹⁴ KGF has also been shown to protect the thymus, another major target of aGVHD.¹⁹⁵

In a follow-up paper, this group determined whether the improvements seen with KGF treatments were due to the growth factor's ability to repair tissue injury caused by the pre-conditioning regimen, or whether KGF could independently ameliorate GVHD. The authors used a non-irradiated mouse model of lethal GVHD; giving C57Bl/6 T cells to BALB/c SCID mice to address this question. Again, KGF treatments improved survival and limited weight loss. Reduced levels of TNF- α and IFN- γ were also observed. Of particular interest, however, this group also looked for the presence of KGFR on the donor alloreactive T cells. There was no KGFR protein detected and no radiolabeled KGF binding to the donor T cells. This indicates that KGF does not moderate the GVH reaction by a direct effect on the donor T cells.¹⁹⁶

The ability of KGF to shield the gastrointestinal tract from the damaging effects of GVHD has recently been demonstrated in an irradiated parent into F₁-hybrid mouse model. B6D2F₁ mice receiving total body irradiation and C57Bl/6 bone marrow grafts

had increased survival and less severe injury to the GI tract when they were treated with KGF. The ability for KGF to protect the gut tissues also resulted in a reduction of serum TNF- α and LPS levels when compared with GVH control mice. Importantly, the ability of KGF to ameliorate GVHD did not hamper the GVL response to administered leukemia cells.¹⁹⁷

1.8 Current methods for the prevention of GVHD:

New understandings in the alloimmune response have helped to define new treatment approaches for ameliorating GVHD. There are three general strategies used to prevent or treat aGVHD. These correspond with three distinct phases in the GVH response: activation of donor T cells, tissue injury and the release of cytokines and inflammatory mediators in response to endotoxin.

Attempts to hinder the T cell alloresponse has been one of the most active areas of research for the prevention of GVHD. Since T cell activation is one of the earliest events occurring in the GVH reaction, any intervention at this stage should prevent the activation of the downstream phases of GVHD. T cells may contribute to the pathogenesis of GVHD via production of IFN- γ and IL-2, or they may cause tissue injury by Fas/FasL mediated pathways. T cell depletion of the graft is an effective means of preventing GVHD however, as discussed earlier, usually results in higher relapse rate of malignant disease or results in a failure to engraft. Since T cells are necessary for proper reconstitution, drugs are often used to block T cell activation. This can be accomplished by inhibiting IL-2 synthesis or by preventing DNA and RNA synthesis. Recent trials with small groups of patients are also evaluating attempts to block T cell co-stimulatory

signals using either CTLA-4 or anti-CD40L.¹⁹⁸ Attempts to isolate and remove specific alloreactive T cells from the graft, or to prevent the presentation of host antigens are also under investigation.^{199,200}

Various cytokine and cytokine receptor antagonists that prevent the cytokine cascade from developing, are being evaluated for their ability to inhibit the second phase in the GVH reaction. The prevention of the production of certain cytokines is a potential means of intervention for impeding the GVH reaction however it is fraught with difficulty as steps must be taken to avoid interfering with other necessary functions of cytokines.

Prevention of the tissue injury and the phase of GVHD where endotoxin begins to play a role is also an active area of research. Clinically, tissue injury is seen even before the BMT has been administered as a result of the treatment regimen used to eliminate the malignant insult or to create room in the marrow for the incoming graft. Further tissue injury can then occur as a result of the GVH reaction. Many growth factors have been appraised for their ability to limit this tissue injury. In particular, protection of the gastrointestinal tract is important in order to restrict the escape of LPS from the gut lumen. Exogenous administrations of KGF or of IL-11 are two such candidates. This form of preventative therapy is ideal because it preserves the T cell and NK cell responses, allowing for normal immune responsiveness while maintaining the GVL effect.¹⁹⁸

1.9 Rationale and Hypothesis:

GVHD remains a serious risk to the BMT recipient. The roles of intestinal injury and LPS in the pathogenetic mechanism of the disease remain poorly understood but ultimately lead to endotoxemic shock and death of the recipient. Investigating this aspect of the disease however is difficult due to the rapidity of the lethal onset and the inability to obtain multiple intestinal biopsies.

We hypothesize that endotoxin acts as a trigger for these terminal events. By administering a dose of LPS that is non-lethal in control mice we will be able to investigate this series of events in a predictable and controllable manner. It is our belief that NO is a key mediator of the intestinal injury. The importance of IFN- γ to NO and the pathogenesis of intestinal GVHD will also be investigated. Furthermore, we predict that the administration of rHuKGF to mice with aGVHD will protect against this lethal outcome.

CHAPTER 2
MATERIALS AND METHODS

2.1 Mice:

Female C57BL/6J (H-2^b, also referred to as wild-type) donors, DBA/2J (H-2^d) donors and (C57BL/6J x DBA/2J)F₁-hybrid (H-2^{b/d}, referred to as B6D2F₁) recipients were obtained from The Jackson Laboratory (Bar Harbor, ME). B6.129S7-Ifng^{tm1Ts} [IFN- γ gene knockout (gko) on a C57Bl/6 background] donor mice were also obtained from The Jackson Laboratory. All mice were 13-16 weeks old when they were used. IFN- γ gko mice were housed in filter-capped cages. All experiments were performed according to the guidelines outlined by the Canadian Council on Animal Care.

2.2 Media and Reagents:

Hanks' balanced salt solution (HBSS) and RPMI 1640 were prepared from pre-packaged ingredients (Gibco BRL, Burlington, ON) and dissolved in water. Complete RPMI 1640 was prepared by the addition of 1% L-glutamine (Sigma, St Louis, MO), (2.92g/100ml dissolved in saline); 1% Penicillin-Streptomycin (10,000IU/ml, Gibco); and 5% Fetal Calf Serum (FCS) (Gibco). FCS was heat inactivated at 56°C for 30 minutes, aliquoted and stored at -20°C until use. 0.1M NaPyruvate (Gibco) and 0.1M HEPES (Fisher Scientific, Nepean, Canada) were also added when RPMI 1640 was used for cell culture. 20x phosphate buffered saline (PBS) was made, the pH adjusted to 7.4 and stored until needed. Chemicals for this recipe were obtained from Fisher. Bovine serum albumin (BSA) (ICN, Aurora, OH) was dissolved in water, sterile filtered and diluted as required.

2.3 Induction of GVHD:

Acute graft-versus-host reactions were induced by injecting spleen and lymph node cells from the C57BL/6J mouse strain into B6D2F₁ recipients. Chronic GVH reactions were induced in a similar fashion using DBA/2J donor mice. IFN- γ gko mice were also used as donors. Donor and recipient mice were age and gender-matched. Grafts were prepared from spleen and lymph nodes, inguinal, axial, cervical and brachial, which were harvested from the donor mice and pooled. A cell suspension was prepared by first mincing the tissue with scissors then pressing it through a stainless steel wire mesh screen using a spatula. The collected cells were washed two times with HBSS and then filtered through gauze to remove any remaining clumps. The cell suspension was counted using trypan blue (Sigma) to exclude dead cells and adjusted to a concentration of 333×10^6 cells/ml. B6D2F₁-hybrid recipient mice were then injected via the tail vein with 100×10^6 donor cells in a volume of 300 μ l of HBSS. All inductions were performed by the candidate.

2.4 Monitoring the GVH reaction:

GVH reactions were monitored for signs of weight loss and mortality in acute GVH mice and rHuKGF-treated GVH mice. Mice were examined for behavioral changes and weighed every two days up until day 10 after which examinations became more frequent and weights were taken daily. Mice were sacrificed when moribund, as determined by hunched posture, piloerection, reduction in coat sheen, general inactivity, particularly when being handled and a loss of 20% of original body weight. As none of

these signs were present in rHuKGF-treated GVH mice by day 30, weights began to be measured only once a week. All monitoring of the animals was done by the candidate.

2.5 Histological analysis of the intestine and other tissues:

Samples of intestine were removed from mice sacrificed by CO₂ asphyxiation. Four centimeter long sections of jejunum, ileum and colon were collected, cleaned and rinsed in HBSS, cut longitudinally, spread open and pinned on Styrofoam™ blocks. The blocks were then placed in 10% neutral-buffered formalin (provided by John Tutt, Health Sciences Centre, Winnipeg, Canada) for 24 hours after which the intestinal sections were cut longitudinally down the centre and stored in a solution of 70% ethanol. These sections were then machine-processed through graded alcohol and embedded in paraffin. Four-micrometer thick sections were cut and stained with hematoxylin and eosin (provided by John Tutt), then examined by light microscopy. Apoptotic intestinal crypt cells were identified as having nuclear condensation, nuclear fragmentation, vacuolation and a loss of cytoplasmic integrity.

Sections of lung, liver, salivary gland, skin, heart and kidney were removed and processed in a similar fashion by fixing overnight in 10% neutral-buffered formalin, machine-processing and staining with H&E. Tissues were collected, prepared for paraffin embedding and examined by the candidate.

2.6 Injections of LPS, L-NAME and L-NIL and rHuKGF:

LPS (Sigma) was dissolved to a concentration of 1mg/ml in PBS. Mice were injected with a single intravenous (i.v.) injection of 10 μ g of LPS in PBS and sacrificed ninety minutes later by CO₂ asphyxiation.

N^o Nitro L-arginine methyl ester (L-NAME, Sigma) was dissolved in PBS and injected at a dose of 5mg/kg intraperitoneally (i.p.) into recipient mice. L-NAME is a non-specific inhibitor of the three NOS enzymes. A second group of mice were injected with a different L-arginine analog, N⁶-(1-Iminoethyl)-L-lysine (L-NIL, Tocris Cookson Ltd., Bristol, UK). L-NIL was injected i.p. at a dose of 5mg/kg. L-NIL is a selective iNOS inhibitor.²⁰¹⁻²⁰³ The NOS inhibitors were administered 60 minutes prior to the injection of LPS.

rHuKGF, 1mg/ml (Amgen, Thousand Oaks, CA) was prepared from sterile lyophilized stock of 6.25mg/vi by the addition of 6.25ml of 0.1% BSA in PBS. rHuKGF was injected sub-cutaneously into the fat pad on the scruff of the neck. The treatment regimen consisted of seven consecutive daily injections beginning three days prior to induction. All injections were performed by the candidate.

2.7 Measurement of Th1 and Th2 cytokines in spleen cell bulk culture:

Spleens were removed aseptically from mice on days 1, 4, 8 and 15. Spleens were minced and pressed through a stainless steel wire mesh screen and cells were collected in HBSS. The cell suspension was washed and resuspended in complete RPMI 1640 medium supplemented with 5% FCS and 1% HEPES (10mM). Cells were counted using trypan blue and resuspended to a concentration of 15 x 10⁶ cells/ml, then serially diluted

to yield two additional concentrations of 7.5×10^6 and 3.75×10^6 cells/ml. Two milliliters (ml) of each concentration was plated in duplicate in 24 well flat bottom Costar 3524 culture plates (Corning Costar, Acton, MA) and incubated at 37°C in 5% CO_2 . Samples of $300\mu\text{l}$ were removed from each well at 24, 48 and 72 hours and frozen at -20°C until assayed by enzyme-linked immunosorbant assay (ELISA). Cultures were visually inspected daily for viability and signs of contamination by phase contrast microscopy.

Cytokine levels produced in spleen cell culture were measured by sandwich ELISA. Supernatants were analyzed after 48 hours in culture for the presence of IFN- γ and IL-4 and after 72 hours for the presence of IL-5, IL-10 and IL-13. Spleen cell cultures were setup, monitored and supernatants harvested by the candidate.

2.7.1 IFN- γ ELISA

ELISA plates, (Costar #3369), for the detection of IFN- γ were coated with $50\mu\text{l}$ of a solution consisting of $3\mu\text{g/ml}$ of purified anti-IFN- γ monoclonal antibody (mAb), XMG1.2 (ATCC), in a carbonate buffer (pH 9.6) over-night at 4°C . The plates were washed two times with wash buffer [(PBS/0.5% Polyoxyethylenesorbitan monolaurate (Tween 20, Sigma))] then blocked with $200\mu\text{l}$ of PBS/1% BSA (pH 7.4) for one hour at 37°C . Plates were washed four times with wash buffer then $50\mu\text{l}$ of standard or $50\mu\text{l}$ of samples were added to the appropriate wells. The standard was obtained from the supernatants of Con-A stimulated mouse spleen cells that was calibrated against World Health Organization-National Institute of Allergy and Infectious Diseases international reagent Gg02-901-533. Dr. C. Laughlin (National Institute of Allergy and Infectious Diseases, National Institutes of Health) provided the IFN- γ standard. The starting

standard concentration was 45U/ml which was added to the first well and then diluted in two-fold serial dilutions over the ensuing six wells in dilution buffer (PBS/0.5% BSA/0.05% Tween 20). Samples were each two-fold, serially diluted, over four wells and incubated for two hours at 37°C. Plates were washed four times with washing buffer and 50µl of a 1 in 2500 dilution of biotinylated R46A2 (ATCC) anti-mouse IFN-γ detection antibody was added. Plates were incubated at 4°C overnight. After four washes with wash buffer, 50µl of streptavidin-alkaline phosphatase conjugate (Jackson ImmunoResearch laboratories Inc., West Grove, PA), diluted 1 in 4000 in dilution buffer, was added to each well and plates were incubated for 45 minutes at 37°C. Plates were washed six times and 50µl of substrate, p-Nitrophenyl phosphate (5mg Sigma 104 phosphatase substrate tablet, Sigma) dissolved in 5ml of substrate buffer pH 9.8, was added. Substrate buffer consisted of 101mg MgCl₂ (Fisher) and 97ml of diethanolamine (Sigma) brought up to a volume of 1 litre with water. Plates were incubated at room temperature and the colour was read at 30 and 60 minutes at 405nm. Amounts were quantified using the linear portion of the standard curve with the lower limit of detection < 0.88U/ml. The IFN-γ ELISA was performed by the candidate.

2.7.2 TNF-α ELISA

TNF-α was measured in the serum of uninjected and LPS-injected mice. Blood was collected from the tail vein 90 minutes after the LPS-injection and allowed to clot overnight at 4°C. The serum was collected the next day and stored at -70°C until measured by ELISA. Plates, (#Costar 3590), were coated with anti-TNF-α mAB G281-2626 (Pharmingen, San Diego, CA) in 0.1M disodium phosphate buffer (pH 6.0)

overnight at 4°C. Plates were washed two times with wash buffer consisting of 0.5ml Tween 20 per litre of PBS. PBS/3% BSA blocking solution was added for a two-hour incubation period at room temperature followed by two additional washes. Standards or serum samples were added to each well and two-fold serial dilutions were performed in PBS/3% BSA. The standard was prepared from recombinant murine TNF- α , 10 μ g/ml in PBS/0.1% BSA, (R&D Systems, Minneapolis, MN) diluted to a concentration of 400pg/ml in PBS/3% BSA. Plates were incubated overnight at 4°C and then washed. Biotinylated rat anti-mouse TNF- α mAB MP6 XT3 (Pharmingen) diluted to 2 μ g/ml in PBS/3% BSA was added for 45 minutes at room temperature for detection. After washing, a 1 in 2000 dilution of conjugate, streptavidin-alkaline phosphatase (Jackson ImmunoResearch laboratories Inc.) was added to each well and the plates were further incubated for 30 minutes at room temperature. The Substrate 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid [(ABTS) Sigma] was prepared by adding 150mg of ABTS to 500ml of 0.1M Citrate buffer (pH 4.35) and stored frozen in 11ml aliquots. 3% H₂O₂ per 11ml substrate was added just before use. The plates were washed and substrate was added to each well. After 30 and 60-minute incubations at room temperature, plates were read at 405nm. Values obtained for the samples were compared to the linear portion of the curve to determine TNF- α concentration. The lower limit of detection was < 0.06ng/ml. The TNF- α ELISA was performed by the candidate.

2.7.3 IL-4, IL-5, IL-10, IL-13 ELISAs:

All Th2 cytokine ELISAs were performed in collaboration with Dr. Kent HayGlass by Bill Stefura as previously described.^{103,105,204} Supernatants to be analyzed

were provided by the candidate. Capture and detection antibodies are as follows: Rat anti-mouse IL-4 mAb 11B11 (Pharmingen) detected with biotinylated rat anti-mouse IL-4 mAb BVD6-24G2 (Pharmingen); rat anti-mouse/human IL-5 mAb TRFK5 (Pharmingen) detected with biotinylated rat anti-mouse IL-5 mAb TRFK4 (Pharmingen); rat anti-mouse IL-10 mAb SXC-1, detected with rat anti-mouse SXC-2 mAb biotinylated by Bill Stefura, [initial hybridomas were provided by Dr. T Mossman (University of Alberta)]; and rat anti-mouse IL-13 mAb 38213.11 (R&D Systems), detected with biotinylated goat anti-mouse IL-13 catalog #BAF413 (R&D Systems).

2.8 Determination of NO levels in the mouse intestine:

Electron paramagnetic resonance (EPR) spectroscopy was used to determine NO levels in the mouse intestine. This technique is based on the ability of electrons to absorb energy and change from a ground state to a higher energy state. EPR signals can only be observed for free radicals which by definition have the unpaired electrons that give rise to such a signal. This is because the molecule must be capable of interacting with a magnetic field, via an electron magnetic moment, in order to be detected. Most molecules, through chemical bonding, contain only paired electrons which are energetically favoured. These paired electrons fill energy levels and spin in opposite orientations that cancel out in the chemical bond and result in no net electron magnetic moment.

Common atoms or molecules that do contain unpaired electrons and thus have an electron magnetic moment are the transition metals and free radicals such as NO. In the absence of a magnetic field, the unpaired electron is free to adopt either of the two

possible spin orientations parallel ($S = +1/2$) or anti-parallel ($S = -1/2$) that are equal in their energy state. However, when a magnetic field is applied, the energy of the parallel spin-orientation becomes lower making it the preferred orientation for the unpaired electron. By applying microwave radiation to this system, the unpaired electrons can absorb this energy and change their spin orientation to the anti-parallel higher energy state. This energy absorption can be measured at resonance and occurs precisely when $h\nu = g\beta H$ where h = Planck's constant, ν = the microwave frequency, g is a constant known as the g -value and represents the absolute magnetic field position of the center of the EPR spectrum, β = the Bohr magneton and H represents the applied external magnetic field. In order to measure the EPR spectrum, the microwave frequency is kept constant and the magnetic field strength is scanned in an appropriate range [500 gauss (G) in this study].

If electron-nuclear spin interactions occur in a free radical, the molecule will produce a unique hyperfine splitting signal in the EPR spectrum that is characteristic of the nuclear spin(s) present in the molecule. Similar to the electron magnetic moments, the nuclear magnetic moments may either oppose or reinforce the magnetic field produced by the spectrometer. In the case of NO, three hyperfine absorption spectral lines are observed due to the three nuclear spin states of the major ^{14}N isotope present in NO.²⁰⁵⁻²⁰⁷

Since NO is a free radical with a short half-life, spin-trapping agents must be used to trap the NO in a more stable compound. The use of the spin-trapping agents diethyldithiocarbamate [DETC, (Sigma)] and iron (Fe^{2+}) result in the formation of Fe^{2+} - $(\text{DETC})_2$ that can trap NO in the tissue. The spin-trapping agents used in these experiments were made fresh shortly before their injection. The first to be injected consisted of 40mg/kg of FeSO_4 (Sigma) and 200mg/kg of sodium citrate (trisodium salts;

formula weight 294.1) mixed with ddH₂O and injected subcutaneously into the mice. The second spin-trapping agent DETC, was dissolved in saline and then administered in a dose of 400mg/kg i.p. These compounds react with each other upon contact *in vivo* to form the insoluble Fe²⁺-(DETC)₂ complex, and they must therefore be injected separately. After thirty-five minutes, the mice were sacrificed and the intestine removed from the distal end of the duodenum to within two centimeters proximal of the ileocaecal valve. The section was cut longitudinally and rinsed in cold HBSS. It was then placed in a cooled Petri dish on ice and cut into fine pieces and placed in a cooled 5cm³ syringe. The tissue was then injected into a pre-cooled Suprasil, synthetic quartz tube (2.4mm inner diameter, Heraeus Amersil, Atlanta, GA). The quartz tubes, once filled with a minimum of 4cm of tissue, were immediately frozen and stored in liquid nitrogen until analyzed.

EPR spectroscopy, used for the measurement of the trapped NO in the ON-Fe²⁺-(DETC)₂ complex, was carried out at 115 K (-158°C; temperature controller Model BVT-3000; Bruker Spectrospin Ltd., Karlsruhe, Germany). The spectra were measured with a Bruker Model EMX EPR X-band spectrometer system operating at 9.25 GHz with 100 kHz modulation. The instrument settings were as follows: microwave power, 5 mW; modulation amplitude, 5 G and signal level, 1 x 10³; and scan range, 500 G. The concentration of the ON-Fe²⁺-(DETC)₂ complex in each sample was assumed to be proportional to the signal amplitude (peak-to-trough) of the triplet-hyperfine structure (hyperfine splitting of 13 G) observed at g = 2.04. Data were expressed as relative EPR signal intensities (arbitrary units) after subtracting the Cu²⁺-(DETC)₂ complex signal observed in all samples -- the copper was present at < 50 ppm in the FeSO₄ salt. WIN-EPR software (Bruker Spectrospin, Ltd., Karlsruhe) was employed for nearly all EPR

data manipulation on a Compaq Deskpro P500 computer. We were able to convert the measured EPR peak-to-trough signal amplitudes into nanomoles of trapped ON-Fe²⁺-(DETC)₂, per gram of wet tissue before freezing. This calculation involved the double integration of the EPR spectrum from a sample in which the ON-Fe²⁺-(DETC)₂ produced a strong signal relative to that produced by a standard sample of a known concentration that had been quantified under similar conditions. The method used for this calibration, which is associated with a 20% error, has been used previously.²⁰⁵ Our results indicate that a value of 495 relative EPR units is equal to a concentration of 100 nanomoles of ON-Fe²⁺-(DETC)₂, per gram of wet tissue.

