THE ROLE OF NK1.1$^+$ CELLS AND TH1 CYTOKINES IN THE PATHOGENESIS OF ACUTE MURINE GRAFT-VERSUS-HOST DISEASE (GVHD)

BY

CYNTHIA A. ELLISON

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University of Manitoba

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ABSTRACT

Previous studies using a murine F_1-hybrid model of acute GVHD have shown that mortality is prevented if the graft is depleted of NK1.1$^+$ cells. The precise role played by these cells in the pathogenesis of GVHD has not been elucidated, but it is thought that they injure host target tissues. Two cytotoxic, NK1.1$^+$ subpopulations are activated during acute GVH reactions: conventional NK cells that lyse NK-sensitive targets, and NK-like cells that lyse both NK-sensitive and -resistant targets. Because the latter is derived largely from the donor, we hypothesized that it may be particularly important in the pathogenetic mechanism. However, because there is also evidence that some NK1.1$^+$ cells have immunoregulatory function, we further postulated that some NK1.1$^+$ cells promote the early Th1 response that underlies development of acute GVHD. Experiments were performed to determine whether (a) γδT cells mediate NK-like activity and are required for acute GVHD to occur (b) depleting the graft of NK1.1$^+$ cells mitigates the development of a Th1-mediated immune response (c) eliminating donor-derived IFN-γ influences the outcome of the disease. Results showed that the NK-like cells express γδTCR, and removing them partially protects recipients against mortality. However, the use of TCRδ (knockout) KO grafts was not protective, and IFN-γ levels were higher. The Th1 response, as measured by IFN-γ production and LPS-induced TNFα release, was abrogated in recipients of NK1.1-depleted grafts, but intestinal lesions and high serum LPS levels still developed. GVHD in recipients of IFN-γ KO grafts was lethal, but more protracted, resembling chronic GVHD. NK1.1 depletion of these grafts was protective. In summary, γδT cells contribute to the demise of GVH mice, but other cells are also involved. In wild-type graft recipients, the protection conferred by NK1.1 graft depletion results from factors other than, or in addition to, abrogated IFN-γ production, which appears to accelerate mortality. The gut injury seen in these mice is mediated by cells other than those in the graft expressing NK1.1. We postulate that these are donor-derived γδT cells.

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This thesis is dedicated to my husband, James, who has always been there for me.

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ABBREVIATIONS

APC; antigen presenting cell

BDF_1; $(C57BL/6 \times DBA/2)F_1$

bg; beige

BMT; bone marrow transplantation

BSA; bovine serum albumin

BW1100; BW5147/M1100.129.237

C; complement

^{51}Cr; $Na_2{}^{51}CrO_4$

CMV; cytomegalovirus

cpm; counts per minute

Con A; concanavalin A

CTL; cytotoxic T lymphocyte

DC; dendritic cell

ELISA; enzyme linked immunosorbent assay

E:T; effector to target ratio

FALS; forward angle light scatter

FasL; Fas ligand

FCS; fetal calf serum

FITC; fluorescein isothiocyanate

gko; gene knockout

GM-CSF; granulocyte/macrophage-colony stimulating factor

GVH; graft-versus-host

GVHD; graft-versus-host disease

HA; minor histocompatibility antigens

HBSS; Hank's balanced salt solution

Hh; hematopoietic histocompatibility antigens

HLA; human leukocyte antigens

HR; hybrid resistance

HSP; heat shock protein

HSV; herpes simplex virus

Ig; immunoglobulin

IFN; interferon

IL; interleukin

KO; knockout

LAL; limulus amoebocyte lysate

LGL; large granular lymphocyte

LPS; lipopolysaccharide

LRW; LAL reagent water

LU; lytic unit

mAb; monoclonal antibody

M-CSF; macrophage-colony stimulating factor

MHC; major histocompatibility complex

MLR; mixed lymphocyte reaction

ND; not determined

NGFR; nerve growth factor receptor

NS; natural suppressor

NK; natural killer

NO; nitric oxide

OD; optical density

PBS; phosphate buffered saline

PE; phycoerythrin

PGE; prostaglandin E

pNA; p-nitroaniline

poly I:C; polyinosinic polycytidylic acid

SA; streptavidin

SAL; small agranular lymphocyte

SALS; side angle light scatter

SCID; severe combined immunodeficiency

SI; spleen index

TCR; T cell receptor

TGF; transforming growth factor

TNF; tumor necrosis factor

TNFR; tumor necrosis factor receptor

TN; triple negative

CHAPTER 1

LITERATURE REVIEW

1.1 Historical perspective.

Acute graft-versus-host disease (GVHD) is a complication of allogeneic bone marrow transplantation (BMT) characterized by severe immunosuppression, extensive tissue damage, cachexia, and a high level of mortality. Graft-versus-host (GVH) reactions occur when lymphoid cells in the graft respond immunologically to alloantigen expressed in recipients that are unable to reject the graft.

Although it was not recognized as such at the time, the GVH reaction was observed by Murphy over 70 years ago. It was not revisited until the 1950's when investigators working in the areas of solid organ transplantation and immunological tolerance saw similarities between their observations and those reported by Murphy decades earlier. This work led to a description of the GVH reaction and the disease that followed. In contrast to experimental GVHD, the clinical form of the disease did not receive much attention until the 1960's when BMT became a method for hematopoietic reconstitution in humans.