All animal injections, tissue collection, EPR spectroscopy and data analysis were performed by the candidate.

2.9 Determination of donor T cells by flow cytometry:

To determine the number of donor T cells present in recipient animals, labeled spleen cells were analyzed by two-colour flow cytometry. Spleens were removed from mice and pressed through a stainless-steel screen with a spatula. The cells were washed through the screen and collected in HBSS, centrifuged at 1000 revolutions per minute (rpm) and re-suspended in 4ml of RPMI.

Columns, for the removal of adherent cells, were prepared by teasing 0.6g of nylon wool (Polysciences Inc., Warrington, PA) and submerging it in RPMI/5% FCS for a minimum of 45 minutes at 37°C and 5% CO₂ to facilitate the removal of air bubbles. A 22G 1 1/2 inch needle was attached to a 20cm³ syringe and the nylon wool was placed inside and covered with RPMI/5% FCS. The medium was allowed to drip as the air

bubbles were removed from the column using a spatula. Following this, the needles were corked, the nylon wool compressed and excess medium was poured off. The spleen cells were added to the column, 2ml of medium was allowed to drip off and the nylon wool was teased up in order to distribute the cells evenly. Columns were topped up with 4ml of RPMI/5% FCS and incubated for one hour at 37°C and 5% CO₂. After the incubation period, columns were allowed to drip to collect the non-adherent cell fraction. Approximately 50ml were collected, the columns were topped up with medium when necessary. The cell suspension was then centrifuged at 1000 rpm for ten minutes and the pellet of cells was resuspended in 4ml of RPMI/5% FCS. The cell suspension was layered over 4ml of Lympholyte™-M (Cedarlane Laboratories Limited, Hornby, ON) and spun for 25 minutes at 1700 rpm with no brake. Cells were collected from the interface, washed two times and counted. Aliquots of 500 000 cells were plated in v-bottom polystyrene Dynex plates (VWR International, Mississauga, ON), topped up with cold PBS/1% BSA and centrifuged at 1300 rpm at 4°C for five minutes. Supernatants were aspirated off and 100µl, at a concentration of 1µg/ml of stock (0.5mg/ml) purified Fc Block™ 2.4G2 (Pharmingen) diluted in cold PBS/1% BSA, was added to each well and incubated for 15 minutes in the dark on ice. Cells were centrifuged for five minutes to remove Fc Block™ and 100µl of antibody was added. Cells were co-incubated with FITC-conjugated mouse anti-H-2D^d 34-5-8S (Cedarlane; 5µg/ml), PE-conjugated rat anti-CD4 CT-CD4 (Cedarlane; 10µg/ml) or PE-conjugated rat anti-CD8 CT-CD8a (Cedarlane; 10µg/ml). FITC-conjugated mouse IgG2a UPC-10 (Caltag Laboratories, Burlingame, CA) or PE-conjugated rat IgG2a LO-DNP-16 (Caltag) were used as isotype controls. Determination of donor T cells was accomplished by co-staining with both anti-

H-2D^d and either anti-CD4 or anti-CD8 antibodies. As H-2D^d is the haplotype of the DBA/2 parent, it will only be expressed on the recipient F₁-hybrid cells. Therefore, all lymphocytes not staining with anti-H-2D^d were determined, by default, to be of donor origin.

An EPICS ALTRA fluorescence-activated cell sorter (Beckman Coulter Canada Inc., Mississauga, ON) was used for flow cytometric analysis with laser excitation set at 488nm. The lymphocyte population was identified in histograms of forward angle versus side light scatter and all acquisition was based on 5000 gated events. The FITC and PE fluorescence signals were split with a 550LP dichroic filter and detected through 525nm and 575nm bandpass filters respectively. Electronic compensation for spectral overlap was adjusted with cell samples labeled separately with either FITC or PE while isotype fluochrome-matched controls were employed to define the level of autofluorescence and nonspecific binding. All data files were collected in listmode format and subsequently analyzed using EXPO32 cytometer software provided with the instrument.

Collection and staining of cells for flow cytometry were performed by the candidate. Analysis of cell samples on the flow cytometer was done by Dr. Edward Rector.

2.10 RNase protection assay for determining intestinal IFN- γ , TNF- α , KGF and KGFR mRNA levels:

Ribonuclease Protection Assays (RPA), run by S.A. Scully of Amgen Inc. (Thousand Oaks, CA), were used to detect IFN- γ , TNF- α , KGF and KGFR mRNA in the mouse intestine. An RPA utilizes bacteriophage, DNA-dependent, RNA polymerases to synthesize a high-specific-activity hybridization probe from a DNA template. Several

properties make bacteriophage RNA polymerases ideal for this procedure. They are stable, single subunit enzymes that are easy to purify and to clone. They polymerize RNA at an exceptionally high rate and are extremely specific for their promoter. A probe, that is complimentary to the distinct sequence of a given mRNA species, is generated and added in excess to a solution of target RNA. The probe will hybridize with complimentary sequences in the sample and protect this RNA from being digested with single-strand-specific RNase. The remaining "protected" sample can then be precipitated, purified and separated on polyacrylamide gel and quantified by phosphorimaging.²⁰⁸

Intestinal samples were collected and snap-frozen immediately post-mortem in liquid nitrogen. Total RNA was extracted from the frozen tissue using STAT 60 (Tel-Test "B", Friendswood, TX) following the manufacturer's protocol. Total RNA was then quantitated by OD 260/280 measurement and an aliquot was evaluated on a denaturing agarose gel to assess RNA integrity. ³²P-labeled murine probes were synthesized from linearized plasmid templates using α^{32} P-UTP and the appropriate RNA polymerase, and gel purified. The following probes were used: IFN- γ , nucleotides 379-557 from Gb: M28621; TNF- α , nucleotides 429-595 from Gb: M13049; KGF, nucleotides 131-337 from Gb: X56551; and KGFR, nucleotides 1270-1399 from Gb: M63503. GADPH and Cyclophilin probes were obtained from Ambion (Austin, TX) and a standard RPA protocol (RPAII kit, Ambion) was used. For IFN- γ and TNF- α , 20 μ g of total RNA and 1.5×10^5 cpm of each probe were hybridized at 55°C overnight followed by RNase digestion and precipitation. For KGF and KGFR, 50 μ g of total RNA and 3.3×10^5 cpm of each probe were used. Samples were run on a 6% TBE urea gel (Invitrogen, Carlsbad, CA), dried at 80°C and exposed on a phosphorscreen overnight. Gels were scanned in a

phosphorimager (Molecular Dynamics, Sunnyvale, CA). Bands were normalized to either GADPH or Cyclophilin and quantified with Image Quant software using local average background correction.

Tissue samples were harvested, labeled and immediately frozen in liquid nitrogen by the candidate, then shipped to Amgen Inc. for the RPA analysis.

2.11 Measurement of serum endotoxin levels:

A *Limulus* Amebocyte Lysate (LAL) assay (Associates of Cape Cod Inc., Falmouth, MA) was used (as described in the accompanying insert) to detect and quantify serum endotoxin levels by Jacqie Fischer. The assay is based on a chromogenic reaction caused by the interaction of an aqueous extract of blood cells from the horseshoe crab *Limulus polyphemus* (the LAL) with endotoxin and a colourless substrate. Factors, present in the Polychrome LAL, become activated by endotoxin to cleave the substrate, producing a yellowish colour that results from the release of p-nitroaniline (pNA). This absorbs at 405nm. A diazo-coupling method can then be used to enhance the sensitivity of the assay. This procedure results in the formation of a diazotized magenta derivative that absorbs between 540 and 550nm.

Mice were first anesthetized with 30mg/ml of stock solution (1.2g/ml in 2-methyl-2-butanol) avertin (Sigma) diluted in PBS. All instruments and glassware were baked for a minimum of two hours at 180°C. Blood was collected aseptically by cardiac puncture and allowed to clot overnight at 4°C. The serum was then aseptically removed and stored at -20°C until used. Samples were thawed, diluted 1:1 in endotoxin free water and boiled for one minute to release bound endotoxin. Samples were plated in quadruplet into 96

well Pyroplates (Associates of Cape Cod) at dilutions of 1 in 50 and 1 in 100, and at a further tenfold dilution to 1 in 500 or 1 in 1000. A standard curve was generated from the Control Standard Endotoxin provided with the kit starting at a concentration of 0.25 endotoxin units (EU) and performing doubling dilutions down to 0.0078EU. Pyrochrome was added to each well as per the manufacturer's instructions. After 30 minutes of incubation at 37°C, the reaction was stopped with HCl reconstituted sodium nitrite and the magenta colour was developed following the addition of ammonium sulfamate and N-(1-Naphthyl)-ethylenediamine (NEDA) as per manufacturer's instructions. Samples were read at 550nm and the amount of endotoxin was quantified using the standard curve. Averages of the quadruplet samples were reported.

Blood was collected from mice and the serum prepared by the candidate for analysis by the LAL assay.

CHAPTER 3

THE ROLE OF IFN- γ , LPS AND NITRIC OXIDE IN INTESTINAL GVHD

3.1 Introduction:

Acute GVHD is a rapidly-progressive form of the disease characterized by cachexia, immunosuppression, widespread tissue injury and a Th1-like immune response. One of the major targeted organs in aGVHD is the gastrointestinal tract where diarrhea and intestinal epithelial cell damage is common. The intestinal injury that occurs in aGVHD is involved directly in the lethality of the disease by providing a portal through which LPS can enter the host and induce septic shock. The LPS level peaks at the same time as it becomes detectable in the serum, coinciding with the onset of mortality.^{118,209} The mechanism as to how intestinal bacteria contribute to the pathogenesis of aGVHD, however, is not completely understood.

In order for these events to take place, LPS must first transit across the gut epithelium. As the GVH reaction proceeds, the gut sustains injury that leads to the formation of intestinal lesions and promote the translocation of LPS into the circulation. Two mediators that have been implicated in the development of this intestinal injury are TNF- α and NO.^{139,140,167,168,210-213} IFN- γ has also been implicated in intestinal GVHD.^{30,107}

Studying the events that lead up to the intestinal injury, as it occurs naturally during the course of a GVH reaction, is difficult and unpredictable leaving the finding of this epithelial crypt cell lesion up to chance. This is because the lesion usually develops shortly before death and the time at which the GVH mice succumb to the disease is variable. Since the intestine undergoes rapid autolysis shortly after death, and since sequential intestinal mucosal biopsies are simply not feasible in mice, it is impossible to study the intestinal lesion in mice dying spontaneously of GVHD.

We have found a means of inducing the lesions by giving mice with GVHD a very low dose of LPS i.v. The dose of LPS we use is non-lethal when administered to control mice. This method provides us with a model to study the pathogenesis of the lesions and the events contributing to their formation. We therefore sought to explore the roles that NO and IFN- γ play in the generation of the intestinal injury by using LPS as a means of inducing the terminal events surrounding lethal aGVHD.

3.2 Experimental design:

GVHD was induced in B6D2F₁-hybrid mice with grafts from either IFN- γ gene knockout or wild-type C57BL/6 donors. Hematoxylin and eosin-stained sections of intestinal tissues were analyzed for the presence of apoptotic lesions in moribund wild-type graft recipients and throughout the GVH time-course for recipients of IFN- γ gene knockout grafts. The ability of a small dose of LPS to promote apoptosis and intestinal lesion formation in recipients of both sets of grafts was investigated. To observe whether iNOS and NO are responsible for intestinal lesion formation, NOS inhibitors L-NAME and L-NIL were administered to GVH mice and LPS-induced lesion formation was examined. Quantification of apoptotic crypt cell numbers was calculated and compared with control mice to ensure that the apoptosis could not be attributed directly to the administration of the NOS inhibitors. Production of NO in the intestine was measured during the GVH reaction. The ability for LPS to increase NO production was also studied. NOS inhibitors were administered to confirm the effects of NO production in GVHD. IFN- γ mRNA levels were also analyzed in the intestines of the GVH mice.

3.3 Results:

3.3.1 Intestinal apoptotic lesions are seen in moribund wild-type graft recipients and are not present in recipients of IFN- γ gko grafts:

Apoptotic epithelial crypt cells appear in the intestine of mice with acute GVHD. Lesions, made-up of these apoptotic cells, form during the end stages of the disease when mice become moribund. Occasional apoptotic cells can be seen in the crypts of B6D2F1 control mice that did not receive grafts. This lesion is shown in figure 3.1B in the jejunum of a B6D2F₁-hybrid GVH mouse on day 17 post-induction. Similar lesions could be seen in samples of ileum and colon taken from moribund, wild-type graft recipient mice (not shown).

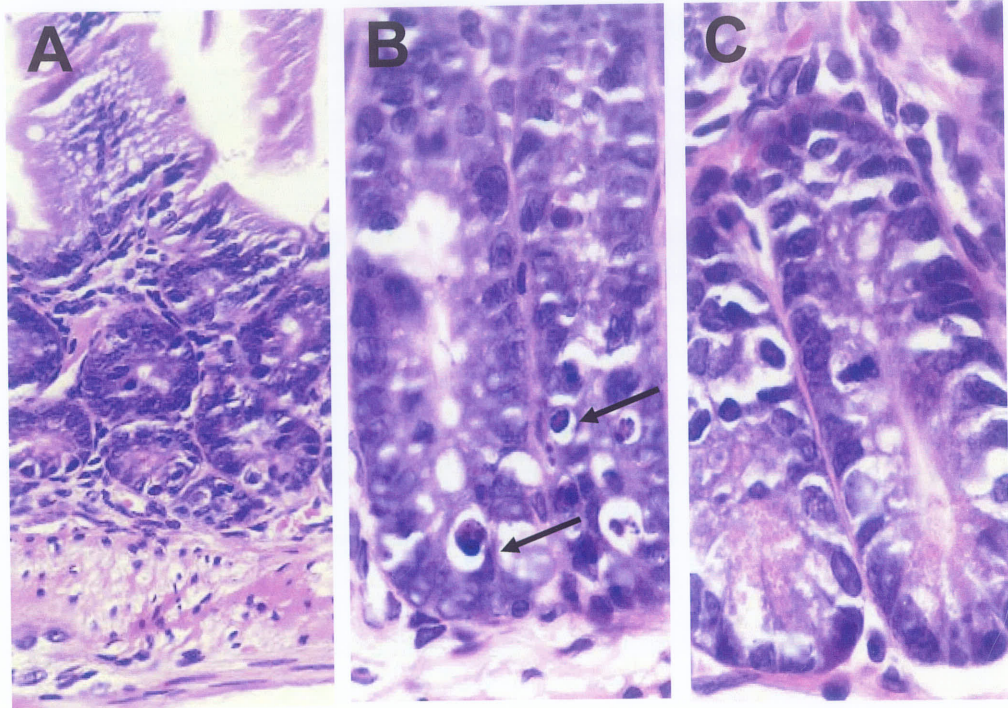
Unlike their wild-type graft recipient counterparts, recipients of grafts from IFN- γ gko donors do not develop any intestinal lesions. Intestinal lesions were never observed in IFN- γ gko graft recipients (Fig. 3.1C).

3.3.2 A small dose of LPS is lethal in recipients of wild-type grafts but not in recipients of grafts from IFN- γ gko donors:

Nestel et al were the first to show that mice with aGVHD become moribund within hours of receiving a dose of 10 μ g of LPS. This dose of LPS has no lethal effects in control mice.¹⁰⁹

The findings we obtained from the C57BL/6 \rightarrow B6D2F1 model of acute GVHD employed in our lab were consistent with those of Nestel and colleagues. Mice were determined to be moribund and were sacrificed when signs of morbidity appeared, such as ruffled fur, hunched posture and a reduced activity level. Using these criteria, the

Figure 3.1: Intestinal histopathology as seen in H&E-stained sections of jejunum from B6D2F1 control mice (3.1A), recipients of wild-type grafts (3.1B), and recipients of IFN- γ gko grafts (3.1C). Both B and C are from sections that were harvested from recipient mice on day 17 post-induction. Arrows indicate apoptotic epithelial crypt cells. Magnifications were X 250 (3.1A) and X 400 (3.1B and 3.1C).



injection of 10 μ g of LPS was determined to be 100% lethal within 24 hours when given to recipients of wild-type grafts. This same dose proved to be non-lethal in healthy B6D2F₁-hybrid control mice that did not have GVHD as all control mice survived the treatment. No mice became moribund when IFN- γ gko graft recipients were injected with a similar dose of LPS, Table 1.

3.3.3 LPS injection induces intestinal apoptotic lesions in wild-type graft recipients but not in recipients of IFN-g gko grafts:

Intestinal lesions develop within 90 minutes when mice with acute GVHD are intravenously injected with 10 μ g of LPS. This was seen in recipients of wild-type grafts as early as day 8 post-induction and was also observed on day 13. This lesion is similar to the intestinal lesion seen in the late-day moribund mice pictured in Fig. 3.1B. An H&E stained section of jejunum, containing the LPS-induced apoptotic epithelial crypt cell lesion, from a day 8 post-induction GVH mouse that received a wild-type graft is shown in Fig. 3.2A.

The lesion was absent from recipients of IFN- γ gko grafts that were treated with the 10 μ g dose of LPS Fig. 3.2B. We have never seen the LPS-induced intestinal lesion in B6D2F₁ control mice.

3.3.4 The effect of NOS inhibitors L-NAME and L-NIL on intestinal lesion formation:

Since NO has been implicated as a mediator of intestinal epithelial cell injury, and treatment with 10 μ g of LPS results in the formation of the intestinal lesion, we wished to

Table 3.1. Number of mice surviving 36 hours after injection of LPS.

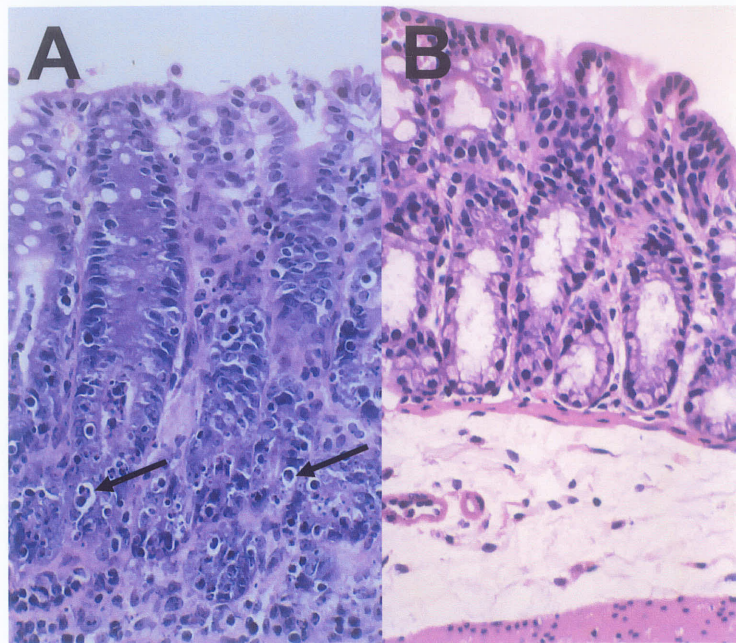
Experimental Group	Number of Survivors
Control B6D2F ₁ -hybrids ¹	6/6
Recipients of wild-type grafts ^{2,3}	0/6
Recipients of IFN- γ gko grafts ²	6/6

¹B6D2F₁-hybrid control mice were age and sex-matched to recipient mice but did not receive grafts.

²Recipient mice were injected with 10 μ g of LPS i.v. on day 15 post-induction.

³Mice were sacrificed when moribund as determined by hunched posture, ruffled fur and reduced activity level.

Figure 3.2. Photomicrographs depicting LPS-induced apoptotic crypt epithelial cells in an intestinal section collected from a recipient of a wild-type graft that received an injection of LPS (10 μ g iv), 90 min before sacrifice (A). Recipients of IFN- γ gko grafts that had received a similar injection of LPS but did not develop intestinal lesions are also shown (B). Arrows indicate examples of apoptotic cells. All sections were collected from recipients on day 8 post-induction. Magnifications were X 250.