1.1.1 Murphy's chicken embryos, 1916.

Murphy's serendipitous discovery of the graft-versus-host reaction occurred during his early studies of cancer. In these experiments, he was implanting either rat tumor tissue, or non-malignant control tissue from adult chickens onto the chorioallantoic membrane of 7-day chicken embryos. Ten days after the transfer, Murphy noticed that the embryos that had received the control tissues displayed disseminated nodules around the sites of implantation, on the skin, and on the spleens, which had become greatly enlarged. He later determined that these changes occurred most frequently when embryos were implanted with spleen tissue, only occasionally when liver was used, rarely following the transfer of kidney fragments, and never following implantation of muscle or cartilage (1).

1.1.2 Deciphering the criteria for a GVH reaction.

Murphy postulated that the changes he had seen were due to cellular stimulation in the spleen and other tissues of mesodermal origin (2). This hypothesis would later be proven incorrect.

In 1957, Simonsen observed infiltrates of small lymphocytic cells, as well as larger cells, within the interstitium of kidneys that had been transplanted into allogeneic dogs. He referred to the larger cells as "pyroninophilic" because they absorbed the histological stain pyronine-methylene green. He also described them as reticuloendothelial cells undergoing mitosis. Based on their appearance and anatomical location, Simonsen thought they had originated in the graft, and that they may be reacting against alloantigen in the recipient dogs (3). Similar observations were made by Dempster (4).

At about the same time, Billingham, Brent and Medewar were attempting to induce immunological tolerance by injecting chicken embryos with adult chicken blood. They hypothesized that these embryos, once mature, would accept a skin graft from the same donor (5). In these experiments, and similar ones performed by Simonsen (3), all but 5% of the chicks died just before hatching. In the investigation that followed, Simonsen showed that splenomegaly, pathological changes and the high level of mortality occurred only when an adult, rather than an embryonic donor was used, and when an embryonic, rather than a newly hatched chick was used as a recipient. He also noted that a positive result was usually observed when blood samples drawn from the deteriorating chicks were analyzed using a Coomb's direct test. Because Billingham, Brent and Medewar had demonstrated that neonates were unable to produce antibodies directed against specific individuals, Simonsen concluded that the donor cells must be responsible for the antibody production. His experiments using inbred strains of mice also indicated that the donors and hosts had to be

genetically different for the reaction to occur (3). For the first time, the conditions of a graft-versus-host reaction had been identified. They were later presented in a Harvey Lecture given by Billingham and Brent in 1966 (6). The criteria are as follows:

1) The graft must contain immunologically competent cells.

2) The host must appear antigenically foreign to the donor.

3) The host must be unable to reject the graft immunologically.

Simonsen was also the first to show that leukocytes mediate GVH reactions (3;7). However, it was the work of several investigators that eventually identified lymphocytes as the cells primarily responsible for this reaction (6;8-13).

By the 1960's investigators began to focus on the pathogenetic mechanism by which GVH reactions develop. One of the most notable experiments, performed by Gowans in 1962, showed that the injection of radiolabeled lymphocytes from parental strain donors into F_1-hybrid recipient mice, or completely allogeneic recipients that had been lethally irradiated, was followed by the appearance of radiolabeled, pyroiniphilic cells in the lymphoid organs of the host. These cells were further shown to be responsive to stimulation by the foreign antigen of the host. (13).

1.2 Murine models.

Four experimental models of GVHD have been established, each with its own advantages and disadvantages.

1.2.1 Runt disease.

Runt disease was the term used by Billingham and Brent to describe the syndrome that developed in newborn mice injected *i.v.* with adult blood from allogeneic donors. It only occurred using some strain combinations and was characterized by a hunched posture, severe weight loss, alopecia, atrophy of the lymphoid organs and diarrhea (6). The experimental model in which Runt Disease develops was originally intended as a method for tolerizing neonates to alloantigen, but it is now known that this can only be achieved if the cells injected into the neonates are devoid of mature T cells (14).

1.2.2 F_1-hybrid model.

This model is commonly used to study GVHD and involves two inbred strains with different major histocompatibility complex (MHC) haplotypes. A lymphoid graft prepared from one parental strain is injected *i.v.* into an F_1-hybrid, which codominantly express the MHC antigens of both parental strains. The cells in the graft respond vigorously when exposed to MHC antigen of the opposite parent, whereas those in the host are unresponsive to MHC antigen from either parent.

The F_1-hybrid model of GVHD is preferred by many immunologists for studying GVHD because it does not require that the recipients be pretreated with immunosuppressive agents in order to prevent rejection of the graft. This avoids the need to differentiate between the effects of the GVH reaction and those of antecedent irradiation or chemoablation of the host's immune system. In addition, Parent→F_1-hybrid strain combinations can be chosen so that donors and recipients differ only with respect to certain MHC antigens, thereby allowing investigators to study the immunogenetics of these reactions. The model is also somewhat flexible in the sense that F_1-hybrid recipients can be irradiated, if necessary, so that the

conditions under which the reaction is observed will more closely resemble those of clinical GVHD.

1.2.2.1 Hybrid resistance.

The phenomenon known as Hybrid Resistance (HR) was first observed over 30 years ago (15). It occurs when parental-strain hematopoietic grafts are rejected by F_1-hybrid recipients. Because the normal laws of transplantation do not govern transplantation of hematopoietic grafts, it was hypothesized that hematopoietic histocompatibility (Hh) antigens were involved in the mechanism of rejection. Unlike MHC antigens, which are codominantly inherited, Hh antigens were believed to be expressed only in homozygous individuals. This would preclude their expression in F_1-hybrids, making a parental strain graft appear "foreign" to an F_1-hybrid animal (16). Further studies suggested that the Hh genes were probably located in the interval between the H-2S and H-2D regions of the MHC (17). The cells responsible for mediating HR were thought to be natural killer (NK) cells because irradiation of the bone marrow microenvironment in an F_1-hybrid resulted not only in a loss of NK cell function, but also in reduced HR (18-20). Although appealing in many respects, this putative mechanism of HR is outdated firstly, because evidence supporting the existence of Hh antigens and their receptors is lacking, and secondly, because recent developments in the area of NK cell receptors have provided a more plausible explanation for the phenomenon.