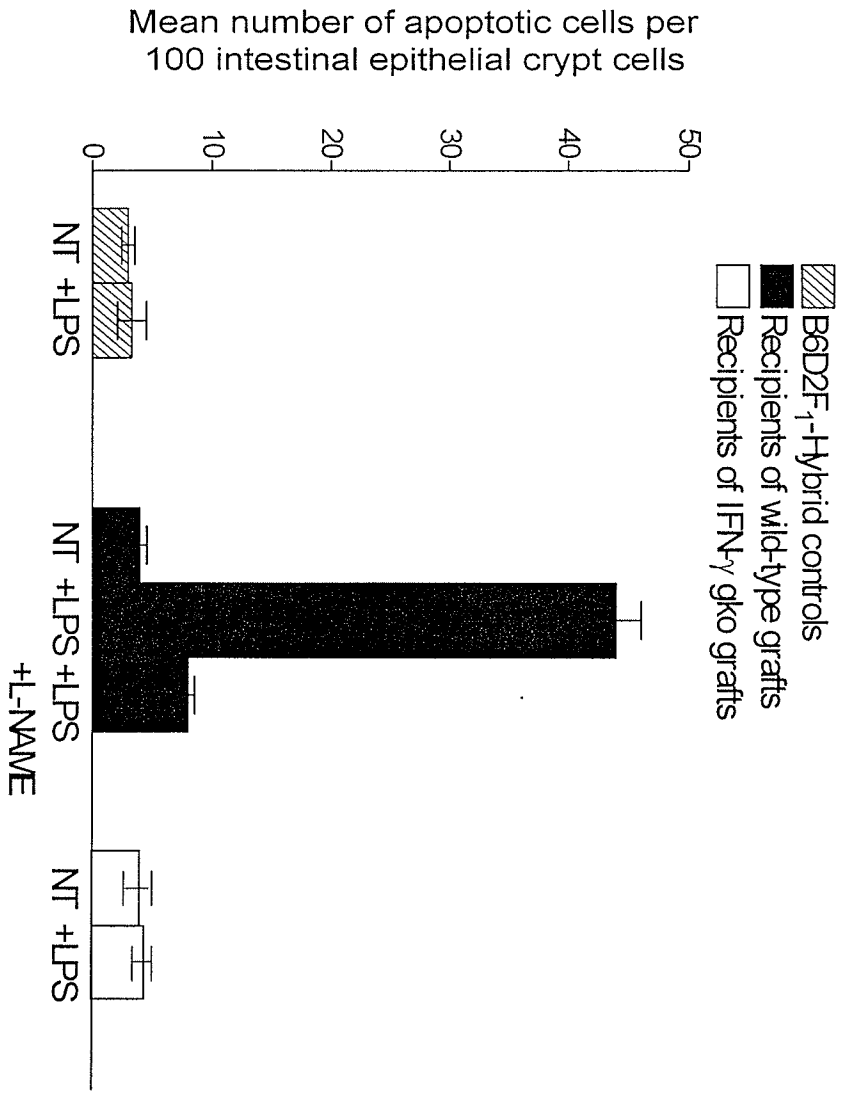
determine whether NO is involved in the mechanism of the LPS-induced gut injury. Two NOS inhibitors were used. Both act as L-arginine analogues that prevent NO production through competitive inhibition of the NOS enzymes for the arginine substrate. The first, L-NAME, is a non-specific inhibitor that acts on both constitutive and inducible NOS enzymes.^{214R} The second inhibitor, L-NIL, is an L-arginine analogue that is specific only for the inducible NOS enzyme.²⁰¹⁻²⁰³ Sixty minutes before the 10µg i.v. injection of LPS we treated recipients of wild-type grafts on the 8th day post-induction with the NOS inhibitors, L-NAME [(5mg/kg i.p.) Fig. 3.3A], or L-NIL [(5mg/kg i.p.) Fig. 3.3B]. Recipients of wild-type grafts that received either of these NOS inhibitors as treatment did not develop the apoptotic intestinal lesion that usually follows LPS injection in GVH mice.

3.3.5 Number of apoptotic crypt epithelial cells in LPS-treated GVH mice:

Because apoptotic epithelial crypt cells can sometimes be seen in the intestinal sections of B6D2F1-hybrid control mice and in both recipients of IFN-γ gko grafts and wild-type graft recipients treated with L-NAME, we wished to quantify the number of apoptotic crypt cells. Apoptotic crypt cells were counted in sections of jejunum from untreated and LPS-treated mice in each group on day 8 post-induction. These values were compared to the numbers of apoptotic cells counted in recipients of wild-type grafts that developed intestinal lesions following treatment with 10µg of LPS. Wild-type graft recipients treated with LPS had significantly higher numbers of apoptotic epithelial crypt cells per one hundred cells counted than all other treatment groups examined ($P < 0.001$) Fig. 3.4.

Figure 3.3. H&E stained intestinal sections depicting the absence of intestinal lesions in wild-type graft recipients that received an LPS injection 90 minutes before sacrifice and were treated with a NOS inhibitor. The section shown in 3.3A is from a wild-type graft recipient that was treated with L-NAME 60 min before the LPS injection. The section shown in 3.3B is from a mouse that received L-NIL 60 minutes prior to the LPS injection. Magnification X 250.

Figure 3.4. The mean number of apoptotic cells counted, per 100 crypt epithelial cells, found in sections of jejunum in B6D2F₁-hybrid control mice, recipients of wild-type grafts and recipients of IFN- γ gko grafts on day 8 post-induction. Mice received either no treatment (NT) or an injection of 10 μ g of LPS (+ LPS). A group of recipients of wild-type grafts also received an injection of L-NAME prior to the LPS injection (+ LPS + L-NAME). Error bars represent the mean standard error determined for three individual mice in each group. The number of apoptotic cells counted in the LPS-injected recipients of wild-type grafts was significantly greater than those counted in all of the other treatment groups with or without LPS ($P < 0.001$) It was also significantly greater than wild-type graft recipients treated with L-NAME before the LPS injection ($P < 0.001$). Statistical analyses were performed by ANOVA followed by a Tukey-Kramer multiple comparison test.



3.3.6 NO levels in untreated and LPS-treated recipients of wild-type and IFN- γ gko grafts:

NO levels were measured in intestinal tissue by an EPR spectroscopic analysis. This technique allows us to directly measure NO in the tissue by injecting mice with the spin trapping agents DETC and FeSO₄. The trapping agents enable us to detect NO by forming a measurable complex, ON-Fe²⁺-(DETC)₂, when they encounter the NO in the tissue. A representative EPR spectroscopic plot can be seen in Fig. 3.5A with the characteristic triplet-hyperfine structure denoting the presence of trapped NO in the sample of intestinal tissue. This plot was obtained from an analysis of intestinal tissue of a recipient of a wild-type graft that was injected with 10 μ g of LPS, followed by NO-trapping agents 60 minutes later on day 8 post-induction. The second plot, Fig. 3.5B is characteristic of the intestines from a B6D2F1 control mouse that did not receive a graft. The triplet-hyperfine structure is absent from this sample indicating that a low background level of NO is present in the intestines of healthy control mice. Similar results were obtained from recipients of IFN- γ gko grafts that were injected with LPS, as an absence of the triplet-hyperfine structure is also seen in these samples Fig. 3.5C. Fig. 3.6 demonstrates the levels of NO detected in recipients of wild-type and IFN- γ gko grafts treated with or without LPS. A burst of NO can be seen in the intestines of wild-type graft recipients when they were treated with LPS. In these mice, LPS induces an approximately six-fold increase in NO production over the NO level seen in their untreated counterparts on day 8 post-induction. Mice from a day 13 post-induction time-point exhibit a five-fold increase in NO production when injected with LPS. These LPS-induced levels of NO are significantly different from the constitutive levels seen in mice

Figure 3.5. Representative plots of the EPR spectroscopy signals observed in intestinal tissue. Mice in 3.5A, 3.5B and 3.5C were injected with the NO spin trapping agents, DETC and FeSO₄, 30 minutes before sacrifice. Figure 3.5A shows the signal from a wild-type graft recipient on day 8 post-induction, following injection of LPS (10 μg, i.v.). The background signal, observed in intestinal tissue from a B6D2F₁ control mouse, is shown in 3.5B. Figure 3.5C shows the signal produced by intestinal tissue from an IFN-γ gko graft recipient that was sacrificed on day 8 post-induction. g represents the absolute magnetic field position of the line of the EPR spectrum.

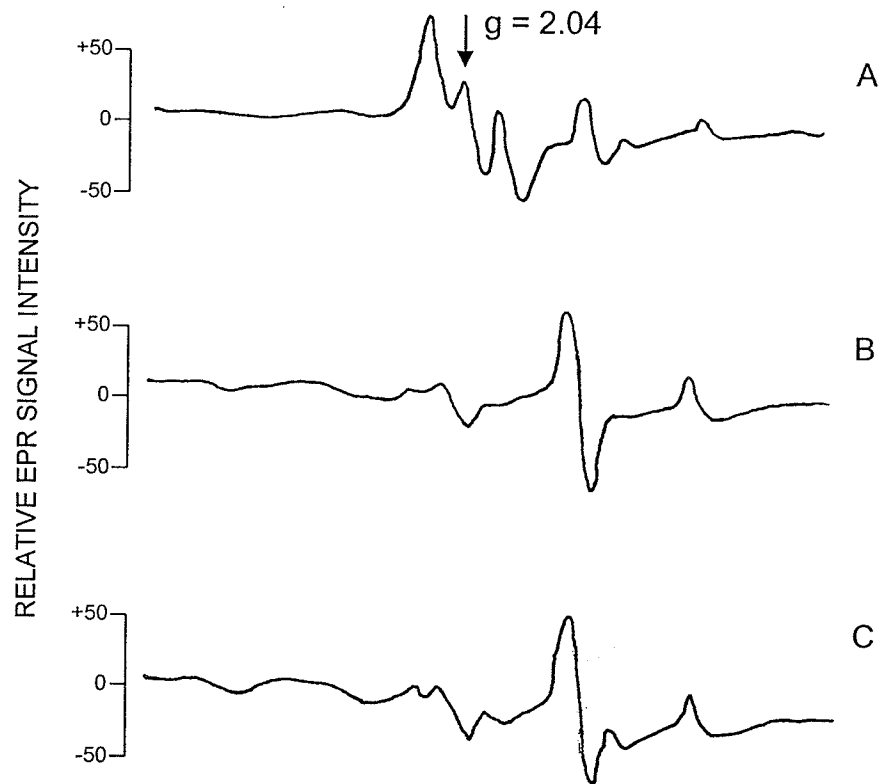
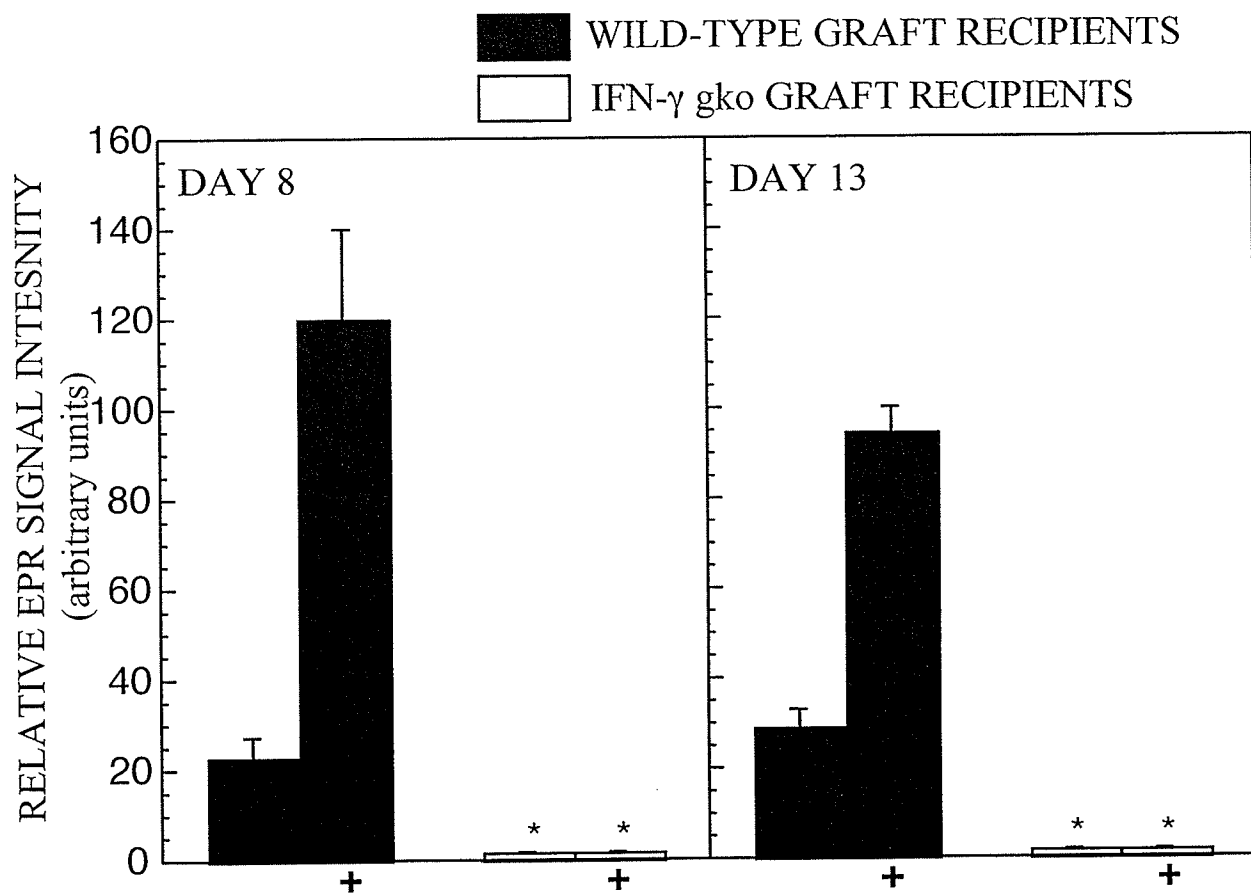


Figure 3.6. NO levels observed in intestinal tissue from recipients of wild-type grafts and recipients of IFN- γ gko grafts on days 8 and 15 post-induction. The relative EPR spectroscopy signal intensities of ON-Fe²⁺-(DETC)₂ complexes are expressed in arbitrary units, which were calculated by measuring the distance from the peak to the trough of the triplet hyperfine structure at the $g = 2.04$ location. The Cu²⁺-(DETC)₂ signal was then subtracted to obtain the final value. The levels seen in recipient mice that either did (+), or did not (-) receive an injection of LPS (10 μ g, i.v.) are shown. Error bars represent the standard error of the mean NO level determined for 3 individual mice. An asterisk indicates that NO could not be detected in the tissue using EPR spectroscopy.

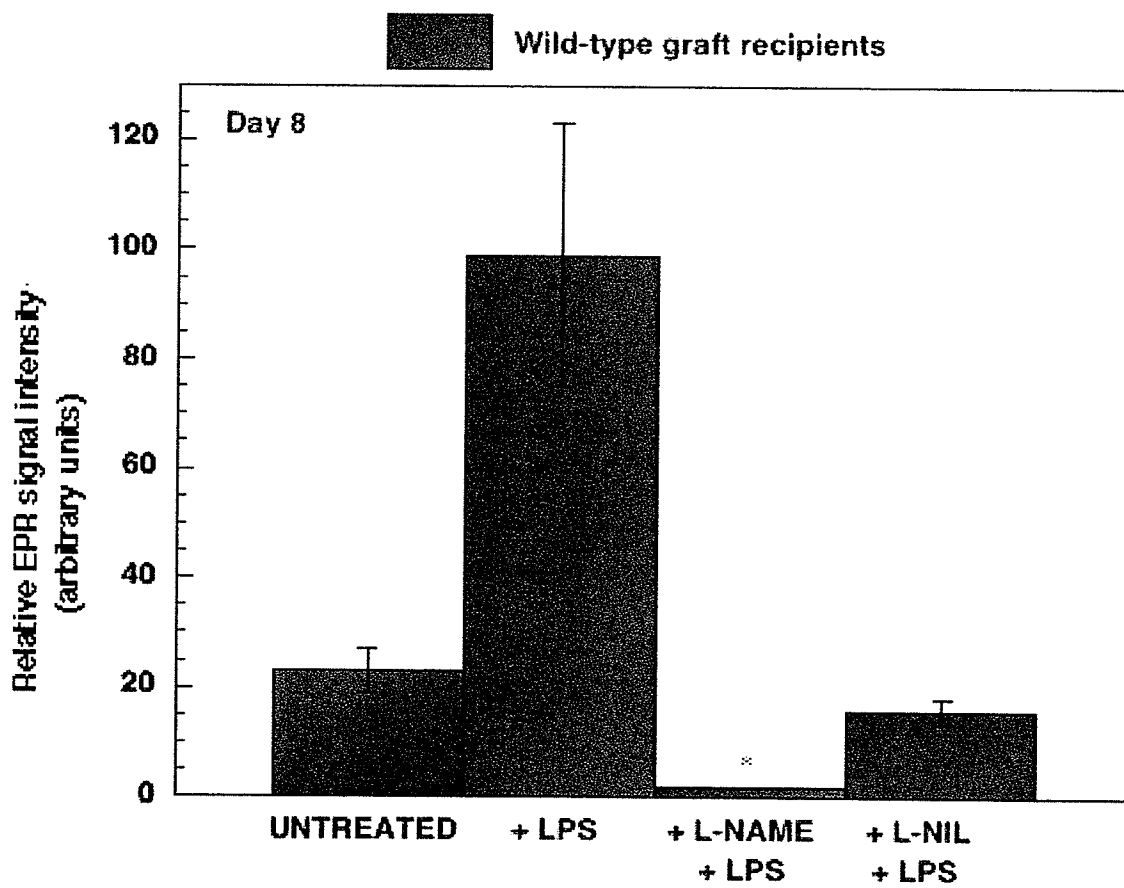


not injected with LPS on both day 8 and day 13 post-induction ($P < 0.001$ and $P < 0.01$ respectively). NO was not detected in recipients of wild-type grafts on day 4 post-induction despite being injected with $10\mu\text{g}$ of LPS. Recipients of IFN- γ gko grafts had no detectable level of intestinal NO on either day 8 or day 13 post-induction. Injection of LPS did not alter this result as no burst of NO was seen and the level remained undetectable. NO production was also absent in recipients of IFN- γ gko grafts on day 70 post-induction (**data not shown**).

3.3.7 Effect of NOS inhibitors L-NAME and L-NIL on intestinal NO production in GVH mice:

Since administration of L-NAME to GVH mice prevented the LPS-induced intestinal epithelial crypt cell apoptosis, we wished to confirm that NO production was being inhibited. An EPR spectroscopic analysis was performed on intestinal tissue from mice that received L-NAME prior to being injected with LPS. Fig. 3.7 demonstrates that intestinal NO levels were undetectable in mice that were treated with the NOS inhibitor L-NAME. We administered the iNOS specific inhibitor L-NIL to mice with GVHD to determine if iNOS was the enzyme responsible for the higher production of NO seen in response to LPS. The level of NO detected in mice that were given L-NIL and LPS was similar to the level seen in untreated, unstimulated GVH mice Fig. 3.7. This suggests that iNOS is responsible for the increase in NO production seen in mice that are recipients of LPS injections. The LPS-induced levels of NO were significantly greater than either level seen when L-NAME ($P < 0.01$) or L-NIL ($P < 0.01$) were administered.

Figure 3.7. Nitric oxide levels observed in intestinal tissue from recipients of wild-type grafts that had been injected with the NO-spin trapping agents DETC and FeSO₄. The first bar represents the mean NO level observed by EPR spectroscopy in tissue from untreated recipients that had been injected with NO spin-trapping agents 30 minutes before sacrifice. The second bar shows the mean level of NO seen in tissue from untreated recipients that had been injected firstly with LPS and secondly with NO spin-trapping agents (60 minutes later), and were sacrificed 30 min later. The third bar shows the mean NO level observed when mice were treated firstly with L-NIL, secondly with LPS (60 minutes later) and thirdly with NO spin-trapping agents (90 minutes later), and sacrificed 30 min later. A similar procedure is represented in the fourth bar, however, L-NAME instead of L-NIL was administered prior to the injection of LPS. An asterisk indicates that a triplet-hyperfine structure produced by the NO-Fe²⁺-(DETC)₂ complex could not be detected by EPR spectroscopy. Error bars represent the standard error of the mean NO level determined for 3 individual mice.



3.3.8 Intestinal levels of IFN- γ mRNA in recipients of wild-type and IFN- γ gko grafts:

This is work that was done in conjunction with Drs. Danilenko and Scully of Amgen Inc. The level of IFN- γ mRNA was measured in the intestinal tissue of recipients of wild-type or IFN- γ gko grafts. Figure 3.8 shows a gel representative of the RPA analysis used to determine IFN- γ mRNA levels. These data are shown graphically in Figure 3.9. The level of IFN- γ mRNA increased on day 8 post-induction in recipients of wild-type grafts. Intestinal IFN- γ mRNA is also seen to increase in recipients of IFN- γ gko grafts on day 8 post-induction. Although this increase is less drastic, it is still significantly larger than the levels seen in B6D2F1 control tissue ($P < 0.001$). On day 15 post-induction, the levels of mRNA in both wild-type and IFN- γ gko graft recipients decreased. At this time-point, the intestinal IFN- γ mRNA in recipients of wild-type grafts decreased by approximately 75% and was no longer significantly different from the level seen in mice that were recipients of IFN- γ gko grafts ($P > 0.05$). By day 60 post-induction, the mean level of intestinal IFN- γ mRNA in recipients of IFN- γ gko grafts was similar to levels seen in control mice.

Figure 3.8. Phosphorimage showing results from an RNase protection assay used to determine the level of IFN- γ mRNA in intestinal tissue from recipients IFN- γ gko grafts (Figure 3.8A) and recipients of wild-type grafts (Figure 3.8B). Tissue was collected from 3 mice at each time-point. Mice were sacrificed on days 1,4, 8 and 15 post-induction in both recipient groups, and on day 80 post-induction in recipients of IFN- γ gko grafts. Control intestinal tissue was harvested from 6 B6D2F₁-hybrid mice that did not receive grafts. Spleen tissue and yeast were run as positive controls. Bands were quantified with Image Quant software using the local average background correction.

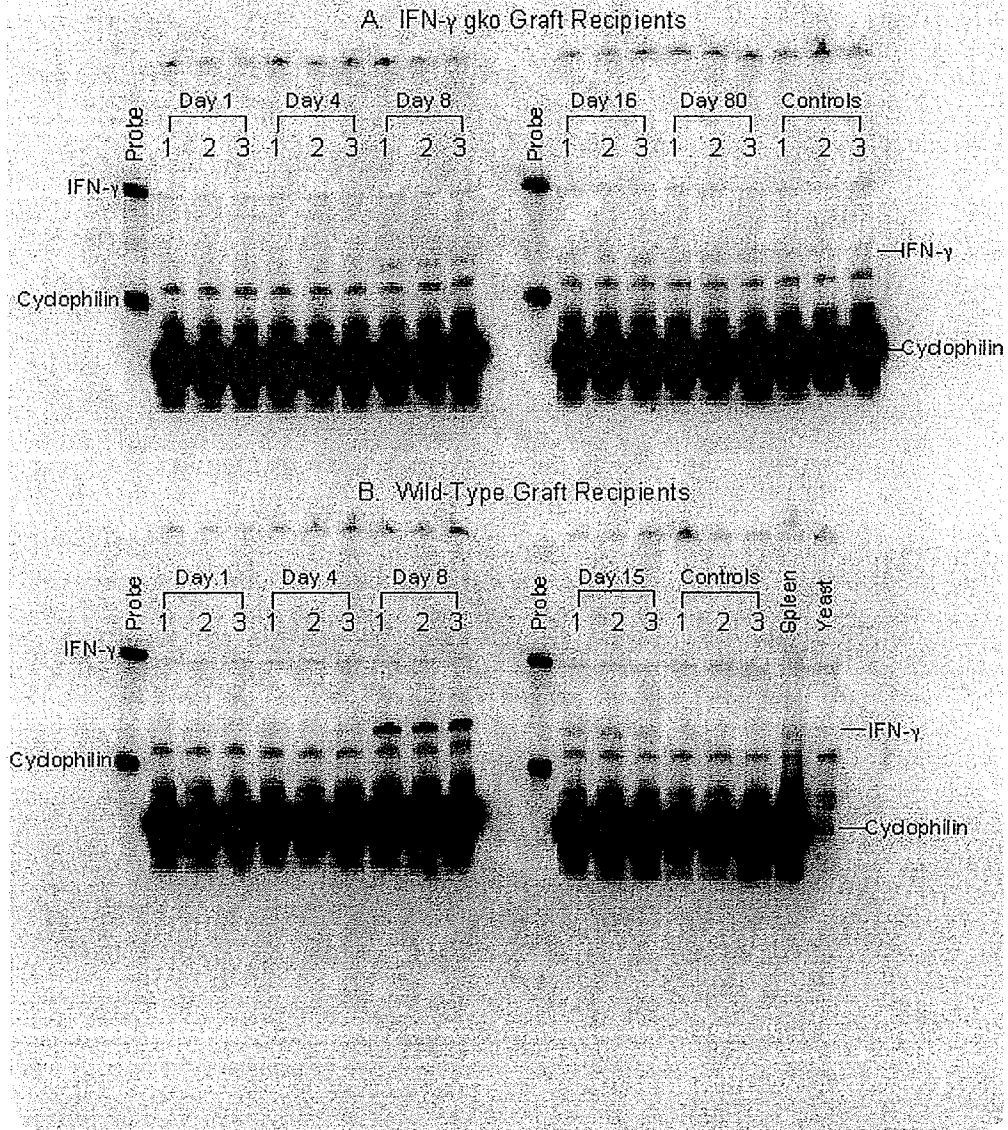
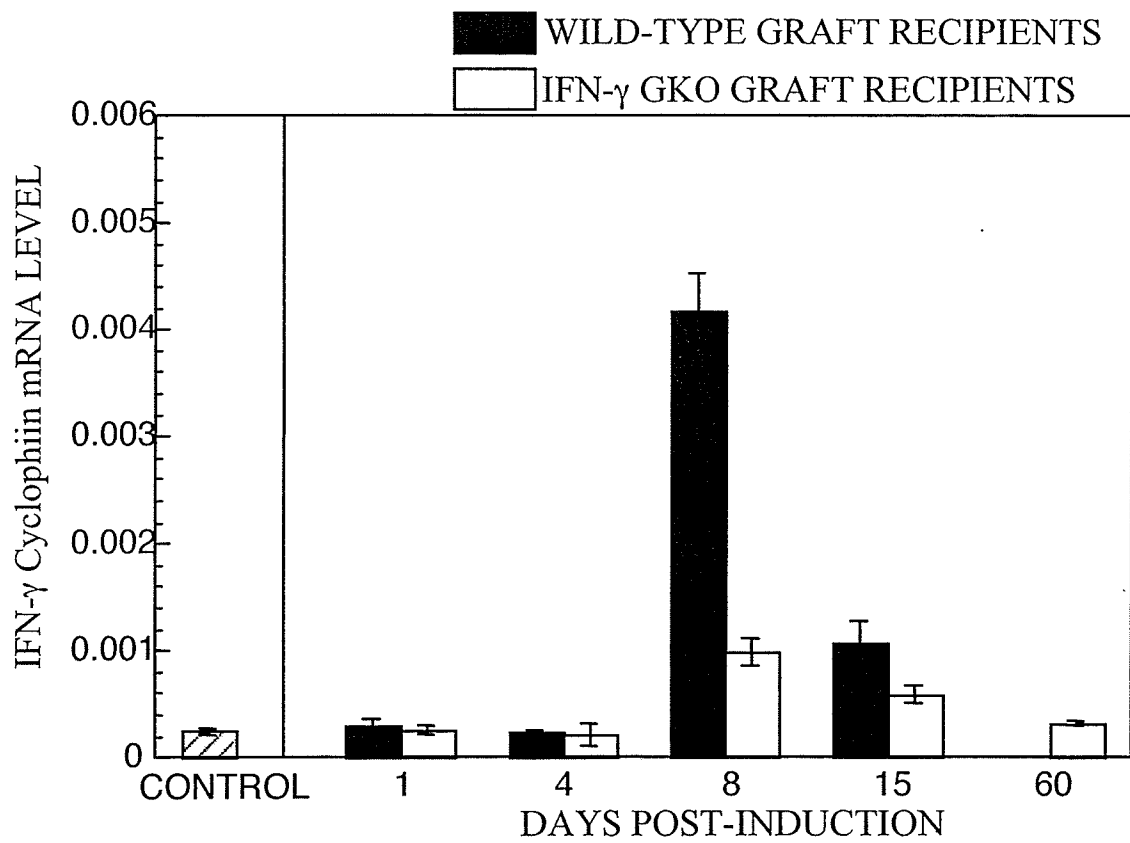


Figure 3.9. Kinetics of IFN- γ mRNA production in intestinal tissue from recipients of wild-type grafts and recipients of IFN- γ gko grafts. IFN- γ mRNA levels were measured using an RNase protection assay. Controls consisted of intestinal tissue from B6D2F₁ control mice that did not receive grafts. Error bars represent the standard error of the mean IFN- γ levels determined for 3 individual mice. On day 8 post-induction, the mean level of IFN- γ mRNA level seen in IFN- γ gko graft recipients was significantly higher than that seen in the control (Student's t test, $p < 0.001$). On day 15 post-induction, the levels seen in recipients of wild-type grafts and recipients of IFN- γ gko grafts were not significantly different (Student's t test $p > 0.05$).



CHAPTER 4
THE EFFECT OF rHuKGF ON GVHD AND THE GVH-INDUCED INTESTINAL
INJURY

4.1 Introduction:

Allogeneic BMT remains an important post-treatment necessity for many patients suffering from a variety of cancers and hematological diseases.^{1,2} While the development of GVHD is a major complication and often the cause of the demise of many BMT recipients, associated with the GVH reaction is a response that may mean the difference between complete remission and a malignant relapse.^{69,70} This graft-versus-leukemia (GVL) effect, if harnessed properly, could represent a new form of cancer treatments for a wide range of patients who are currently excluded due to age or advanced disease. One difficulty that remains is that the GVL effect has not been separated from the GVH reaction. As such, many attempts have been made to limit GVHD while maintaining the GVL effect but none to date have been entirely successful.^{65,66} While studies to protect against GVHD through depletion of lymphoid cells from the graft, immunosuppression and by impeding various cytokine responses are underway,^{60-66,103,104,141,209,215} each of these procedures have the obvious caveat in that they may interfere with the normal immune response.

One important aspect to consider when attempting to limit the GVH reaction is the role that endotoxin plays in the course of the disease. During the GVH reaction, the gut becomes permeable to endotoxin leading to macrophage activation and the release of inflammatory mediators.^{109,118} As demonstrated in section 3.3, one important inflammatory mediator whose release is triggered by endotoxin is NO. The LPS-induced burst of NO coincides with the formation of intestinal apoptotic epithelial cell lesions. These lesions, when they appear naturally, are found in late-day, moribund mice that

suffer from aGVHD. If the gut could be protected from this injury, perhaps this lethality of GVHD would be mitigated.

The use of rHuKGF as a candidate for preventing GVH-related mortality has only recently become apparent. KGF is a paracrine mediator of epithelial cell growth.¹⁷⁰ The KGFR has also been found to be expressed on the epithelial cells found within the gut, a major target of the GVH reaction.¹⁸⁰ As epithelial cells appear to be an important target in the GVH reaction,⁶⁷ KGF may represent a means of protecting against lethal GVHD while maintaining the beneficial GVL effect.

The ability for KGF to protect against deadly tissue damage has been demonstrated in a variety of lethal models of oxidant-^{187,216} radiation-^{181,182,185,188,189} and chemical-induced injuries.^{184,188}

In irradiated models of GVHD, KGF has been shown to improve survival and to limit weight loss as well as limit tissue injury to the lungs, skin and thymus.^{194,195} Aside from its ability to mitigate the injury caused by the GVH reaction, KGF can also promote IL-13 production and a lower Th1 cytokine response in a SCID mouse model of GVHD.¹⁹⁶ Another group has also demonstrated that KGF can shield the gastrointestinal tract from the damage associated with an irradiated, pre-conditioned mouse model of GVHD.¹⁹⁷

While many studies have focused on the ability for KGF to ameliorate tissue injury due to the BMT conditioning regimen, few have directly examined the ability of KGF to protect against the injury induced by the GVH reaction itself. As the gut is a major target in the GVH reaction, and its injury promotes LPS leakage critical to the

lethal outcome we embarked on a study to determine the effects of rHuKGF on GVH-induced injury.

4.2 Experimental design:

KGF-treated mice were given daily sub-cutaneous injections of rHuKGF for a seven-day time-course beginning 3 days prior to induction. GVHD was induced in B6D2F₁-hybrid mice with grafts from wild-type or IFN- γ gene knockout donor C57BL/6 mice. KGF-treated GVH mice were monitored and weighed to predict the onset of mortality. To ensure that the effects of the rHuKGF were not due to a failure to engraft, the number of donor T cells was measured by flow cytometry. We specifically focused on the ability of rHuKGF to protect the gastrointestinal tract during the GVH reaction. IFN- γ mRNA, TNF- α mRNA and NO levels were measured in the intestine of KGF-treated mice. The ability for LPS to induce increased intestinal NO and serum TNF- α was also monitored. LPS-induced intestinal lesion formation was studied in the KGF-treated GVH mice. We also measured serum endotoxin levels in the KGF-treated mice and compared them with untreated control mice and mice with GVHD. Intestinal KGF and KGFR mRNA were measured in GVH mice and compared with the levels seen in recipients of grafts from IFN- γ gko mice. Since treatment with KGF lowers serum and intestinal IFN- γ levels, the ability for rHuKGF-treatment to restore KGFR levels in GVH mice was also examined. Cytokine levels, IL-4, IL-5, IL-10, IL-13 and IFN- γ , were measured in spleen cell cultures from KGF-treated GVH mice.

4.3 Results:

4.3.1 The effect of rHuKGF on GVH-induced mortality and weight loss:

The ability of rHuKGF to protect against the mortality and wasting associated with acute GVHD is shown in Figure 4.1A. Mortality was 100% in the untreated recipients of C57BL/6 grafts, with the first deaths occurring on day 19 post-induction. All of the mice in this group had succumbed by day 68. On the other hand, only one mouse died during a similar period of time in the group that received the rHuKGF treatment regimen. The remaining rHuKGF-treated mice survived beyond day 89.

Both groups of mice began to lose weight beginning on the twelfth-day post-induction and continued to do so until day 20 where the weights began to stabilize, Fig. 4.1B. At this time-point, untreated mice with GVHD had lost 22.7% of their initial weight. On the other hand, rHuKGF-treated GVH mice had lost only 7.7% of their initial body weight by this day. In the following weeks, the rHuKGF-treated mice also recovered some of the weight that they had lost during the course of the reaction. This weight-gain, however, never reached the level that was measured in the mice pre-induction.

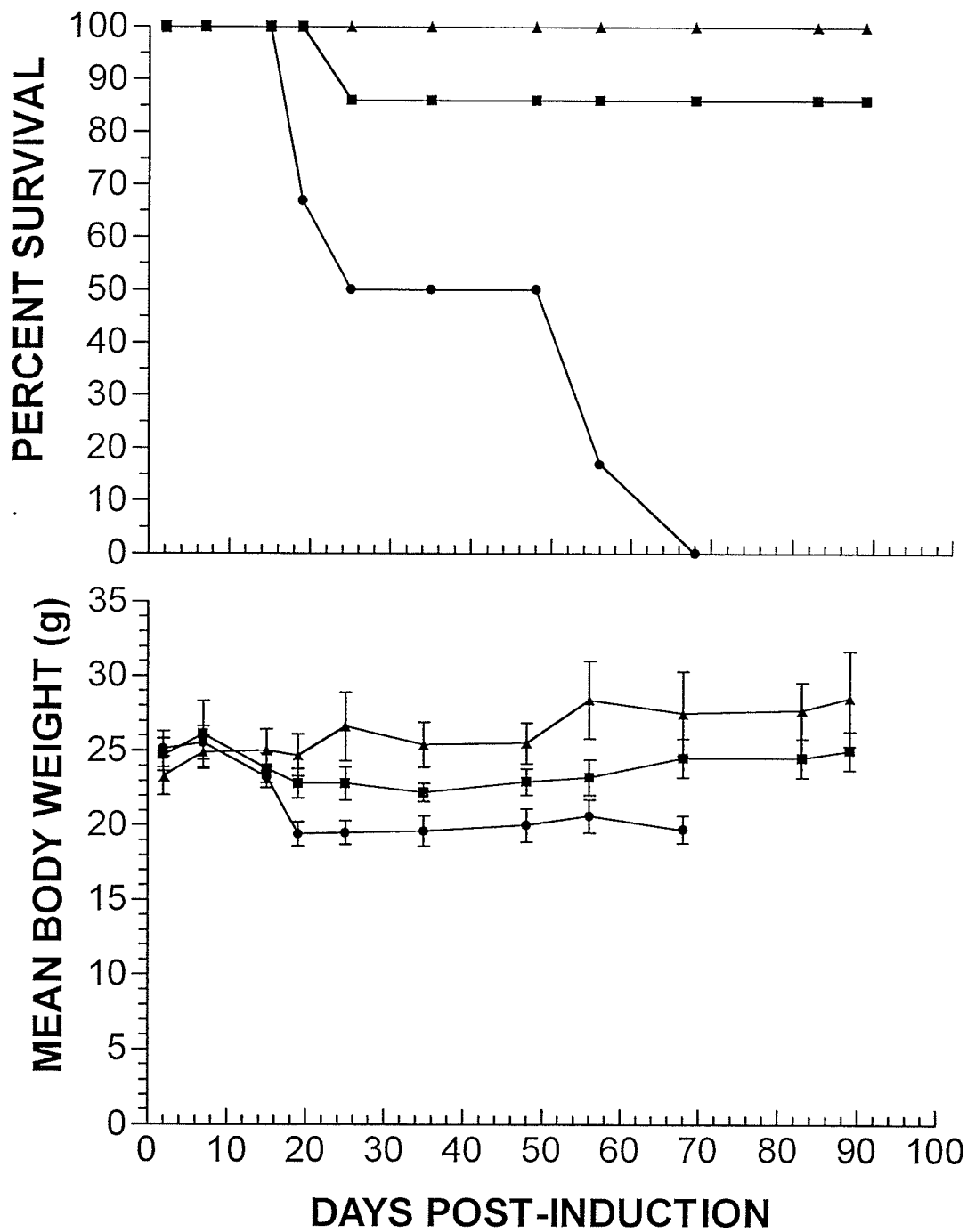
Both weight loss and mortality were absent from B6D2F₁-hybrid control mice that did not receive a graft but were treated with rHuKGF.

4.3.2 Engraftment of CD4⁺ and CD8⁺ T cells:

Flow cytometry was used to determine the percentage of spleen cells that were of donor origin. Spleen cells were analyzed for the expression of the H-2D^d haplotype. Since this haplotype is expressed only on B6D2F₁-hybrid host cells, any cell not

Figure 4.1.

The number of mice surviving on various days post-induction is demonstrated in Figure 4.1A. This can be seen for untreated, C57BL/6 graft recipients (●, n=7), rHuKGF-treated recipients of C57BL/6 grafts (■, n=6) and rHuKGF-treated B6D2F₁-hybrid control mice that did not receive grafts (▲, n=3). Figure 4.1B graphs the changes in weight seen in these mice, untreated GVH mice (●), rHuKGF-treated mice (■) and in the B6D2F₁-hybrid controls (▲). Error bars represent the standard error associated with the mean body weights of all surviving mice, in each group, with the last recorded weight from mice that died of GVHD (phantom weights).



expressing this marker must, by default, be of donor origin. Figure 4.2 shows representative histograms of the flow cytometric data for one mouse in rHuKGF-treated GVH group on day 8 post-induction. Histograms for comparison with untreated GVH mice can be seen elsewhere.¹⁰³

A comparison of the numbers of engrafted cells in both untreated and rHuKGF-treated GVH mice is shown in Figure 4.3. The percentage of donor derived cells were similar in both groups at the day 8 time-point. By day 13 post-induction, rHuKGF-treated mice with GVHD had significantly more total CD4⁺ and CD8⁺ cells when compared with the untreated GVH group ($P < 0.05$). Engrafted donor cells were also present, day 116 post-induction in rHuKGF-treated mice.

4.3.3 Cytokine measurements from spleen cell cultures:

The Th1 cytokine IFN- γ and the Th2 cytokines IL-4, IL-5, IL-10 and IL-13 were measured in spleen cell cultures from both untreated and rHuKGF-treated GVH mice. Th2 cytokine ELISAs were performed by Bill Stefura. Cultures were set up on days 1, 4, 8 and 15 post-induction. For comparison, spleen cell cultures from B6D2F₁ control mice that did not receive grafts were also sampled at the same time points, Figure 4.4.

Culture supernatants from untreated graft recipients contained IFN- γ only on day 8 post-induction. This is consistent with previous findings.¹⁰⁵ KGF-treated recipients, on the other hand, had measurable IFN- γ in cultures from each of the four days tested. However, the only level that was significantly greater than control BDF₁ levels was the value obtained on day 8 ($P < 0.001$). This day 8 level of IFN- γ from rHuKGF-treated GVH

Figure 4.2.

A representative histogram, used to identify the lymphocyte population, depicts the forward angle versus side light scatter of spleen cells in Figure 4.2A. The population of cells determined to be lymphocytes can be seen gated. Histograms based on 2-colours from one day 8 post-induction rHuKGF-treated mouse are seen in Figure 4.2B, C and D. H2-D^d FITC labeled cells are depicted on the horizontal axis for histograms 4.2B, C and D. Figure 4.2B demonstrates the number of donor and recipient non-adherent cells present in the spleen. Donor cells appear as H2-D^d negative cells in the lower left quadrant. Host cells are H2-D^d positive and appear in the lower right quadrant. PE-conjugated rat IgG_{2a} is used as an isotype control for anti-CD4 and anti-CD8 binding. Figures 4.2C and 4.2D show the proportions of CD4⁺ and CD8⁺ PE-stained host and donor cells. FITC-conjugated mouse IgG_{2a} was used as an isotype control for anti-H2-D^d labeling where non-specific labeling was less than 1% (data not shown). Each histogram was drawn from 5000-gated events.