The "missing self" hypothesis proposed by Karre in 1990 is currently the most widely accepted mechanism through which NK cells recognize potential targets (21). It also provides a better explanation of how they may mediate HR. The hypothesis is based on the idea that NK cells function *in vivo* to eliminate tumorous or virally-infected cells that have down-regulated expression of class I molecules in an attempt to evade recognition by

cytotoxic T cells (CTL). Experiments performed in the last decade provide considerable support for this idea (22-28). One showed that tumor cells failing to express H-2 molecules were less tumorigenic in a syngeneic host than their H-2-expressing counterparts, and that the rejection response was non-adaptive (22). Another showed firstly, that bone marrow cells deficient in MHC class I expression due to a mutation in the B_2-microglobulin gene were rejected by syngeneic hosts, and secondly, that the rejection process was mediated by NK1.1$^+$ cells (23).

Recent landmark studies have shown that the Ly49 family of genes encode receptors on murine NK cells that bind to class I molecules and, in most cases, deliver inhibitory signals to the NK cells (29;30). There are eight or more genes within the Ly49 multi-gene family, which is located in the NK gene complex on mouse chromosome 6 (31-34). It is thought that unless an NK cell receives an inhibitory signal via an Ly49 receptor-class I MHC interaction, it can become activated to kill a target cell through multiple interactions between non-polymorphic receptors expressed on the NK cell and highly conserved determinants present on the target cell surface (35).

In 1995, it was shown that at least two members of the family, Ly49A and Ly49C, are subject to allelic exclusion (36). This would suggest that the NK cell population in F_1-hybrid mice consists of 2 distinct subsets, each with inhibitory receptors for the class I molecules expressed by each of the parental strains. When exposed to a hematopoietic graft from one parental strain, one subset would receive inhibitory signals via its Ly49 receptors while the other would reject the graft. Indeed, it has been shown that the ability of H-$2^{b/d}$, F_1-hybrids to reject bone marrow cells from H-2^d parental strain mice is abrogated if the F_1-hybrids are treated with anti-Ly49C monoclonal antibody (mAb) *in vivo*. This treatment is thought to be effective because it eliminates the Ly49C$^+$ NK cell subset in the F_1-hybrid, which fails to lyse H-2^b-expressing cells, but rejects H-2^d-expressing, parental strain

hematopoietic grafts (29;37). Although these findings represent a good beginning, a better understanding of the mechanism of HR will await complete characterization of the Ly49 family of molecules. In particular, it would be interesting to know whether peptide binding to class I molecules influences their ability to deliver an inhibitory signal through the corresponding Ly49 receptor. One hypothesis suggests that the genes within the H-2S/D interval in the MHC, formerly thought to encode Hh antigens, may actually encode peptides capable of influencing class I MHC-Ly49 interactions (35).

Other experiments investigating the mechanism responsible for HR employed D8 mice which express the transgene for the class I molecule H-2D^d, but are otherwise C57BL/6 (H-2^b). In this situation, the "missing self" hypothesis would dictate that different subsets of NK cells from D8 mice would receive inhibitory signals from cells expressing H-2K^b, H-2D^b, H-2D^d, and H-2L^b. Experiments showed that D8 mice could reject hematopoietic grafts from normal, C57BL/6 mice. This effect was abrogated if the D8 mice were depleted of NK1.1$^+$ cells, indicating that NK cells mediated the rejection process. It was postulated that the subsets of NK cells that receive inhibitory signals from H-2^d molecules were responsible. The same investigators observed similar results when the reverse experiment was performed and hematopoietic grafts from D8 donors were injected into wild-type, C57BL/6 recipients (38). These grafts were rejected and it was also determined that the rejection could be abrogated by using NK1.1-depleted B6 recipients. Because the D8 cells would have expressed H-2^b antigen inhibiting all NK cells in the B6 mice, it was proposed that the "missing self" paradigm cannot completely explain the mechanism by which NK cells recognize and respond to target cells, and that NK cells can, in some instances, reject target cells following the positive recognition of alloantigen (39). These findings are particularly relevant to our understanding of the possible mechanism by which donor-derived, H-2^b NK cells in the graft may reject

allogeneic target cells in H-$2^{b/d}$, F_1-hybrid recipient mice during the development of acute GVHD.

In summary, it is clear that a high level of HR in an F_1-hybrid model of GVHD could lead to rejection of the graft, thereby complicating the study of GVHD. However, this difficulty can be overwhelmed by increasing the cell dose used to induce the GVH reaction, so that the graft's ability to reject cells expressing the MHC haplotype of the opposite parent is greater than the recipient's ability to mediate HR. In addition, there are methods for studying engraftment that would provide verification that the graft had not been rejected.

1.2.2.2 Acute versus chronic GVHD.

When lymphoid cells in parental strain grafts are introduced into F_1-hybrid hosts, two forms of GVHD can result. The first, referred to by Gleichmann as "stimulatory" GVHD, is characterized by lymphoid hyperplasia, hypergammaglobinemia and autoantibody formation (40-44). These conditions, as well as severe immune complex glomerulonephritis, result in GVHD resembling systemic lupus erythematosis (40;45). Other pathological changes such as a Sjogren's-like syndrome, collagen vascular disease, scleroderma-like lesions and primary biliary cirrhosis have also been observed (40;41;46). Today, we commonly refer to "stimulatory" GVHD as the chronic form of the disease.

Acute GVHD, previously referred to by Gleichmann as the "suppressive" form of the disease, is characterized by a transient period of intense stimulation of the lymphopoietic system, which is followed by severe hypoplasia. F_1-hybrid mice with "suppressive" GVHD eventually develop aplastic anemia, hypogammaglobinemia and sepsis (41;43;44;47;48). The latter is invariably fatal and occurs in mice that are not maintained in pathogen-free conditions (47-49).

1.2.2.3 Disparities in MHC class I and II antigens.

Gleichmann wished to identify the cellular mechanism that would determine whether a GVH reaction would follow an acute or chronic course. Together with his colleagues, he hypothesized that the different pathological signs of acute and chronic GVHD in (B10 x DBA/2) F_1-hybrid recipients, which are derived from parental strains with different class I and II MHC antigens, resulted from the activation of different populations of alloreactive T cells in the B10, parental strain graft. Results from their experiments showed that acute, lethal GVHD was best induced using grafts containing both alloreactive T helper (Lyt-1$^+$/Lyt-2$^-$) and alloreactive "suppressor" cells (Lyt-1$^-$/Lyt-2$^+$). Under these conditions, removal of Lyt-2$^+$ T cells from the donor inoculum resulted in the development of chronic GVHD, whereas Lyt-1-depletion of the graft prevented the development of a GVH reaction (44;50). Additional experiments showed that chronic GVHD could be induced in class I and II disparate strain combinations by using only the Lyt-1$^+$ subset, (44) and that this form of the disease would also occur if donors and recipients were only different with respect to class II expression (43). In contrast, disparity in class I antigens alone did not produce GVHD with pathological signs of either the acute or chronic form of the disease. Overall, these results suggest that Lyt-1$^+$ T cells recognize and become activated by class II differences in the host, and are required for the development of either acute or chronic GVHD. In contrast, Lyt-2$^+$ T cells respond to class I differences in the host (50), and are required for the development of acute GVHD.

Experiments performed by Korngold and colleagues were similar to those of Gleichmann, but provided different results. Using a CBA (H-2^K)→ (CBA X B6) F_1-hybrid (H-2$^{k/b}$) strain combination, they showed that purified CD4$^+$ T cells could cause acute, lethal GVHD as severe as that seen when unseparated T cells were used. Alternatively, chronic GVHD developed when purified CD8$^+$ cells were used (51). In performing several of these

experiments, the investigators concluded that the ability of either the $CD4^+$ or $CD8^+$ T cell subset to cause lethal GVHD depends on the particular strain combination used. For example, $CD4^+$ cells are superior to $CD8^+$ cells in their ability to cause acute, lethal GVHD in the C3H ($H\text{-}2^k$) →(C3H xDBA/2) F_1-hybrid ($H\text{-}2^{k/d}$) strain combination, whereas both subsets are equally potent in the B6 ($H\text{-}2^b$) →(CBA x B6) F_1-hybrid ($H\text{-}2^{k/b}$) strain combination (52). This observation, and the fact that these investigators irradiated the F_1-hybrid recipients may help to explain why these results are somewhat different than those of Gleichmann and colleagues. More importantly, these inconsistencies draw attention to the fact that functional overlap exists in the $CD4^+$ and $CD8^+$ T cell subsets. Because both are capable of cytokine production and cytolysis, and GVHD-associated target organs express both class I and II antigens, Korngold and Sprent proposed that either T cell subset could mediate the development of lesions in the gut and elsewhere in the host (52).

One interesting exception to the finding that acute GVHD develops from reactions induced across class I and II barriers is the Parental Strain→(C57BL/6xDBA/2) F_1-hybrid ($H\text{-}2^{b/d}$) model. When this strain combination is used, grafts from C57BL/6 ($H\text{-}2^b$) donors produce acute, lethal GVHD and grafts from DBA/2 donors produce a chronic form of the disease. In their attempts to understand this anomaly, Gleichmann and colleagues showed that the inability of DBA/2 cells to cause lethal GVHD is associated with a gene locus located outside the class I region. This suggested that cells other than CTL are required for the development of acute, lethal GVHD (53). Another study suggested that the very low frequency of CTL precursors in DBA/2 mice could diminish their ability to cause acute GVHD (44;50).

1.2.2.4 Disparities in non-H-2 antigens.

In 1979, Rappaport and colleagues gave a pathological description of what they referred to as a "minor GVHR". These reactions were induced using B10.D2 (H-2^d) parental strain grafts that expressed several non-H-2 antigens incompatible with the irradiated (B10.D2 x DBA/2)F_1-hybrid (H-2^d) recipients. The disease they observed was characterized by severe sclerosis and glomerulonephritis, signs that are consistent with chronic GVHD (54). In another study, Miconnet and colleagues used the same F_1-hybrid strain combination and established T cell clones from the recipient mice following the induction. Most of these were non-cytolytic, $CD4^+$ clones responsive to non-H-2 Ag. The ability of some of these clones to cause acute lethal GVHD was demonstrated by using them to induce a secondary GVH reaction in a separate group of recipients. Interestingly, only the clones derived during the early stages of the primary reaction were able to mediate a similar secondary reaction (55).