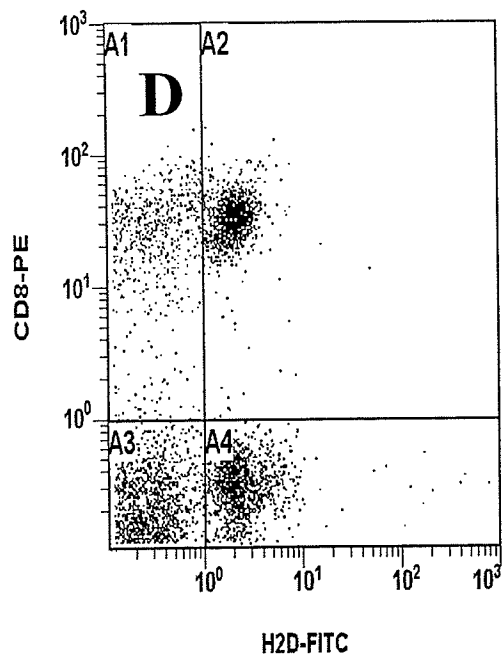
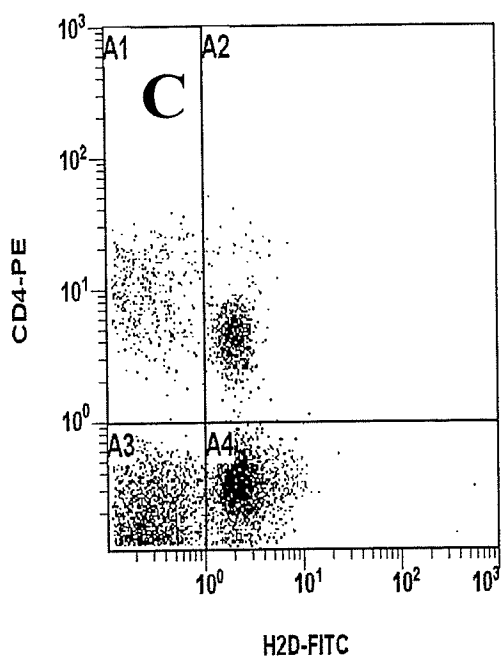
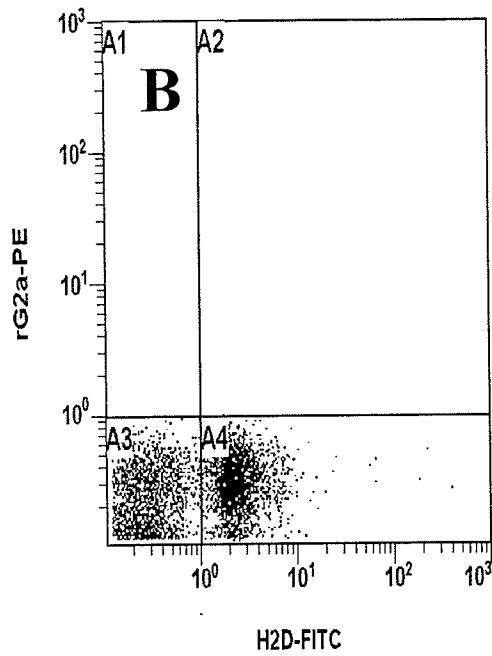
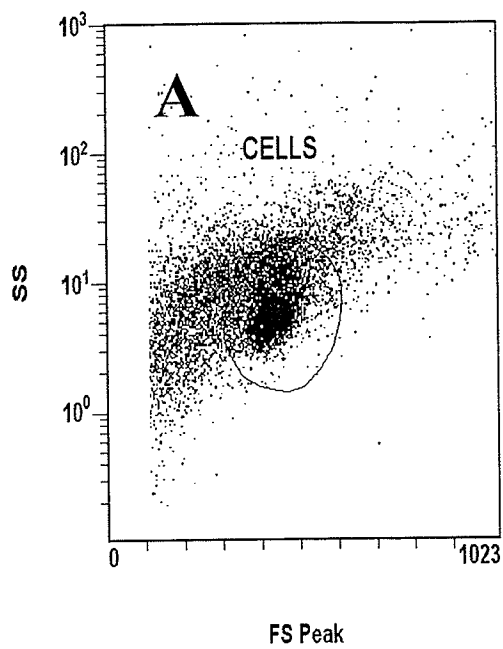
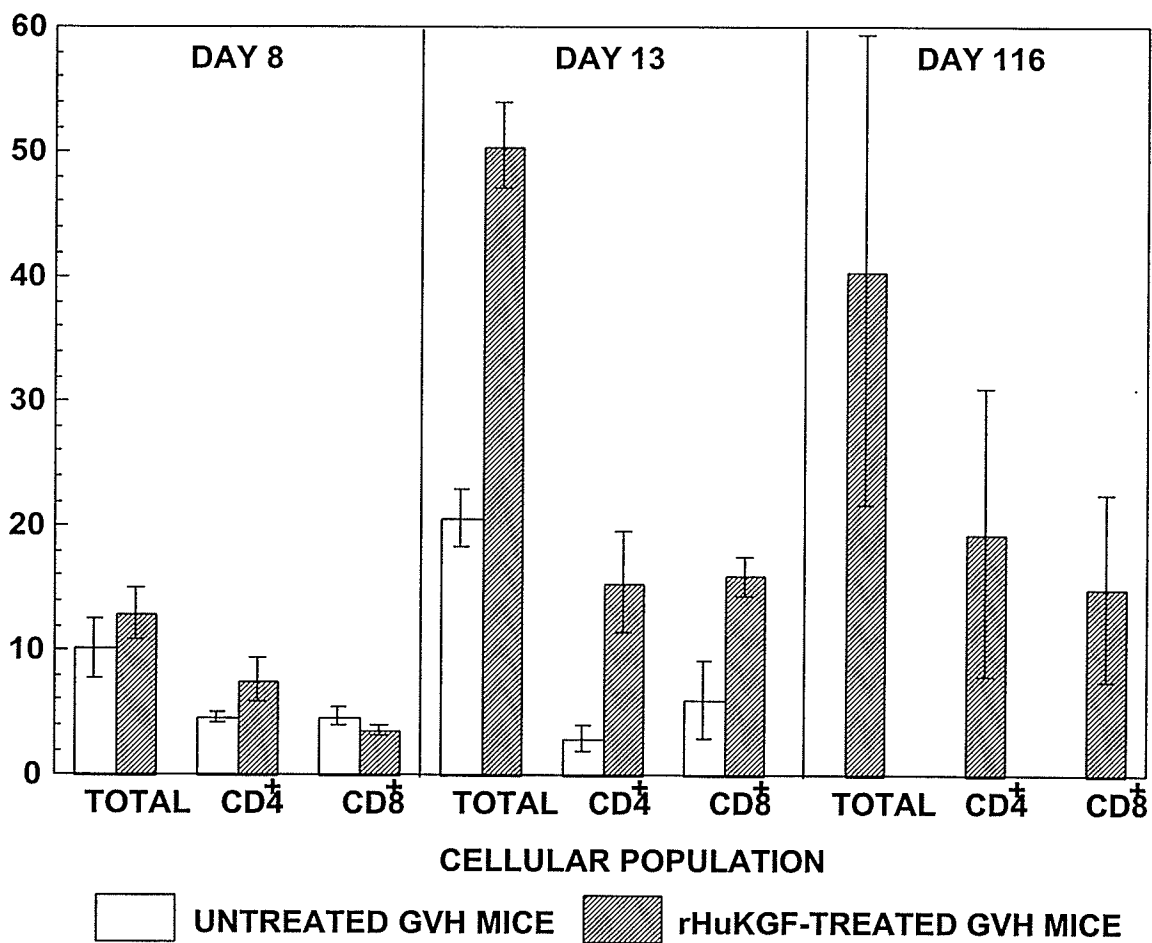


Figure 4.3.

The level of donor cell engraftment is compared in Figure 4.3, untreated mice with GVHD (open bars) and rHuKGF-treated mice (hatched bars). The percentages of total cells, CD4⁺ and CD8⁺ cells are shown for days 8 and 13 post-induction for both groups of mice as well as for day 116 post-induction for rHuKGF-treated GVH mice. Error bars demonstrate the standard error found for the mean percentages of three mice in each group. The percentage of total donor-derived cells for rHuKGF-treated GVH mice was found to be significantly greater on day 13 ($p < 0.05$, student's T test).



mice was not greater than the day 8 level measured in the untreated graft recipients ($P < 0.001$).

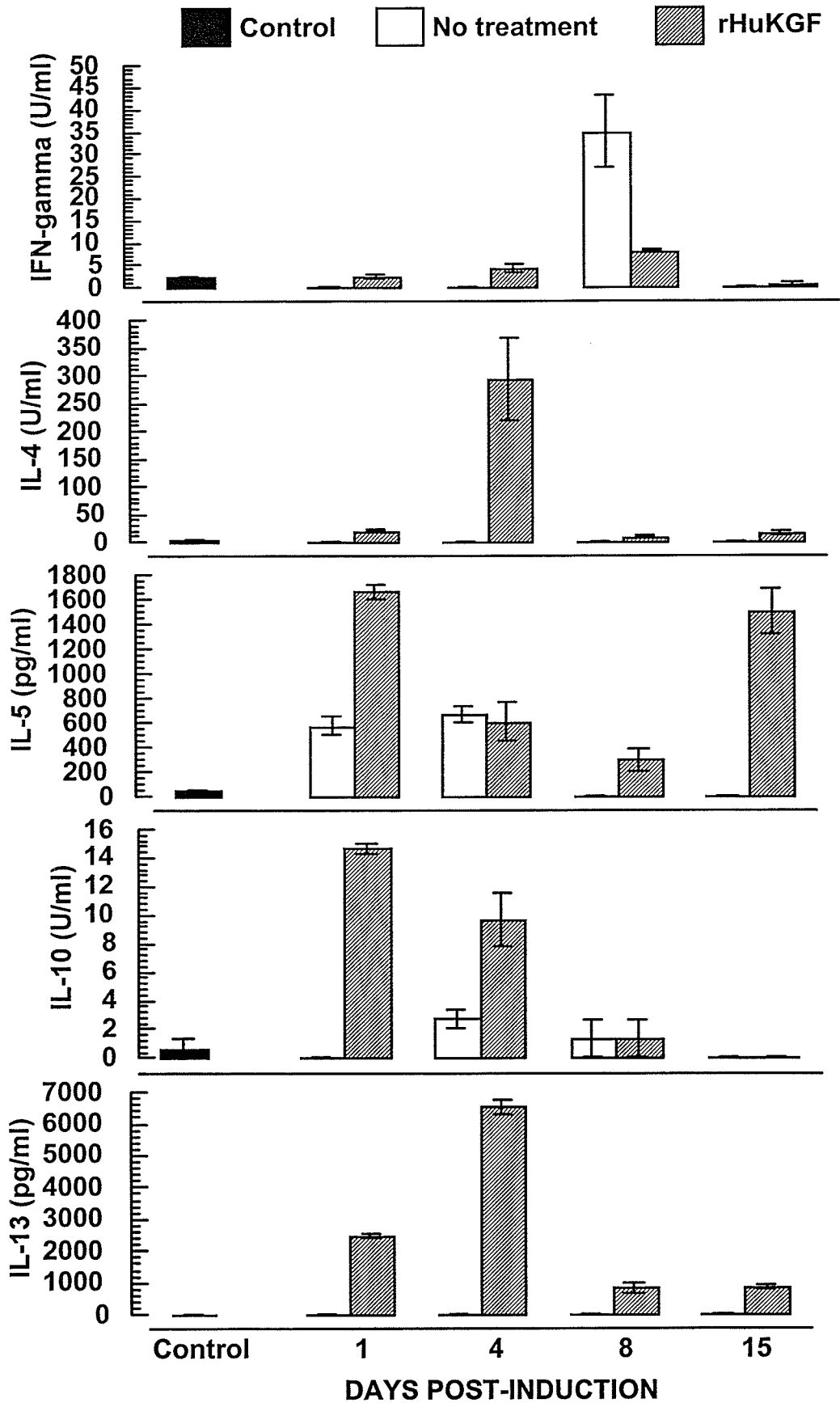
The Th2 cytokines IL-4 and IL-13 could not be detected in cultures from untreated GVH mice. These cytokines were however present in rHuKGF-treated mice with GVHD (Figure 4.4). Although present in culture on all days tested, IL-4 was only significantly different from the control value on day 4 in these mice. IL-13 levels were significantly different from BDF₁ control levels on each and all of the days tested in the rHuKGF-treated group. IL-5 production was also observed on all days tested in the GVH group treated with rHuKGF but this cytokine could only be detected on day 1 and day 4 cultures from untreated GVH mice. All of these measurements were significant when compared to normal BDF₁ mice. On the two days where IL-5 was detected in untreated GVH mice, only the day 1 values were significantly lower than their rHuKGF-treated counterparts ($P < 0.001$). The cytokine IL-10 was only seen on days 4 and 8 in both groups of mice. No IL-10 could be detected in untreated mice on day 1 or in either group on day 15. IL-10 levels were only significantly greater in the rHuKGF-treated group, when compared to the untreated mice, on day 4 ($P < 0.01$).

4.3.4 The effect of rHuKGF on intestinal IFN- γ mRNA and TNF- α mRNA levels:

Because the intestine is a primary target in aGVHD, we performed assays to determine whether the levels of intestinal IFN- γ and TNF- α were affected by the rHuKGF treatment. IFN- γ and TNF- α mRNA levels were measured in the intestine of untreated or rHuKGF-treated mice with GVHD on days 1, 4, 8 and 15 post-induction (Figure 4.5). A significant increase over BDF₁ controls was seen in the IFN- γ mRNA

Figure 4.4.

Th₁ cytokine IFN- γ and Th₂ cytokines IL-4, IL-5, IL-10 and IL-13 were measured in spleen cell culture supernatants on days 1, 4, 8 and 15 by ELISA. Cytokines were measured in both untreated and rHuKGF-treated recipients of C57BL/6 grafts as well as B6D2F₁-hybrid control mice. Error bars represent the standard error of the mean cytokine levels obtained from 3 individual mice. The differences between the IFN- γ levels seen in both groups on day 8 and the control values were statistically significant ($p < 0.001$, untreated GVH mice; and $p < 0.05$, rHuKGF-treated GVH mice). The differences between IFN- γ levels on day 8 in untreated and rHuKGF-treated GVH mice were statistically significant ($p < 0.001$). The difference between IL-5 levels in the rHuKGF-treated group and control values were statistically significant on all days except for day 8. The difference between the IL-5 levels on day 1 in the two groups were statistically significant ($p < 0.001$). Levels of IL-10 were significantly higher in the rHuKGF-treated group on day 4 ($P < 0.01$). All analyses were by ANOVA followed by a Tukey-Kramer multiple comparison test.



levels on day 8 post-induction in both untreated and rHuKGF-treated mice ($P < 0.001$) as shown in Figure 4.5A. A nearly two-fold difference in IFN- γ mRNA was seen on this day in the untreated GVH mice when they were compared with the rHuKGF-treated group. By day 15, intestinal IFN- γ mRNA levels had decreased in the untreated GVH group and were no longer significantly greater than those seen in the rHuKGF-treated mice with GVHD. However, IFN- γ levels were still significantly greater than control values ($P < 0.05$).

TNF- α mRNA appeared to increase in both GVH groups on days 8 and 15 post-induction when compared with B6D2F₁-hybrid control mRNA levels (Figure 4.5B). Only the day 15 value for TNF- α in the rHuKGF-treated group was found to be significantly greater than the control value ($P < 0.05$). No significance could be detected between the untreated and the rHuKGF-treated groups of GVH mice on any of the observed time-points.

The detection of the tissue mRNA levels, by RPA, was completed by S.A. Scully of Amgen Inc.

4.3.5 Comparison of TNF- α serum levels in untreated and rHuKGF-treated mice with GVHD following injection with LPS:

Serum TNF- α levels were measured on day 8 and day 13 post-induction in both rHuKGF-treated and untreated mice with GVHD (Table 4.1). Sera from normal control mice were also assayed. No TNF- α could be detected in the BDF₁ control mice even after injection of 10 μ g of endotoxin. Untreated mice with GVHD also demonstrated an absence of TNF- α in the serum on day 8 but a small amount could be detected on day 13.

Figure 4.5.

RPA was used to measure cytokine mRNA levels in untreated and rHuKGF-treated acute GVH mice, as well as in B6D2F₁-hybrid control mice. GVH reactions were induced using grafts from C57BL/6 donors. Error bars represent the standard error of the mean cytokine levels obtained from 3 individual mice. The cytokine mRNA levels were normalized to Cyclophilin mRNA. The difference between the IFN- γ mRNA levels in untreated and rHuKGF-treated GVH mice was statistically significant on day 8 ($p < 0.001$). The increase in the IFN- γ mRNA level seen on day 8 in rHuKGF-treated acute GVH mice, when compared to the control level, was statistically significant ($p < 0.001$). The IFN- γ mRNA levels seen in untreated and rHuKGF-treated GVH mice on day 15 were significantly higher than the control level as well ($p < 0.01$ and $p < 0.05$, respectively). The increase in TNF- α mRNA expression in rHuKGF-treated GVH mice on day 15, when compared to control level, was statistically significant ($p < 0.05$). None of the other TNF- α mRNA levels were significantly different from control values ($p > 0.05$).

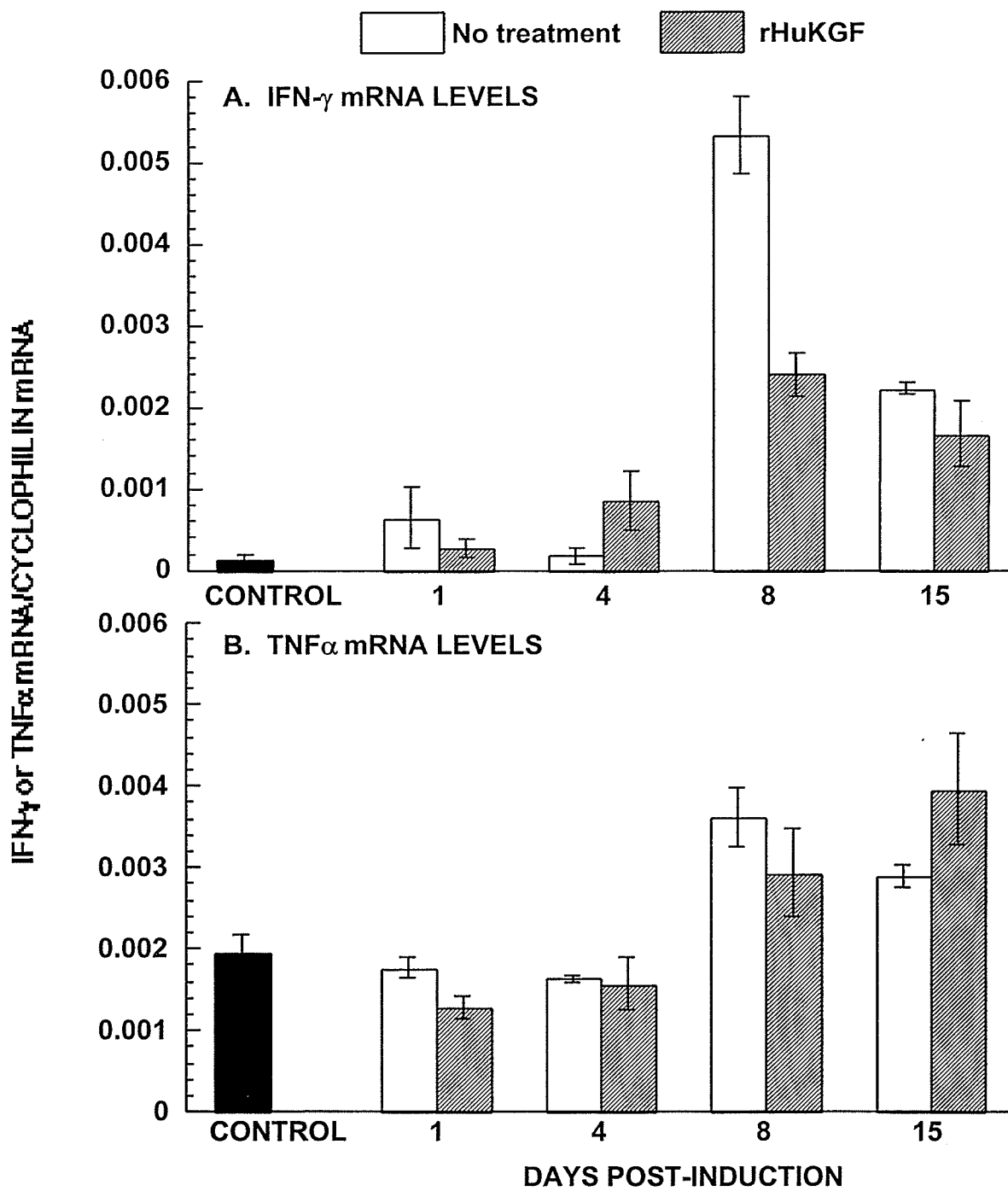


Table 4.1. Constitutive and LPS-induced TNF- α levels in serum of untreated and rHuKGF-treated acute GVH mice.

Experimental Group ^{1,2}	TNF- α (ng/ml)	TNF- α (ng/ml)	TNF- α (ng/ml)	TNF- α (ng/ml)
	No LPS	+ LPS	No LPS	+ LPS
	Day 8	Day 8	Day 13	Day 13
GVHD	<0.06 ³	75 \pm 35	75 \pm 25 ⁴	340 \pm 25
GVHD + rHuKGF	<0.06	> 1150 ³	<0.06	>1150

¹n=3 for each experimental group

²A third group consisting of normal control mice was also included. These mice (n=3) showed no detectable TNF- α in the serum (i.e., values <0.06) whether or not they had been injected with LPS.

³Serum TNF- α levels were measured by ELISA. Values shown as <0.06 indicate a level below which cytokine could not be measured reliably. Values shown as >1150 indicate a level of cytokine above the highest value in the linear portion of the standard curve.

⁴The increase in the serum level of TNF- α following LPS injection was statistically significant compared to the constitutive level (p<0.02; Student's *t* test).

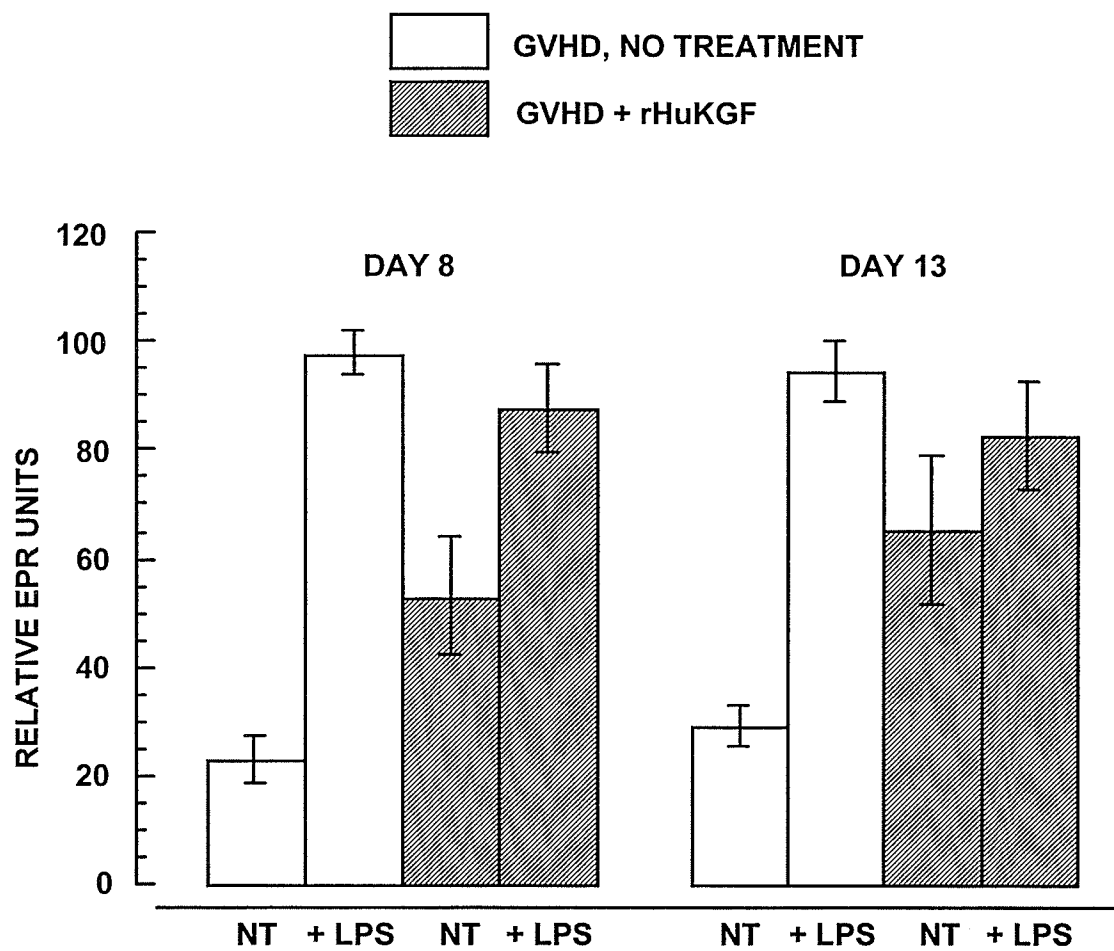
An increase in TNF- α levels was observed after injection with LPS. This increase was found to be significantly greater than the constitutive values ($P < 0.02$). Sera from the rHuKGF-treated group that were not injected with LPS contained no measurable TNF- α . The effect of the LPS injection on this group was similar to that seen in the untreated GVH group. However, rHuKGF-treated mice demonstrated significantly higher serum levels of LPS-induced TNF- α when compared to their untreated counterparts.

4.3.6 The effect of rHuKGF on intestinal Nitric Oxide levels:

As demonstrated in chapter 3, NO is a key mediator of intestinal injury. We therefore performed experiments to determine whether the ability for rHuKGF to prevent early-day mortality was due to an effect on intestinal NO production. The bar graph in Figure 4.6 compares the NO levels seen in untreated GVH control mice with the levels observed in rHuKGF-treated mice with GVHD, on both day 8 and day 13. LPS-induced NO levels are also shown. Interestingly, the rHuKGF-treated mice had higher constitutive levels of NO in their intestines on both day 8 and day 13 when compared with the untreated GVH group ($p < 0.01$). NO levels rose significantly above the constitutive level when LPS was injected into day 8 mice of both groups, ($p < 0.001$) for the untreated group and ($p < 0.05$) for the rHuKGF-treated group. The level of NO, in the untreated group, appears to demonstrate a larger increase. Similar results were seen on day 13 GVH mice; however, LPS only induced a significant increase over the constitutive NO level in the untreated GVH mice ($p < 0.05$). NO could not be detected in intestinal tissue collected from B6D2F₁-hybrid control mice (data not shown).

Figure 4.6.

NO was measured in wild-type and rHuKGF-treated recipients of C57BL/6 grafts on day 8 and day 13 post-induction. The bar graph shows intestinal NO levels for both constitutive and LPS-induced NO production. NO was undetectable in the intestines of B6D2F₁-hybrid control mice (n=3, data not show). Error bars represent the standard error of the mean NO level in three individual mice. Constitutive NO levels were significantly greater in the rHuKGF-treated group on day 8 post-induction when compared to the untreated GVH mice ($p < 0.01$). Both untreated and rHuKGF-treated GVH mice had significantly greater NO levels on day 8, upon stimulation with LPS, when compared to their respective constitutive levels ($p < 0.05$ and $p < 0.001$ respectively). NO levels produced after LPS injection were also significantly greater on day 13 post-induction in the untreated GVH group ($p < 0.05$). Comparisons were done using ANOVA followed by A Tukey-Kramer multiple comparison test.



4.3.7 The effect of rHuKGF on intestinal epithelial cell apoptosis:

KGF has been demonstrated to be a potent stimulant of epithelial cell growth capable of limiting the tissue injury associated with radiation, chemotherapy and other toxic agents whose mechanisms promote apoptosis.^{181,182,184,185,187-189,216} Since NO has been implicated in the formation of the lethal, LPS-induced intestinal lesion, (chapter 3) and since the production of NO in response to LPS is not inhibited by rHuKGF treatment, it seemed likely that the growth factor's protective effect was due to its ability to prevent intestinal cell apoptosis in mice with aGVHD. Figure 4.7 demonstrates this effect. The LPS-induced lesion can be detected as early as day 8 post-induction in untreated graft recipients Fig. 4.7 C-D. This lesion can be found throughout the jejunum, ileum and the colon of all untreated mice suffering from acute GVHD. It is, however, never seen in the rHuKGF-treated GVH mice Fig. 4.7E-H. LPS-induced epithelial cell apoptosis was also absent in sections of B6D2F₁-hybrid control mice. Fig. 4.7A-B.

4.3.8 rHuKGF prevents the accumulation of endotoxin in the serum of GVH mice:

We hypothesized that treatment with rHuKGF should prevent the accumulation of endotoxin in the serum because it prevents intestinal epithelial cell apoptosis and preserves the integrity of the intestine. Serum endotoxin levels were therefore measured in untreated, untreated moribund and rHuKGF-treated GVH mice using the LAL assay Table 4.2. As expected, all four untreated mice, sacrificed when moribund, had detectable levels of endotoxin in their sera. Endotoxin was also detected on day 15 post-induction in 6 of 8 mice from the untreated group, in 2 of 6 rHuKGF-treated GVH mice and in 1 of 3 control mice. When quantified the level of endotoxin in the rHuKGF-treated mice was

Figure 4.7.

H&E-stained sections of jejunum, from B6D2F₁-hybrid control mice (4.7A and 4.7B), untreated day 8 GVH mice (4.7C and 4.7D) and rHuKGF-treated GVH mice on day 8 post-induction (4.7E and 4.7F) and day 116 post-induction (4.7G and 4.7H) are shown. GVHD was induced using C57BL/6 donor mice. Mice in 4.7B, 4.7D and 4.7F were infected with 10 μ g of LPS and sacrificed 90 minutes later. Examples of apoptotic crypt cells are indicated by arrows in 4.7D. Magnifications are 250x.

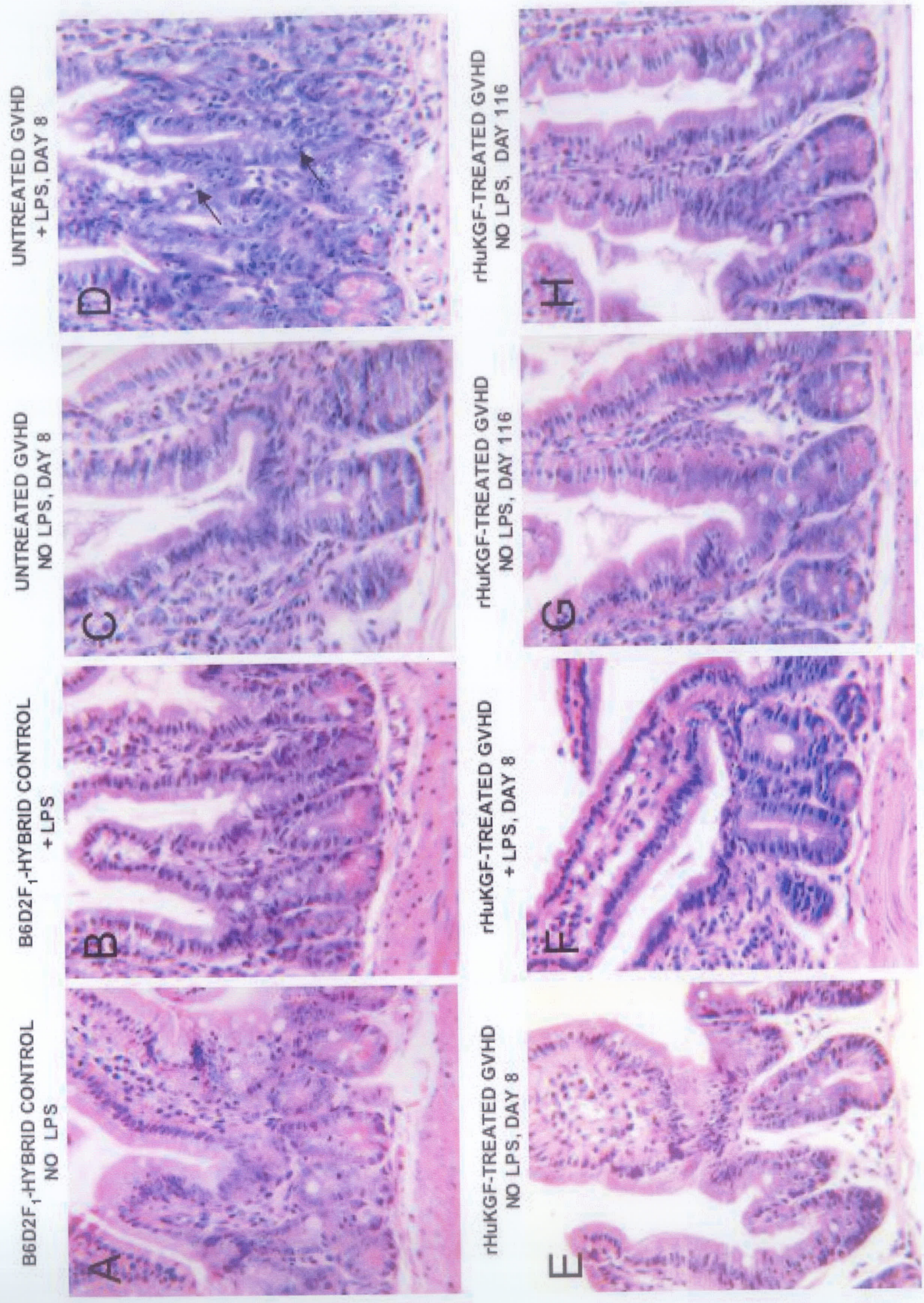


Table 4.2. Serum endotoxin levels in untreated and rHuKGF-treated acute GVH mice.

Group	Mean Level \pm SEM EU $\times 10^2$
Untreated GVH Mice, Day 15	49.6 \pm 11.1 ¹
rHuKGF-treated GVH Mice, Day 15	3.1 \pm 2 ²
Moribund, Untreated GVH Mice ³	61.0 \pm 7.5
B6D2F ₁ -hybrid Control Mice	8.2 \pm 8.2

¹The differences in the mean endotoxin level observed on day 15 in untreated GVH mice (n=8) was significantly different from that seen on day 15 in rHuKGF-treated GVH mice (n=8) and in B6D2F₁ control mice [(n=3) p<0.01]. The difference in endotoxin levels observed on day 15 in untreated GVH mice and moribund, untreated GVH mice (n=4) was not statistically significant (p>0.05). For all comparisons, ANOVA followed by a Tukey-Kramer multiple comparison test was used.

²The difference in the endotoxin levels observed in rHuKGF-treated GVH mice and control mice was not statistically significant (p>0.05).

³Moribund untreated GVH mice were sacrificed on days 16 and 18 post-induction.

found to be significantly lower than the levels seen in the moribund and in the day 15 untreated GVH group ($p < 0.01$). It was also found to not be significantly different from control mice ($p < 0.05$).

4.3.9 Histopathology in the tissues of rHuKGF-treated GVH mice:

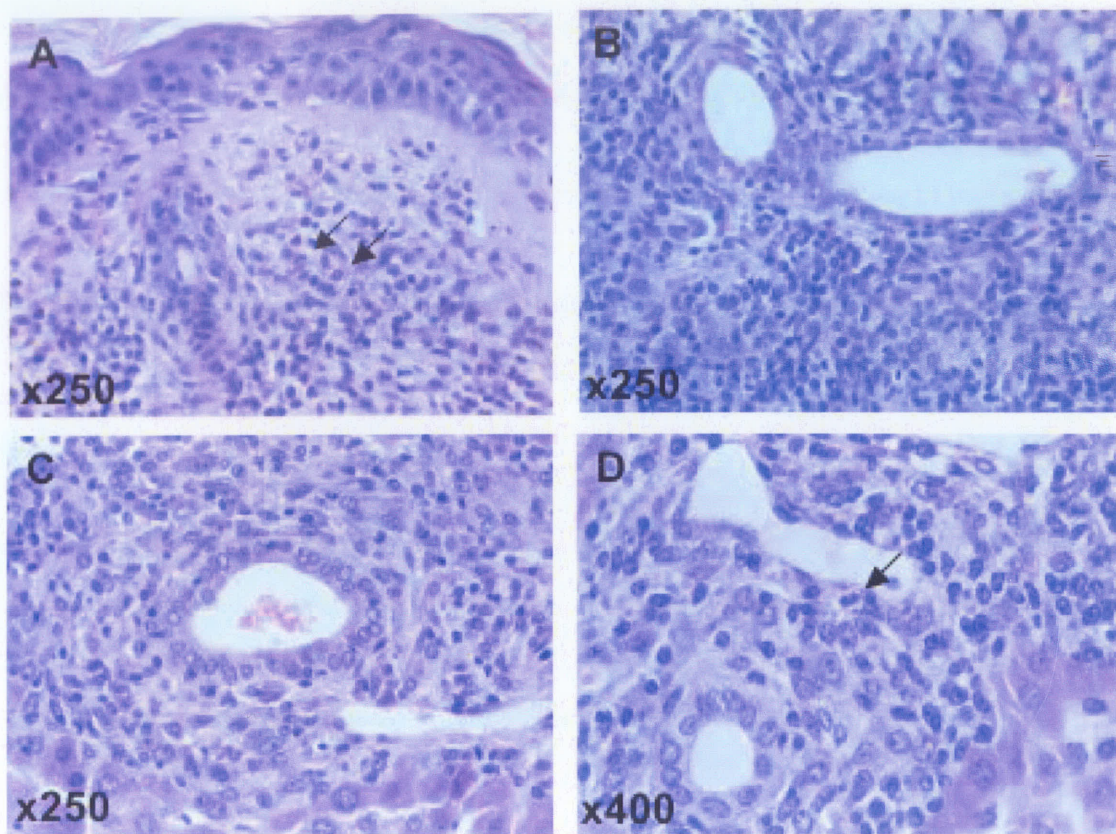
To further characterize the effect of the rHuKGF treatment on the GVH response, we examined tissue from various organs that are targeted in the acute GVH reaction (Figure 4.8). Infiltrates of lymphocytes admixed with eosinophils were observed as early as day 13 post-induction and are depicted on day 125 in the skin (Figure 4.8A). More extensive lesions were observed around the excretory ducts in the salivary gland (Figure 4.8B) and in the portal areas of the liver (Figures 4.8C-D). These infiltrates were also present on two other days tested, day 15 and day 116 post-induction. These changes were not identified in rHuKGF-treated control mice that did not receive a graft.

4.3.10 Levels of KGF and KGFR mRNA expression in the intestines of mice with GVHD:

KGF and KGFR mRNA levels were measured in the intestines of GVH mice by an RNase protection assay, performed by S.A. Scully. Figure 4.10 demonstrates these levels in a representative gel from a group of untreated mice with GVHD. Figure 4.11A summarizes this data graphically. The level of KGF mRNA expression decreases significantly on day 8 post-induction when compared to control levels. This drop in intestinal KGF expression is also significantly lower than values obtained on day 1 and day 4 ($p < 0.001$ and $p < 0.05$ respectively) post-induction. The intestinal KGFR mRNA

Figure 4.8.

Photomicrographs of skin (4.8A), salivary gland (4.8B) and liver (4.8C and 4.8D) were taken from H&E-stained tissue of day 125 rHuKGF-treated GVH mice. GVH reactions were induced using grafts from C57BL/6 donors. Arrows point to eosinophils present in the infiltrates.



levels were also significantly lower day 8 post-induction when compared to control mice ($p < 0.05$) and to the day 1 ($p < 0.05$) and day 16 ($p < 0.01$) values for untreated GVH mice. Intestinal KGF and KGFR mRNA expression levels were also determined for mice that received IFN- γ gko grafts (Figure 4.11B). KGF mRNA expression showed a similar decrease on day 8 to that seen in wild-type graft recipients. However, unlike wild-type graft recipients, KGFR mRNA did not decrease below control levels on day 8 in the recipients of IFN- γ gko grafts. Instead, it remained similar to the values observed in the B6D2F₁-hybrid control mice.

4.3.11 Ability of rHuKGF treatment to restore intestinal KGF and KGFR expression in mice with aGVHD:

The level of intestinal IFN- γ mRNA is lower in rHuKGF-treated GVH mice. Since IFN- γ may be responsible for the lower level of KGFR expression in the intestines of mice with GVHD, we performed experiments to determine whether rHuKGF-treatment could avert this outcome. A comparison of KGF and KGFR expression in the intestines of untreated and rHuKGF-treated GVH mice is shown in Figure 4.12. Interestingly, despite receiving the rHuKGF treatment regimen, KGFR levels are reduced to the same level, on day 8 post-induction, as that seen in the untreated GVH group.

Figure 4.9.

Phosphorimage showing results from an RNase protection assay used to determine the levels KGF and KGFR in the intestinal tissue of untreated GVH mice that received grafts from C57BL/6 donors. Tissue was collected upon sacrifice from three mice on days 1, 4, 8 and 16 post-induction. Control tissue was collected from B6D2F₁-hybrid mice that did not receive grafts. Expression levels were normalized to either Cyclophilin or GADPH, both of which provided similar results. Bands were quantified using Image Quant software using the local average background correction. P/C denotes positive controls for KGF and KGFR.

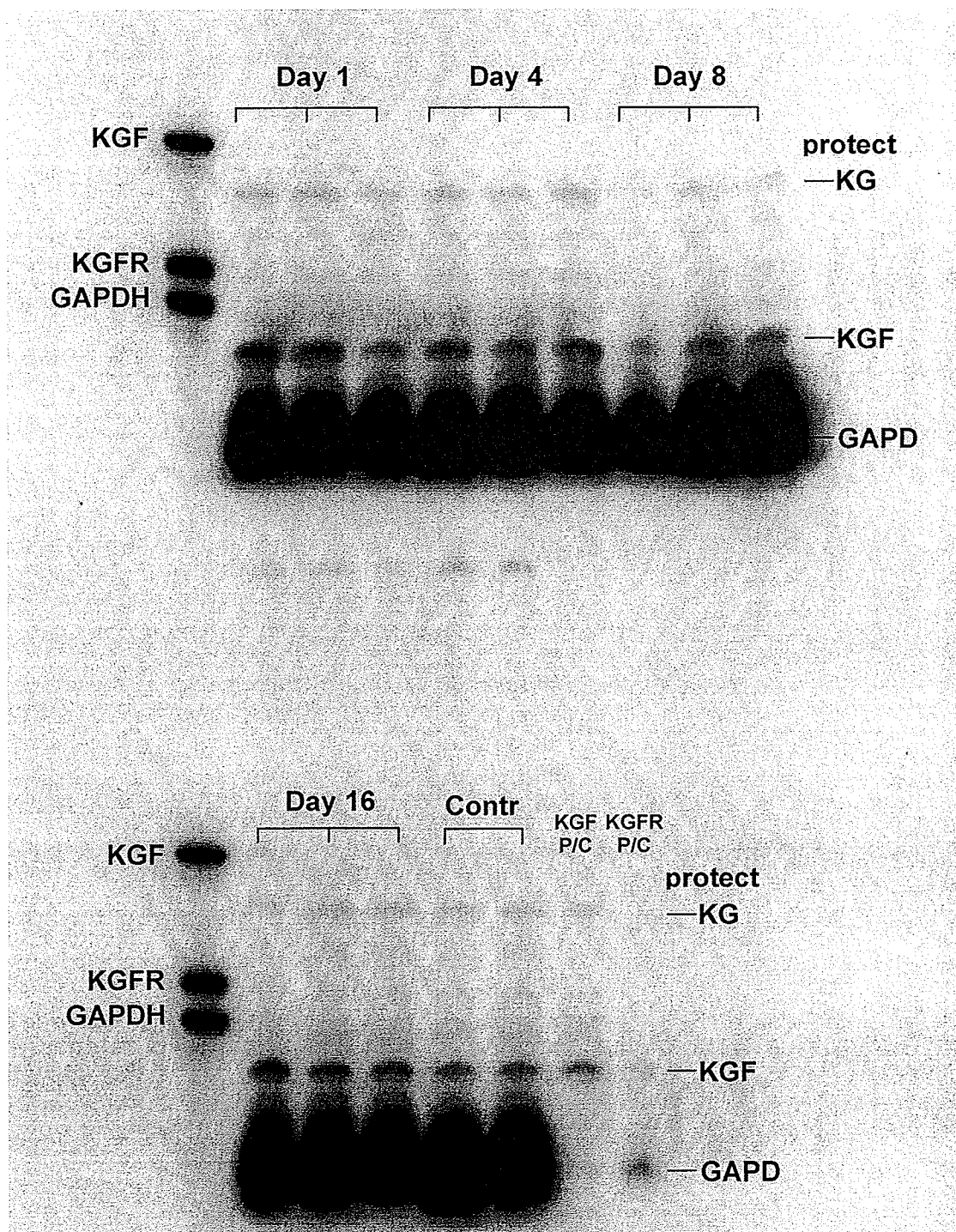


Figure 4.10.