Other experiments performed by Korngold and Sprent showed that GVH reactions directed against minor histocompatibility antigens (HA) develop into acute GVHD when high doses of donor-derived T cells are used, and into chronic GVHD when lower doses are used (56). These investigators used a Secondary Disease model of GVHD in which donors and recipients were of different strains that expressed the same MHC haplotype. Using 6 strain combinations, they found that $CD8^+$ T cells were consistently capable of mediating the response to minor HA, but with variable intensity. In contrast, $CD4^+$ T cells were only involved in two of the six strain combinations (57). Similar experiments performed by Hamilton showed that grafts containing both the $CD4^+$ and $CD8^+$ T cell subsets produced GVHD more severe than that seen when either subset was used, and that the subset playing a more dominant role depended on the particular strain combination used (58). Overall, these results suggest that the dose of alloreactive T cells in the graft and the particular non-H-2

antigens recognized by the cells determine whether GVHD follows an acute or chronic course.

1.2.3 Secondary disease.

Secondary Disease is the experimental model that most closely resembles clinical GVHD because recipients receive a lethal dose of irradiation prior to transplantation. It was observed and described by Trentin in 1956 while he was performing experiments aimed at developing a method for hematological reconstitution following severe irradiation injury. Using inbred strains of mice, he noticed a high incidence of mortality in recipients of allogeneic grafts, beginning several days or weeks after the lethal effects of the antecedent irradiation became apparent. He found that the degree of protection conferred by the graft was directly proportional to the genetic relatedness of the donor and recipient. When inbred stains received marrow from either another inbred strain, or from a partially-mismatched F_1-hybrid, they showed high levels of circulating erythrocytes, white blood cells and platelets, but rapidly lost weight. They also demonstrated "pulmonary congestion and consolidation" (59). These results were consistent with those of Van Bekkum and colleagues who used the term "Secondary Disease" to describe a syndrome resulting from an ongoing GVH reaction in lethally irradiated recipients of allogeneic marrow. These mice displayed signs including wasting, diarrhea, dermatitis and alopecia (60).

Irradiation injury in humans prior to allogeneic BMT undoubtedly complicates any study of the ongoing GVH reaction. However, the relationships between these two events can be studied by using models of Secondary Disease, and the survival of recipient mice beyond the time-point at which graft rejection would normally occur can provide direct evidence of engraftment.

1.2.4 Parabiosis intoxication.

Van Bekkum and colleagues also used a method known as parabiosis to study GVHD. The technique involves surgical connection of the circulatory systems of two individual mice. Using parental strain-F_1-hybrid pairs, they observed that the F_1-hybrid mouse was the first to die in 19 of 20 parabiotic pairs, and that the parental strain mouse died shortly thereafter. The F_1-hybrid mice experienced severe weight loss, skin lesions, hair loss and atrophy of the lymphoid organs, whereas the parental strain mice were unaffected by these pathological changes. In fact, the only abnormalities seen in the parental strain mice were abdominal and thoracic hemorrhages, which became apparent only when both partners died simultaneously (60). A more detailed comparison of parabiosis intoxication and the graft-versus-host reaction was made by Good and colleagues in 1968. Using the same model of GVHD, they showed that the intoxicated F_1-hybrid parabionts displayed pathological changes consistent with those seen in acute GVHD. These included transient splenomegaly followed by atrophy of the lymphoid organs, atrophy of the thymus, dermatitis, liver necrosis and vascular damage. The non-intoxicated parental-strain partner showed none of these signs but did become polycythemic (61).

Because of the obvious technical difficulties, this model is not commonly used to study GVHD. It does, however, provide a method for a producing a very severe form of acute GVHD, probably due to the very high dose of mature T cells that are exposed to host alloantigen.

1.3 GVHD in humans.

1.3.1 Bone marrow transplantation and GVHD.

In the mid-1950's Mathe performed the first clinical bone marrow transplant. It was the only hope for victims of a serious radiation accident in the former Yugoslavia (62).

Another pioneer in this area, E. D. Thomas, used BMT as a final attempt to save patients who failed to respond to conventional treatments for end-stage leukemia or aplastic anemia (63). Unfortunately, many of the very ill patients died before the graft could be properly evaluated and those that did survive beyond this time-point eventually succumbed to lethal GVHD (64).

Today, candidates for a bone marrow transplant include those diagnosed with severe combined immunological deficiency, aplastic anemia, hematological disorders, and malignant disorders such as leukemia or lymphoma (63). The application of this therapy is also expanding to include the treatment of some solid tumors such as breast cancer and neuroblastoma (65;66). If a patient is about to receive a bone marrow transplant, he or she is subjected to an immunosuppressive conditioning regimen before the marrow is infused. This is necessary firstly, because the host must not be able to reject the graft, and secondly, because it facilitates the elimination of malignant cells from the host in cases of leukemia or lymphoma. The treatment usually involves drugs such as cyclophosphamide and methotrexate, which may be delivered in combination with total body irradiation.

The graft is harvested by aspirating marrow from the pelvic bones of the anesthetized donor into tissue culture medium containing heparin. The recipient is then infused with 2-6 x 10^8 cells per kg *i.v.* Once within the host, these cells migrate to the bone marrow where they proliferate and differentiate. Within two to four weeks, peripheral blood counts in the patient should begin to rise as hematologic reconstitution is achieved (67). Following an allogeneic bone marrow transplant, patients are further treated with immunosuppressive drugs to prevent both rejection of the graft and GVHD. Methotrexate, cyclosporin, anti-thymocyte globulin, prednisone and cyclophosphamide are some of the drugs used for this purpose (68). Unfortunately, this therapy makes the patient very vulnerable to infection with bacterial, fungal and viral organisms.

It is well known that histocompatibility of the donor and host will reduce the risk of GVHD in recipients of hematopoietic grafts (64;69). In humans, the tissue type of an individual is determined by the human leukocyte antigens (HLA). These are encoded by the human major histocompatibility complex located on chromosome 6. Serological testing can be used to determine whether the donor and recipient express the same HLA-A and HLA-B haplotypes. The absence of a mixed lymphocyte reaction (MLR) when donor and recipient lymphocytes are co-cultured indicates compatibility with respect to HLA-D. Because it is known that other non-HLA antigens can also influence the development of GVHD, the best donors, in order of decreasing suitability, are as follows: a syngeneic twin, an HLA-matched sibling, another HLA-matched relative, and an unrelated, HLA-identical donor. Due to advances in our ability to manage GVHD, a partially-matched donor can now be selected if a more histocompatible donor cannot be found (67). However, results from an extensive clinical study performed in 1988 indicate that the probability of GVHD increases from 60% to 75% when one or two HLA antigen differences exist, respectively, and to 90% when all three HLA antigens are mismatched. Mortality, on the other hand, increases only when two or more mismatches were present (70). Another study found that differences in both HLA-B and HLA-DR provide the worst prognosis for survival, suggesting that some of the class III antigens encoded on chromosome 6 in the region between HLA-B and HLA-DR may be associated with the development of GVHD (71). One of the best understood minor histocompatibility antigens (HA) involved in GVH reactions is the H-Y antigen located on the Y chromosome. This is evidenced by a higher risk of GVHD in male recipients of grafts from female donors (72).

It is well known that reducing the number of mature T cells in the graft can reduce the incidence of GVHD. However, T cell depletion of bone marrow grafts prior to transplantation is also associated with reduced levels of engraftment (73-75). This is further

complicated by a decrease in the graft-versus-leukemia effect, a phenomenon in which activated cells in the graft appear to prevent leukemic relapse in recipients of bone marrow transplants (68). Older patients also appear to be at higher risk of developing acute GVHD following allogeneic BMT (76). The reasons have not been identified, but it is thought that achieving immunological tolerance of the graft may be more difficult with an aging thymus. In addition, a reduction in tissue repair mechanisms in older patients may also contribute to a lower survival rate in these patients. The effect of increasing donor age on the development of GVHD is less well established with some studies showing a correlation between these two variables, and some finding no relationship (76;77).

Although BMT is now the most common situation in which GVHD occurs in humans, others do exist. For instance, pediatricians determined in the 1960's that many cases of severe immunodeficiency in newborns appeared to have no genetic cause. They also found that infants with hydrops fetalis developed a syndrome similar to Runt Disease if they were given intrauterine or post-natal blood transfusions. It was later determined that paternal antigen expressed by the fetus can trigger a GVH-like reaction in the infant following materno-fetal transfusion (65;78-80). Graft-versus-host disease is also seen in immunosuppressed patients following transfusion of non-irradiated blood from a histoincompatible donor, or following transplantation of solid organs containing lymphoid tissue. These include intestine, liver and lung (81-84).

1.3.2 Clinical features of acute GVHD.

If GVHD develops, it is clinically classified as "acute" if signs appear very soon after transplantation, and "chronic" if they do not develop until 100 or more days post-BMT. However, these designations are rather imprecise because the pathological features of chronic

GVHD can develop soon after transplantation and signs consistent with acute GVHD can develop in human recipients of bone marrow transplants after a considerable delay (70).

Although epithelial tissues of skin, gastrointestinal tract, liver, conjunctivae, mucous membranes, exocrine glands and bronchial tree are often affected by acute GVHD, injury to the lymphoid organs is also seen. In patients with acute GVHD a maculopapular rash on the palms, soles, ears, back, shoulders or face is often the first sign that the disease is present. This may be associated with fever, and can, in severe cases, lead to denudation that resembles a burn injury. Opportunistic infections and protein loss can occur under these conditions, and represent serious problems for patients faced with cutaneous GVHD. Pathological changes in the liver include necrosis of both hepatocytes and the small bile duct epithelium. Symptoms of intestinal GVHD include diarrhea, nausea and abdominal pain. When present immediately after BMT, these symptoms are usually a consequence of the BMT conditioning regimen; however, if they persist and are not associated with infection, it can be assumed that the patient has acute GVHD. Histological analyses have shown that these lesions may be limited to destruction of single intestinal epithelial cells, or may involve the entire intestinal crypt. Mucosal denudation may also be apparent in some cases (85).

Grading acute GVHD is based on pathological changes observed in the skin, GI tract and liver. An individual showing only a transient skin rash is considered to have grade I GVHD. Grades II and III have multiple organ involvement, and Grade IV GVHD is terminal. The expected survival rates are as follows: grade I; 90%, grades II-III; 60%, and grade IV; 0% (86).

It is important to note that acute GVHD does not occur exclusively in recipients of allogeneic grafts. It frequently develops in recipients of HLA-matched grafts, due to differences in non-H-2 antigens, and in recipients of either syngeneic or autologous marrow (87-89). In the latter two cases, the GVHD is thought to result from alterations in the

antigenicity of host by chemotherapy or radiotherapy, infections, or leukemia-associated antigens. Alternatively, it is possible that the non-specific effects of cytokines produced during hematological reconstitution contribute to the development of a GVH-like reaction (87).