Histogram depicting KGF and KGFR mRNA levels, detected by RNase protection assay in recipients of grafts from wild-type C57BL/6 donors (4.11A) or grafts from IFN- γ gko mice (4.11B). Error bars represent the standard error of the mean mRNA levels observed in intestinal tissue from 3 individual mice. The levels of KGF mRNA expression observed on day 8 in both recipient groups were significantly less than those measured on day 1 ($p < 0.001$ for both groups of recipients) and day 4 ($p < 0.05$ and $p < 0.01$ for recipients of C57BL/6 and IFN- γ gko grafts respectively). The level of KGFR mRNA expression in recipients of wild-type grafts on day 8 post-induction was significantly less than that seen in control mice ($p < 0.05$) and in GVH mice on days 1 ($p < 0.05$) and 16 ($p < 0.01$) post-induction. Expression of KGFR in recipients of grafts from IFN- γ gko donors was greater than control values on days 1 and 4 post-induction, but this difference was statistically significant only on day 1 ($p < 0.05$). The level on day 1 was also significantly greater than that seen on day 8 ($p < 0.05$). There was no difference between levels on day 8 and controls. The data were analyzed by ANOVA, followed by a Tukey-Kramer multiple comparison test.

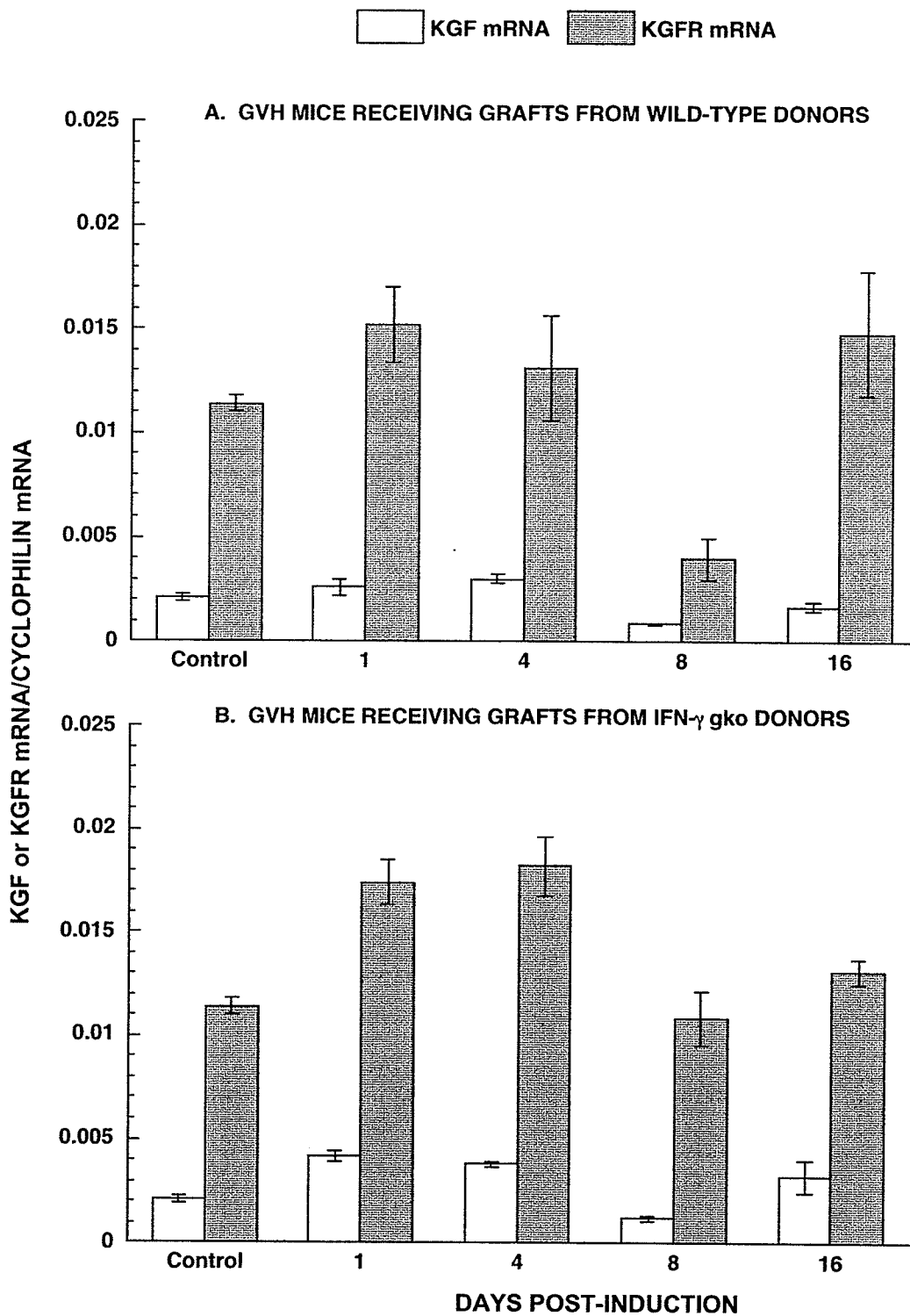
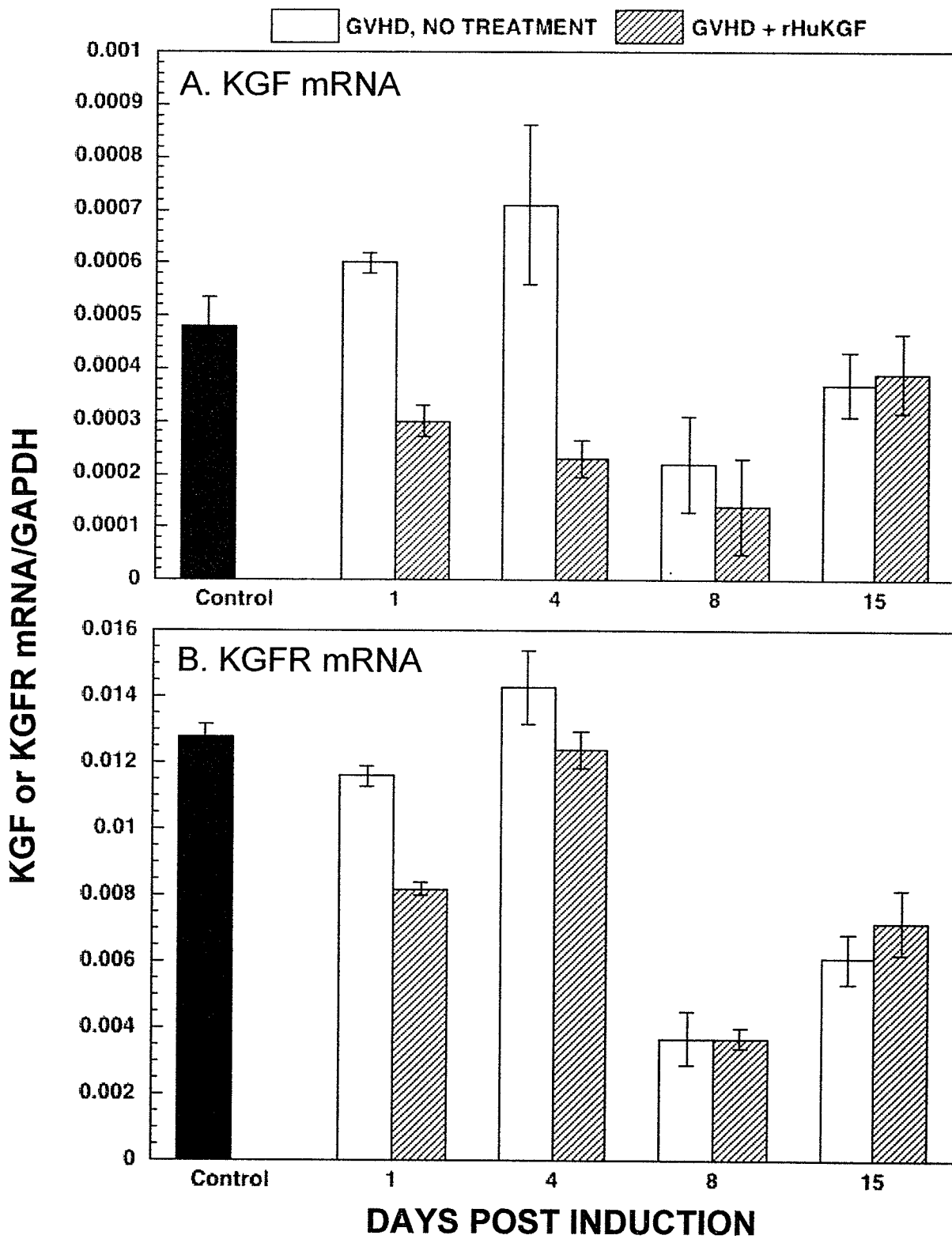


Figure 4.11.

Intestinal KGF and KGFR mRNA levels were compared in untreated and rHuKGF-treated GVH mice using RNase protection assays. A difference in KGF mRNA expression was found to be significant only on day 1 post-induction ($p < 0.01$). There were no significant differences between KGF mRNA levels in either GVH group and levels seen in control mice on all days tested ($p > 0.05$). The KGFR mRNA levels in the KGF-treated group on days 1, 4, 8 and 15 were significantly lower than the level in control mice ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively). The difference between the level in control mice and those in the untreated group was only significant on days 8 and 15 ($p < 0.001$). All statistical analyses were done by ANOVA followed by a Tukey-Kramer multiple comparison test.



CHAPTER 5:
DISCUSSION

5.1 The role of IFN- γ , NO and LPS

The results of this study show that, in contrast to their wild-type counterparts, recipients of IFN- γ gko grafts do not develop intestinal apoptotic epithelial cell lesions and do not succumb to a sub-lethal injection of 10 μ g of LPS. By comparison, intestinal lesions were observed in late-day, moribund mice that had received wild-type grafts. The sub-lethal injection of LPS was also found to be 100% lethal in the recipients of wild-type grafts.

It was also found that the injection of 10 μ g of LPS, to wild-type graft recipients on either day 8 or day 13 post-induction, induced the formation of intestinal apoptotic lesions 90 minutes after its administration. The administration of LPS to these mice also resulted in a burst of NO production in the intestine. This NO burst could be prevented by injection of mice with the NOS inhibitors L-NAME or L-NIL. We observed that these NOS inhibitors also prevented the development of LPS-induced apoptotic lesion. These findings indicate that NO is involved in the formation of intestinal lesions. The ability for L-NIL to prevent this LPS-induced NO burst and the ensuing intestinal lesion suggests that iNOS is a key enzyme in mediating these lethal events of intestinal GVHD

Different results were obtained when these same experiments were performed in recipients of IFN- γ gko grafts. In this model, it was found that the injection of 10 μ g of LPS did not induce intestinal lesion formation, nor did the administration of this dose of LPS trigger a NO burst in the intestine. These results therefore suggest that IFN- γ is also critical to the mechanism of intestinal GVHD.

In an attempt to further understand the role of this cytokine, experiments by Mowat *et al.* showed that administration of anti-IFN- γ antibodies to unirradiated F₁-hybrid recipients of parental strain grafts resulted in a marked reduction in the enteropathy associated with the GVH reaction. While this study did not determine the mechanism by which anti-IFN- γ antibodies protected mice from intestinal injury associated with GVHD, the authors of this study suggested that IFN- γ primes macrophages for TNF- α release which causes the ensuing injury to the gut. Our results however indicate that NO either alone or concurrent with TNF- α is responsible for the intestinal injury. Mowat also suggested that cytotoxic T cells (CTL) cannot be responsible for the intestinal injury seen in GVHD since these cells remain present despite the treatment with anti-IFN- γ antibodies. The presence of the CTLs and the absence of the intestinal enteropathy after the administration of the anti-IFN- γ antibodies suggests that this is indeed the case.¹⁰⁷

Our results also demonstrate that donor IFN- γ is critical to the induction of apoptotic crypt epithelial cells supporting Mowat's finding that IFN- γ is important for the development of intestinal injury in GVHD. We did not observe the intestinal lesion in recipients of IFN- γ gko grafts nor could we induce the formation of intestinal apoptotic lesions with LPS. Similar results were obtained using a different mouse strain combination of sub-lethally irradiated recipients of IFN- γ gko grafts. Interestingly, the authors of this paper also showed that recipients of IFN- γ gko grafts, pre-conditioned with lethal total-body irradiation, experienced a more severe form of acute GVHD.²¹⁷ A survival comparison done by Murphy *et al* using lethally irradiated recipients of IFN- γ gko grafts and control wild-type graft recipients also observed a depression in the

survival rate of the IFN- γ gko recipient mice.²¹⁸ This suggests that the presence of donor derived IFN- γ can have opposing effects that are dependent on the intensity of the pre-conditioning regimen.

A different study however indicated that IFN- γ normally plays a protective role in GVHD. In this irradiated model, Murphy demonstrated that recipients of IFN- γ gko grafts had accelerated mortality and liver pathology. The spleens from these GVH animals produced significantly more IL-2 than did mitogen-stimulated splenocytes from the IFN- γ gko mice. This may explain why the GVH reaction was accelerated, because IL-2 is known to induce the proliferation of donor T cells.^{30,218} Since we never observed an accelerated form of GVHD in our IFN- γ gko model^{103,104} the conditioning regimen and the inflammation it produces may be altering the role IFN- γ plays in the GVH reaction.

The heightened sensitivity to LPS that we observed in our model was originally thought to result from the secretion of TNF- α . One theory of the role that TNF- α plays in acute GVHD was originally proposed by Nestel and colleagues.¹⁰⁹ In this paper, the authors observed macrophage priming by the cytokine IFN- γ . These primed macrophages would then release large amounts of TNF- α if exposed to endotoxin. In GVH mice, death would ensue shortly after an injection of endotoxin at a dosage that was sublethal in normal mice. Other studies also support a role for TNF- α in lethal GVHD.^{30,40,139} Our results however do not support this conclusion entirely. For instance, we observed high levels of TNF- α in response to an injection of LPS in our recipients of IFN- γ gko grafts.¹⁰³ Recipients of IFN- γ gko grafts do not succumb to an injection of LPS despite this large increase in serum TNF- α . This suggests that something other than IFN- γ can prime macrophages for LPS-induced TNF- α release and that TNF- α is not solely

responsible for the lethality of GVHD. While many studies agree with these findings, our own results indicate that IFN- γ is not always necessary to prime macrophages for TNF- α release. Our current results also demonstrate that TNF- α is not by itself responsible for the LPS-induced apoptosis of intestinal epithelial cells. This is because the recipients of IFN- γ gko grafts, despite having high levels of TNF- α , do not die or develop intestinal lesions when injected with sub-lethal amounts of LPS.

In addition, a study using TNF receptor knock-out mice further established that TNF- α cannot be responsible for the GVH-induced tissue injury. The binding of TNF- α to the receptor, TNFR p55, normally induces cell death. The lethally irradiated TNF receptor p55-deficient recipients of bone marrow transplants used in this study demonstrated improved survival when compared to wild-type recipients. Despite this observation, typical GVH lesions continued to develop in the gut and liver. This suggests that while TNF- α plays an important role in GVHD, it is not responsible for the production of tissue injury and intestinal lesion formation.²¹⁹

In a contrasting study done by Stüber *et al*, it was shown that the intestinal crypt cell apoptosis seen in acute GVHD is due to TNF- α production. The authors used an irradiated model of GVHD where C57BL/6 donor cells were injected into irradiated B6D2F₁-hybrid recipient mice. Recipients were further treated with either anti-TNF- α antibodies, one of two anti-Fas ligand antibodies or pentoxifylline.¹⁴⁰ It was observed that pentoxifylline and anti-TNF- α but not anti-Fas ligand antibodies inhibited epithelial cell apoptosis in the jejunum. However, these results were observed on day 3 post-induction, substantially earlier than when apoptotic intestinal lesions were found in our model. It must also be mentioned that, while pentoxifylline is an inhibitor of TNF- α secretion, it is

not specific for this cytokine and has been shown to inhibit NOS2 suggesting that pentoxifylline may be affecting NO production as well.²²⁰

Despite the apparent effect that TNF- α appears to have, attempts to prevent the lethal effects of acute GVHD through various inhibitors of this cytokine have met with some success.^{30,141,219} A recent animal study however cautions against such an intervention. This study demonstrates that the neutralization of this cytokine in the GVH reaction can lead to the development of humoral autoimmunity and chronic GVHD. This was thought to be caused by the inhibition of a mechanism whereby activated cytotoxic T cells eliminate autoreactive B cells from the acute GVH reaction. It was also observed that IFN- γ production was impaired by in vivo treatment with anti-TNF- α antibodies. TNF- α may therefore be contributing to the ensuing chronic GVH reaction by preventing the development of the Th1-dominated response that is necessary for acute GVHD. Consequently, this study cautions against the therapeutic use of anti-TNF- α antibodies as autoimmunity may develop.²²¹

Other animal studies have also attempted to prevent GVHD by blocking the effect of TNF- α from the GVH reaction. Piguet *et al* showed in their irradiated model of GVHD that using anti-TNF- α antibodies prevented the development of skin and gut lesions.¹³⁹ Vallera *et al*, on the other hand, failed to protect against lethal GVHD when using a TNF- α antagonist in an irradiated, fully MHC disparate model of GVHD.²²²

While augmented TNF- α , along with the human and animal inhibition studies, suggest that TNF- α does play a role in the GVH reaction, this role may be to act synergistically with other mediators produced during the course of the disease. For this reason, the timing of the administration of anti-TNF- α antibodies may be critical. The

treatment with anti-TNF- α antibodies, administered at an appropriate point, may limit the effects of the GVH reaction by interfering with the downstream events in the reaction such as by preventing NO production. This could then prevent the intestinal injury from developing.

One hypothesis that resulted from our research is that TNF- α works synergistically with NO in aGVHD bringing to light a fresh area for future investigation. An association between NO and TNF- α has been noted in other disease processes. For example, NO has been implicated in TNF- α -induced hypotension. Hypotension, induced in dogs by treatment with TNF- α , was mitigated by the administration of N^G-methyl-L-arginine (L-NMA), an inhibitor of nitric oxide synthesis. While pre-treatment with anti-TNF- α antibodies alone could protect against deleterious endotoxic shock, the ability for these antibodies to protect against this hypotension was lost when they were administered after the exposure to endotoxin.²²³ These findings implicate NO as a possible downstream mediator and contributor to endotoxic shock in which TNF- α plays a pivotal role. Further support for this stems from the fact that administration of NOS inhibitors: N^G-monomethyl-L-arginine (L-NMMA) and L-NMA causes blood pressure to return to near normal values in patients and animals suffering from septic shock if administered after the exposure to endotoxin.^{151,224-227}

LPS can also synergize with IFN- γ to increase NO production.¹⁵⁸ Since NO production is increased by LPS, NO may be contributing to the development of septic shock. This may be how the appropriately-timed administration of anti-TNF- α antibodies could prevent NO from participating in shock. The antibody treatment would eliminate the synergistic action of TNF- α and IFN- γ on NO production. As IFN- γ alone can

stimulate NOS2, some NO could still be produced and cause the tissue injury seen in GVHD; but this level of NO, with the absence of TNF- α , may not be enough to cause septic shock and death.

While TNF- α appears to synergize with other cytokines to induce NO production, IFN- γ appears to be critical for NO production in GVHD.²²⁸ Our data support this conclusion as we were unable to detect NO in the intestines of IFN- γ gko graft recipients even after the injection of LPS. Further support for this conclusion can be found in the study by Dalton that characterized the IFN- γ gko mouse. He noticed that macrophages from these mice lost their ability to produce NO in response to LPS.²²⁹ Hoffman *et al* and others have also concluded that IFN- γ is necessary for LPS-induced NO synthesis.^{111,159}

Although there appear to be many roles that NO may be playing in the GVH reaction, its effects can be categorized into two general functions. One is its cytostatic ability which results in widespread immunosuppression in animals suffering from acute GVHD.^{159,165,167,228} Another function is related to cytotoxicity and its ability to mediate intestinal epithelial cell injury and promote the development of endotoxemia in GVH mice.^{30,167,168} The ability for NO to alter intestinal permeability has been shown in several other studies. In vitro studies have demonstrated that NO can affect tight junction permeability to macromolecules in a model using intestinal epithelial cell monolayers.²³⁰ Unno and colleagues demonstrated permeability changes in the rat intestine following LPS treatment. This treatment led to increased bacterial translocation and increased leakage of fluorescein-labeled dextran, a molecule similar in size to several components of the bacterial cell wall, from the gut lumen. NO and iNOS were also found to increase in these LPS-treated rats. While LPS causes the upregulation of a variety of inflammatory

mediators that may be responsible for such an effect on the gut, NO was shown to play a key role since the administration of aminoguanidine, after the LPS injection, prevented the increase in NO production and lowered the intestinal barrier dysfunction.²³¹ Similar results, using aminoguanidine, were demonstrated by another group.²³² Decreases in intestinal permeability have also been observed directly in models of GVHD following NO inhibition.¹⁶⁴

Results from Paul Kubes group however do not support the notion that NO can increase intestinal permeability. Their in vivo cat studies show that the inactivation of NO via L-NAME results in an impaired intestinal barrier and that NO donors return the barrier to normal rather than enhancing intestinal permeability. While Kubes' data contradicts many of the aforementioned studies, he points out the argument that the ability for the overproduction of NO to cause deleterious effects to intestinal epithelial cells resides with the reaction of NO with the superoxide anion to produce peroxynitrite (ONOO⁻).^{146,147,233,234} It would therefore be interesting to see if ONOO⁻ was being produced in our model of GVHD and intestinal injury.