Because patients with acute GVHD are severely immunosuppressed, they are susceptible to a number of opportunistic infections. Bacteria, normally present in the gut and skin flora, many infect the host three to four weeks post-BMT. There is heightened susceptibility to fungal infections, particularly *Candida albicans* and *Candida tropicalis*, which is worsened by the antibiotic therapy used to treat the bacterial infections, and also by the absence of intact epithelial boundaries in the host. Viral infections are also common during this period. An example is cytomegalovirus (CMV) which represents one of the most serious infections affecting patients with acute GVHD, sometimes leading to complications such as enteritis or pneumonia. Epstein-Barr virus infection has also been associated with acute GVHD, and with a lymphoproliferative disorder developing in the first 3 months post-BMT (90;91). Patients may also develop Herpes Simplex Virus (HSV) infections which probably resulting from reactivation of a latent virus (92).

The relationship between herpes viruses and the development of GVHD has been investigated for over a decade, particularly since it has been shown that infection with these viruses often precedes the development of GVHD (93). Indeed, there is considerable evidence for an increased risk of GVHD if either the donor, or the donor and recipient are seropositive for HSV (94;95). Similarly, a positive correlation has been observed between the presence of anti-CMV antibodies in the donor and/or recipient and the development of GVHD (96;97).

In 1990 an extensive clinical study showed that donor seropositivity for at least 3 herpes viruses significantly increased the risk of acute GVHD (98). How and why this

association exists in not entirely clear, but it has been postulated that the virus itself may predispose the host to a severe GVH reaction. For example, it has been hypothesized that the reversal in the T4/T8 ratio and decreased macrophage function caused by a host HSV infection may weaken the ability of the residual, host immune system to eliminate effector T cells from the graft (98). This could be exacerbated if the donor is also infected, since it is thought that donor-derived mononuclear cells immune to a particular herpes virus may initiate a GVH reaction if they are exposed to the same virus in the host (95). This putative mechanism is supported by evidence that the V-protein of CMV has homology to the α-chain of HLA class I molecules and can bind to β_2-microglobulin with high affinity. Similarly, there is evidence for homology between the CMV antigen IE-2, which is expressed by cells during active or latent CMV infections, and HLA-DR. If this hypothesis is correct it would further suggest that the interferon (IFN)-γ produced during an active viral infections could further exacerbate the reaction by increasing expression of both class I and II molecules on a variety of cell types (99).

1.3.3 Clinical features of chronic GVHD.

In the 1970's it was reported that recipients of bone marrow transplants from HLA-matched siblings developed a protracted, autoimmune-like form of GVHD (100;101). This chronic form of GVHD develops in 30-50% of all patients who have experienced long-term survival after receiving a bone marrow transplant from an HLA-identical sibling (102). Because the host's immune function is severely suppressed, up to 80% of those with extensive tissue damage will succumb to infection (103;104). In patients treated for aplastic anemia, chronic GVHD has been associated with previous episodes of acute GVHD and increasing recipient age (105;106). There is also some evidence that this disease may be

associated with a CMV infection in the recipient, or the use of marrow from a CMV-seropositve donor (107;108).

Chronic GVHD is referred to as "progressive" if it directly follows an episode of acute GVHD, "quiescent" if it occurs after a period of remission from acute GVHD, and "de novo" if it occurs in individuals who have not previously had acute GVHD (103). Patients with chronic GVHD usually develop changes in the skin including lichenoid papules, areas of hyper- or hypopigmentation, and localized erythema. Similar changes can also be observed in the oral mucosa, and the mouth can be unusually dry due to a sicca syndrome that affects some of the salivary glands and ducts (70). Because dryness of the eyes is also characteristic of chronic GVHD, patients are sometimes described as having a Sjogren's-like syndrome in which keratoconjunctivitis sicca of the eyes and oral dryness are seen simultaneously. If chronic GVHD becomes extensive, patients can develop localized subcutaneous fibrosis, sclerosis and ulceration (103). Jaundice may also occur due to bile duct abnormalities and liver dysfunction. Pathological changes in the liver include fibrosis, hepatocellular cholestasis and bile duct destruction (70). The signs of GI tract involvement in chronic GVHD are similar to those seen in acute GVHD and include nausea, vomiting, diarrhea and abdominal pain. If the disease becomes extensive, fibrosis of the lamina propria, submucosa and serosa may develop, which may also be associated with a malabsorption syndrome (109). Cough, dyspnea and pneumothorax can indicate bronchiolitis obliterans, often after an episode of acute bronchitis or pneumonia. Some other changes associated with this disease include vaginal strictures and stenosis, eosinophilia, thrombocytopenia and neuropathy. Some patients also develop myasthenia gravis as a result of intense autoantibody production. Cases of chronic GVHD are classified as "subclinical" if histological signs are present, but the patient has no symptoms, or "clinical" if there is localized skin involvement and liver dysfunction. Chronic GVHD is considered to be "extensive" if there is generalized skin

involvement, more severe liver changes showing chronic aggressive hepatitis and bridging necrosis or cirrhosis, or if there is involvement of the eyes, salivary glands, or any other target organ such as lung or kidney (70).

Like acute GVHD, chronic GVHD is also associated with the development of bacterial, fungal and viral infections, which usually occur 6 or more months post-BMT (110).

1.4 Tissue injury in acute GVHD.

Acute GVHD affects a number of organs including the skin, liver and gastrointestinal tract. An inflammatory syndrome mediated by a number of cytokines and effector cells is largely responsible for this damage.