This conflicting information regarding the role of NO in intestinal permeability has been addressed with the use of iNOS deficient mice. Such a model would have no need for the use of pharmacological NOS inhibitors that result in unwanted, non-specific, inhibition of other non targeted NOSs. In this study, cNOS would remain active allowing for a less complicated interpretation of the results. Using these iNOS knockout mice Mishima *et al* showed that iNOS does indeed appear to play a role in endotoxin-induced intestinal injury. LPS-treated iNOS ^{-/-} mice had lower tissue and plasma NO₂⁻/NO₃⁻ levels and were resistant to enterocyte apoptosis and subsequent bacterial translocation

that would normally be seen in control mice.²³⁵ Other studies have also demonstrated that macrophages, cultured from iNOS knockout mice, do not demonstrate enhanced NO production in response to IFN- γ and LPS. Diminished peroxynitrite production was also observed in these cells.¹⁴⁶

Our results support a role for NO in the development of the intestinal injury that occurs in GVHD. We show for the first time that the systemic administration of LPS to mice with aGVHD results in a NO burst in the small intestine and subsequent epithelial cell injury that can be blocked by the administration of NOS inhibitors. We therefore propose that IFN- γ can prime macrophage for LPS-induced NO release and that this NO, rather than TNF- α , is the principal mediator of the intestinal epithelial cell apoptosis and ultimate death of the host.

Garside *et al* also believe that NO causes the intestinal injury seen in the GVH reaction. They used a model of GVHD that is known for producing the early proliferative events in intestinal GVHD. Treatment of their mice with the NOS inhibitor L-NMMA resulted in the inhibition of the events that normally take place early in the gut.¹⁶⁸

Hoffman, on the other hand, did not observe any improvements in intestinal pathology when aminoguanidine was given to GVH mice. Survival was however ameliorated in this model. These opposing results could be due to the differences in the model used or the different NOS inhibitors and doses that were used in comparison to our results and those of Garside.²¹²

A role for LPS in acute GVHD has been demonstrated in many studies.^{125-128,236} Using LPS, we have developed a novel approach that allows us to study the events surrounding the intestinal injury in aGVHD. Further evidence implicating LPS as a key to

the GVH reaction comes from a study involving LPS-sensitive and LPS-resistant donor mice. While the production of IFN- γ was similar in both LPS-resistant and LPS-sensitive GVH mice, the amount of TNF- α produced by LPS-stimulated cultures of splenocytes from the LPS-resistant mice was half that of the level seen in similar LPS-sensitive cell cultures. LPS-resistant recipient animals also had significantly less mortality and suffered from significantly less GVHD when compared to the LPS-sensitive animals. Improvements included a reduction in intestinal injury and lower levels of LPS in the serum. Neutralizing TNF- α antibodies further reduced mortality and intestinal injury in the GVH animals.²¹¹ This study did not however look at NO production which can also exacerbate the intestinal injury. It would be interesting to measure NO while blocking TNF- α to observe any possible direct response this cytokine may have on NO production in GVHD.

While these studies indicate that LPS is present during the end stages of the disease, just how it initially escapes the intestinal lumen remains to be answered. Our data argues that NO causes the destruction of the intestinal epithelium which results in the formation of large lesions through which LPS can exit. Our results also indicate, however, that LPS must be present in order to trigger the release of large amounts of NO to cause the lesion formation. We believe that some factor produced during the GVH reaction causes the intestine to become permeable to small, non-lethal, amounts of LPS. This low level of LPS would trigger the release of some NO that could then go on to injure the intestine and increase its permeability to more endotoxin. As more endotoxin leaks out, more NO gets produced and creates further intestinal injury and the cycle continues until the death of the host. While this study did not address just what molecule

initiates the initial translocation of LPS across the gut wall, several possible candidates exist.

First of all, the conditioning regimens may be responsible for enhancing intestinal permeability, due to their ability for enhancing GI tract damage and increasing serum levels of TNF- α and LPS.^{30,40,237} The cause of the initial loss of gastrointestinal tract integrity in non-irradiated models, such as the one we employ, however is unknown. Several studies suggest that cytokines may be responsible for this phenomenon.

It is unlikely that IFN- γ is responsible for this early increase in intestinal permeability. While several studies have demonstrated that IFN- γ can affect the barrier function of intestinal epithelial cells, the resulting increase in tight junction permeability is not large enough to permit the passage of large macromolecules such as LPS.^{238,239}

One other possibility is IL-4. IL-4 is present early in the acute GVH response and may be responsible for allowing enough LPS to cross the intestinal barrier. The resulting switch to a Th1 cytokine response and acute GVHD could eventually lead to the release of large amounts of NO and TNF- α and severe intestinal lesions. In vitro model systems of human colonic epithelial cell monolayers have been used to study the effects of IL-4 on intestinal permeability. In these studies, the addition of IL-4 to the monolayers resulted in a reduction of barrier function allowing significant amounts of horse radish peroxidase (HRP) to transport across. HRP is a relatively large, 44,000 KDa, macromolecule comparable in size to the reduced form of LPS.²⁴⁰ Both transcellular and paracellular movement of HRP was seen indicating that non-specific leakage of macromolecules can occur in response to IL-4.²⁴¹⁻²⁴³

5.2 The protective capabilities of rHuKGF

The intestine is a primary target of acute lethal GVHD. It seems logical that the protection of the gut must be taken into consideration when addressing GVHD prophylaxis. rHuKGF is a molecule that may protect the intestine from the effects of the GVH reaction. Our results demonstrate that rHuKGF can protect against the lethal effects of acute GVHD in an unirradiated parent into F₁-hybrid model was demonstrated in chapter 4. A treatment regimen of daily rHuKGF injections, for seven consecutive days, beginning three days before induction resulted in a significant reduction in weight loss and mortality when compared to untreated controls. It was also demonstrated that rHuKGF did not prevent T cell engraftment as donor cells were detected in rHuKGF-treated mice on days 8,13,116. Mice treated with rHuKGF did not develop intestinal apoptotic lesions despite having high levels of intestinal NO and high levels of intestinal TNF- α mRNA. Intestinal lesions were absent when 10 μ g of LPS was administered even though this dose caused an increase in intestinal NO and serum TNF- α levels. The ability for rHuKGF to further protect the gut from GVH-induced injury was demonstrated by the lack of detectable endotoxin in the serum of mice treated with rHuKGF when compared to untreated GVH control mice. This is the first time that rHuKGF had been investigated for its ability to prevent intestinal GVHD in an unirradiated model.

Other studies also demonstrate that rHuKGF can protect the gut from injury. KGF was shown to protect against radiation and chemotherapy-induced intestinal damage in an *in vivo* mouse model of GVHD. Significant improvements in survival, weight loss, intestinal mucosal thickness and crypt size were noted when rHuKGF was administered as a pre-treatment. These authors also noted that enteric bacteria were detected in the

liver of mice not treated with rHuKGF and that the rHuKGF pre-treatment prevented this and maintained mucosal integrity. Interestingly, pre-treatment with rHuKGF was necessary to protect against the chemotherapy or radiation-induced injury since rHuKGF post-treatment did not have any amelioratory effects on survival and weight loss.¹⁸² The importance of administering rHuKGF both before and after the allogeneic bone marrow transplant was also observed by this group.

Despite the ability for rHuKGF to prevent the lethal component of acute GVHD, other important side-effects were noted. Treatment with this growth factor resulted in a switch to a Th2 dominated cytokine profile consisting of higher levels of IL-4, IL-5, IL-10 and IL-13 and lower levels of IFN- γ when compared with untreated GVH control mice. These results show for the first time that the cytokines IL-4, IL-5 and IL-10 are present in rHuKGF-treated mice with GVHD. The Th2-dominated cytokine profile resembles, to some extent, that seen in the GVH model using IFN- γ gko mice as donors and are commonly associated with models of chronic GVHD.¹⁰³ They suggest that rHuKGF may have an immunoregulatory effect on the GVH reaction. Much like our model, Panoskaltsis-Mortari *et al* observed reduced TNF- α and IFN- γ levels and higher IL-13 levels in the serum of rHuKGF-treated GVH.¹⁹⁶ We also detected TNF- α in the sera of rHuKGF-treated GVH mice indicating that the LPS-induced TNF- α release was preserved. This occurred despite a predominantly Th-2 cytokine pattern and low IFN- γ . Intestinal IFN- γ and TNF- α mRNA levels were also compared for the first time in untreated and rHuKGF-treated GVH mice. While IFN- γ levels were elevated in both groups, this increase was greater in the untreated group. No differences in TNF- α levels were seen in the treated and untreated group. This data suggests that rHuKGF protects

mice from the effects of acute GVHD by playing an immunoregulatory role, in contrast to studies suggesting that KGF works directly by protecting epithelial cells from injury.

Many recent studies demonstrated that KGF-treatment can protect against epithelial cell injury. Noting that reactive oxygen species (ROS) have been shown to affect the epithelial barrier and increase permeability in several organs, including the lung and the intestine, Chapman *et al.* tested the effects of KGF-treatment on lung epithelium after long-term ROS exposure. Using a lung epithelial cell line, they demonstrated that KGF treatment maintained tight junctions following continuous H₂O₂ exposure. In control mice, H₂O₂ exposure interfered with tight junctional protein interactions. It was also observed that KGF pre-treatment enhanced the recovery of the epithelial layer integrity after H₂O₂ exposure.²¹⁶ A similar study demonstrated that KGF can reduce albumin flux across epithelial cell monolayers following H₂O₂ exposure.¹⁸⁶ If epithelial tight junctions become disrupted in the intestine early in the GVH response and allow a small amount of LPS to leak from the intestine, a Th1 immune response may develop resulting in acute GVHD. KGF may protect by preventing this from occurring.

Both irradiation and reactive oxygen intermediates kill cells by damaging DNA. One possible explanation for the protective mechanism of KGF on epithelial cells may be by its ability to enhance the inherent DNA repair mechanisms found in the cell. A study, using type II alveolar epithelial cells, supports this hypothesis. The cells were examined for signs of apoptosis that resulted from hyperoxic-induced lung injury. DNA strand breaks were absent in epithelial cells when KGF was added to the culture medium and expression of apoptotic associated proteins such as Bax, p53 and p21 were reduced. It was also observed that the expression of an auxiliary protein associated with DNA

polymerase δ was increased. This suggests that KGF may prevent DNA damage by influencing the polymerases involved in repair and replication.²⁴⁴

This same conclusion may be drawn from several other studies as well. Administration of H_2O_2 to culture caused DNA strand breaks and reduced double-stranded DNA levels in ATII rat alveolar epithelial cells and A549 bronchoalveolar carcinoma cells. These effects were again prevented by treatment with KGF. The authors found that KGF did not protect by increasing the activity of the anti-oxidants catalase and glutathione but rather observed that the utilization of inhibitors for DNA polymerases ϵ , δ , α and β completely prevented the protective capabilities of KGF.¹⁸⁷

Similar results were obtained in an in vitro model of γ -radiation induced damage. KGF ameliorated the DNA strand breaks caused by the radiation treatment. Its protective ability was blocked by the addition of inhibitors of DNA polymerases α , δ and ϵ . Inhibition of DNA polymerase β did not however prevent the effects of KGF at protecting the cells suggesting that this enzyme may not be involved.¹⁸⁵

Another protective mechanism of KGF may be its ability to enhance cell proliferation however several lines of evidence indicate this does not happen. For instance, the protective effects of KGF can occur within as short a time as one hour of incubation. This is substantially beneath the amount of time necessary for cells to divide or double.¹⁸⁷ Increased proliferation was also ruled out as a cause of KGF-mediated protection from radiation-induced damage in an alveolar epithelial cell line as KGF did not augment the number of cells in the proliferative phase of the cell cycle.¹⁸⁵ As this evidence stems from only in vitro models, we cannot necessarily exclude the possibility that KGF may exert its protective effect through this mechanism in vivo.

While the immediate protective effects of KGF on epithelial cells may not be due to the induction of proliferation, intestinal crypt cell populations have been shown to increase within 24 hours after KGF administration and goblet cells increase after four to seven days of treatment with KGF.¹⁸⁰ Furthermore, increases in homodeoxyuridine and Ki-67 or tritiated thymidine demonstrates substantially more mitotic events occurring in the stem cell zone of the crypts. This suggests a protective role for KGF whereby it increases the proliferative events in the intestine.¹⁸³ Other studies also support the suggestion that KGF has an effect on gastrointestinal epithelial cell proliferation.²⁴⁵

This study observed for the first time KGF and KGFR mRNA expression levels in the intestine of mice with GVHD. While this data is important to the study, it was in part, dependent upon the RPA experiments performed in collaboration with Amgen Inc. Interestingly, these results show that the levels of KGFR expression are reduced in the intestine of GVH mice on day 8 post-induction suggesting a period of temporary susceptibility in the gut. This happens to coincide with the peak in IFN- γ seen in the spleens and intestines of these animals. We also observed that there was no reduction in KGFR in recipients of IFN- γ gko grafts. Another role for IFN- γ in GVHD may therefore be to reduce KGFR expression, thereby impairing the gut's ability to withstand various injurious agents like NO. It would be expected that KGFR expression levels be higher in the GVH mice that were treated with rHuKGF since this treatment was shown to result in a reduction in IFN- γ levels. We found this, however, to not be the case as no difference in KGFR expression could be seen when comparing rHuKGF-treated and untreated GVH mice.

KGF may also protect by increasing antioxidant activity. While KGF may not induce the expression of catalase, superoxide dismutase or glutathione, it has been demonstrated to induce the expression of a human gene in the skin that is 85% homologous in sequence and identical in the size of the protein to a bovine non-selenium glutathione peroxidase. This evidence suggests a different means for KGF in protecting against oxidative stress whereby anti-oxidants are produced.²⁴⁶ KGF was also shown to affect glutathione in the gut of malnourished rats as well as preventing the glutathione depletion that is normally seen in the lung and liver of TBI and cyclophosphamide-treated BMT mice.²⁴⁷ Furthermore, antioxidant glutathione-S-transferase and non-selenium-dependent glutathione peroxidase gene expression were upregulated in mouse tongue following three consecutive days of KGF treatment. Epithelial thickness was augmented by the KGF-treatment in mouse tongue, as were crypt depth, villus height and goblet cell number in the small intestine. KGF-treated animals also had increased DNA, protein and tissue wet weights after radiation-treatment when compared with irradiated controls indicating that the small intestine was protected from the injurious radiation.²⁴⁸ Goblet cells may also play a role in protecting the GI-tract from injury by producing trefoil proteins which aid in stimulating injury repair.²⁴⁹

Finally, the ability for KGF to protect when administered many days before GVH associated lesions develop suggests that it plays a role in regulating the immune response rather than having a direct repair mechanism of GVH tissue injury. However, the mechanism through which this occurs is unknown. Panoskaltsis-Mortari *et al.* were unable to demonstrate the existence of KGFR on T cells. This suggests that KGF does not protect by directly interacting with T cells.¹⁹⁶

It has been shown that treatment with rHuKGF appears to have no impact on the GVL effect. In this study, rHuKGF-treated mice were followed for their ability to survive a lethal dose of P815 leukemia cells at the time of BMT. While rHuKGF both pre and post-induction significantly ameliorated the severity of GVHD by improving survival and decreasing the level of gastrointestinal injury and it did not interfere with any GVL effects. As a result, the leukemia-free survival in mice suffering from acute GVHD and treated with rHuKGF was significantly improved.¹⁹⁷

5.3 Summary and final thoughts

This thesis has presented several notable findings that are important to the study of aGVHD. In order to explore the roles of NO and IFN- γ it was first necessary to develop a model that allowed us to better observe the terminal events surrounding aGVHD. This was achieved with the administration of LPS. Using this method, we confirmed findings made in other labs that IFN- γ is necessary for the development of the intestinal injury in aGVHD (Fig. 3.1 and 3.2). We found that this mechanism did not appear to involve TNF- α but instead relied on the ability of IFN- γ to synergize with LPS and cause a NO burst in the intestines of LPS-treated mice with GVHD (Fig. 3.5 and 3.6). Apoptotic intestinal epithelial cells were also found in this tissue suggesting that NO is responsible for the gut injury that occurs in aGVHD. Supporting these findings are the observations that pre-treatment with the NOS inhibitors L-NAME or L-NIL prevented both the intestinal NO burst and intestinal lesion formation (Fig. 3.3 and 3.7). We also demonstrated for the first time that while rHuKGF protected against the lethal effects of aGVHD, it did not block the LPS-induced NO burst (Fig. 4.1, 4.6 and 4.7). To date, no

other studies have used rHuKGF to investigate its ability to protect against intestinal GVHD in an unirradiated parent into F₁-hybrid model. We also showed for the first time that the Th2 cytokines IL-4, IL-5 and IL-10 are produced in this model of GVHD along with IFN- γ (Fig. 4.4 and 4.5). It was also found that the observed reduction in KGFR mRNA expression coincided with the peak in IFN- γ production suggesting that these two may be linked (Fig.4.5, 4.9, 4.10 and 4.11). We finally suggest that the mixed cytokine pattern (Fig. 4.4) and the tissue infiltrates, rich in eosinophils, (Fig. 4.8) imply that rHuKGF is immunoregulatory and, although it protects against aGVHD, may be causing a switch to a chronic GVH reaction.

Although the mechanism leading to the development of aGVHD is not clearly defined, we believe that the intestine, and the LPS contained within it are two of the most important players. We propose that LPS acts as an adjuvant promoting a Th-1 type of immune response resulting in the development of acute GVHD. The fact that LPS plays a role in GVHD has been clearly demonstrated in both murine studies and in clinical trials.^{30,40,125-128,236} The importance of it acting as an adjuvant is based on the different cytokine patterns expressed in acute versus chronic GVHD. Studies by Rus *et al* suggest the GVH reaction appears to start out with Th-2 cytokine production¹⁰² that would normally lead to the development of the chronic form of the disease. Some event must occur during the early stages of this reaction that causes a switch from a Th2-type to a Th1-type immune response, allowing for the development of acute GVHD. LPS may be such an adjuvant. The intestinal damage observed in the later stages of the disease allows for the leakage of large amounts of endotoxin into the circulation. We propose that the

intestine becomes permeable to small amounts of endotoxin at an earlier time point when LPS can influence the developing immune response. This hypothesis is supported by numerous studies in the field of allergy, asthma and the hygiene hypothesis which suggests that the exposure to endotoxin during early childhood is important for the development of Th-1 mediated immunity. This exposure results in a skewing of the immune response away from the Th-2 phenotype associated with allergy towards a more Th1 dominated immunity.²⁵⁰

Interestingly, the introduction of bacteria into healthy decontaminated recipients of bone marrow grafts does not always result in the deterioration of their condition. When bacteria were re-introduced into similar mice on day 8 or 15, the ensuing GVH reaction develops as it would in control, non-decontaminated, recipients. Reconstitution on day 26 results in some mortality whereas mortality is not enhanced in mice reconstituted on day 40. This provides further support for the ability of LPS to influence the early immune response that results in aGVHD.²⁵¹ Several other studies in GVHD also indicate that LPS may have an adjuvant effect in the immune system.²⁵²⁻²⁵⁴

The mechanism as to how the early LPS gets out of the gut has not been elucidated. Clinically, LPS begins to leak out of the damaged intestine as a result of the conditioning regimen. A similar mechanism cannot however be claimed for the unirradiated parent into F₁-hybrid model. The end result, however in both of these models, is the same with the detection of LPS in the serum and the death of the host. We believe that IL-4 is responsible for initiating the early intestinal permeability to LPS. IL-4 is present at the beginning of the GVH reaction and then disappears once LPS has induced the IFN- γ -dominated Th-1 response. Although IL-4 is present in chronic GVHD,

other Th-2 cytokines somehow prevent acute GVHD and the Th-1-mediated cascade of events from occurring so that large amounts of LPS cannot exit the gut to kill the host. The Th-1 cytokine IFN- γ is likely involved in this.¹¹¹

The ability for rHuKGF to protect stems from its ability to block the intestinal epithelial cell apoptosis which prevents the flux of endotoxin out of the gut lumen, thereby preventing aGVHD. While many places for intervention exist to ameliorate acute GVHD, rHuKGF remains ideal as it does not impair T cell engraftment or interfere with the GVL effect. Current treatments that prevent aGVHD by blocking inflammatory mediators may also be inhibiting the immune response making them less than ideal. This strategy possesses other difficulties as well since there does not appear to be one single cytokine or molecule whose sole inhibition can prevent GVHD. A cocktail of antibodies against these mediators would therefore seem more appropriate but again might interfere with the immune response. Preliminary trials indicate that low levels of rHuKGF appear to be well tolerated with no obvious adverse effects in healthy human controls.²⁵⁵ The doses used however were lower than the effective doses used in most rHuKGF animal studies including our own. One must also be cautioned about the potential indirect immunoregulatory effects KGF may have. In this thesis, rHuKGF while protecting against acute GVHD, seemed to elicit some chronic GVHD characteristics and further careful evaluation should therefore be made.

6.1 References

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