1.4.1 Histopathology.

1.4.1.1 Skin.

During the early stages of acute GVHD, a small number of lymphocytes can be seen adhering to the postcapillary venules in the uppermost dermis. These cells eventually extravasate and migrate into the epidermis, or remain at the dermal/epidermal junction. It is also common to observe the migration of lymphocytes into the upper third of the hair follicle resulting in "cytotoxic folliculitis". Later in the disease, another phenomenon known as "satellite cell dyskeratosis" can develop. When this occurs, lymphocytes, often containing cytoplasmic granules, can be seen surrounding an apoptotic keratinocyte. As the disease progresses, basal-cell-layer vacuolation can also develop, causing the epidermis to be "sloughed off". Although these changes normally occur during the first month post-BMT, the time interval between which the initial inflammatory infiltrates are seen and tissue damage is observed can be as short as 7 days (111).

1.4.1.2 Liver.

The most characteristic sign of acute GVHD in the liver is the presence of lymphocytic infiltrates and cellular damage in the bile ducts, particularly those of small diameter. The infiltrates appear to be in very close proximity to dying bile duct epithelial cells that are undergoing cytoplasmic vacuolization and nuclear pleomorphism. Dead epithelial cells are sloughed into the lumen of the duct, whereas those that remain viable assume a squamous shape. Hepatocytes also become necrotic or apoptotic during the course of the disease, causing the liver to have a hepatitis-like appearance. Endothelialitis is also common in GVHD of the liver, particularly in the terminal hepatic venules. However, this usually occurs only in more severe cases of the disease, and is thought to be mediated by lymphocytes that attach to and damage the endothelium (112).

1.4.1.3 Intestine.

Intestinal crypt cells are the primary targets of acute GVHD in the gut. The first phase, referred to as "proliferative", is characterized by lymphocytic infiltration of the intestinal mucosa, an increased rate of mitosis in epithelial cells, and hypertrophy of the crypts since the rate at which enterocytes in the crypts migrate to the tips of the villi increases. In the second, "destructive" phase of the disease, the crypts undergo intense hyperplasia and the villi become damaged, as the basal crypt enterocytes become apoptotic. Necrotic lesions can then be observed in the mucosa, as inflammatory infiltrates appear and lymphocytes become less abundant. A third phase of intestinal GVHD, characterized by atrophy of both the crypts and villi and the complete absence of local lymphocytes can also develop in some hosts (113).

1.4.2 Mechanisms of cell death.

The pathogenetic mechanism by which tissue injury develops in individuals with acute GVHD is not well understood, but it is likely that effector cells play a major role by killing target cells through either direct contact, or the release of cytotoxic cytokines. The type of cell death that occurs may be apoptotic or necrotic, as both have been observed in tissues targeted by an acute GVH reaction.

1.4.2.1 Programmed cell death versus necrosis.

Kerr and colleagues were the first to use the term "apoptosis" to describe the morphological changes seen in a cell undergoing programmed death. This was in contrast to necrosis, which they considered to be unintentional or accidental cell death. It is well known that apoptotic cells shrink, sometimes losing as much as 30% of their volume, and that the plasma membrane becomes detached from the cytoskeleton, a process known as blebbing or "zeiosis". Nuclear collapse also occurs as condensed chromatin localizes to the nuclear envelope and becomes cleaved by endonucleases that act at susceptible sites between the nucleosomes. Because one nucleosome is located approximately every 180 base pairs (bp), the DNA fragments from apoptotic cells produce a characteristic ladder pattern following electrophoresis. Also helpful in the identification of tissues undergoing apoptosis are "apoptotic bodies" which appear as these cells break apart into smaller, plasma membrane-bound particles that are visible by light microscopy (114).

The morphological changes associated with apoptosis dramatically contrast that which is seen during necrosis. Necrotic cells do not undergo zeiosis, but instead proceed to swell and lose the integrity of their plasma membrane. Nuclear chromatin undergoes irregular clumping, densities appear in the matrix of the mitochondria, cytoplasmic compartments

swell, and cellular membranes become discontinuous. Eventually, the chromatin disappears and the organelles disintegrate (115).

Today, it is well known that programmed cell death plays an important role in normal development and homeostasis. However, it can also contribute to the pathogenesis of several diseases. Much of the work done *in vitro* has shown that many different events can trigger apoptosis in different cell types, but the intracellular processes that follow are very similar. These events include adverse environmental conditions such as the withdrawal of growth factors, exposure to certain chemicals, irradiation and ischemia. However, apoptosis can also be induced by specific molecular interactions at the cellular surface (116). These include the binding of tumor necrosis factor (TNF) to TNF receptor-1 (TNFR1), the binding of FasL to Fas antigen, and the delivery of granzymes through perforin pores and/or the binding of granzymes to receptors (117).

1.4.2.2 Perforin and granzymes.

In the immune system, both NK cells and CTL can induce granule-mediated apoptosis of non-self and malignant target cells, as well as those infected with intracellular pathogens. The process by which this occurs involves the synergistic effects of perforin, a pore-forming protein released by the cytotoxic cell, and a family of granule-associated serine proteases known as granzymes (117). Of these, granzyme B has been shown to induce a rapid type of apoptotic cell death. This is in contrast to the slower, or delayed response that is sometimes seen when other granzymes are involved (118;119). The mechanism by which granule-mediated apoptosis is initiated and executed has been studied for some time. It is currently held that the effector cell is stimulated by the target cell to release granules into the intercellular space between the two cells. Perforin molecules then assemble to create a pore in the target cell. In the case of granzyme B, it has been shown that specific binding sites for

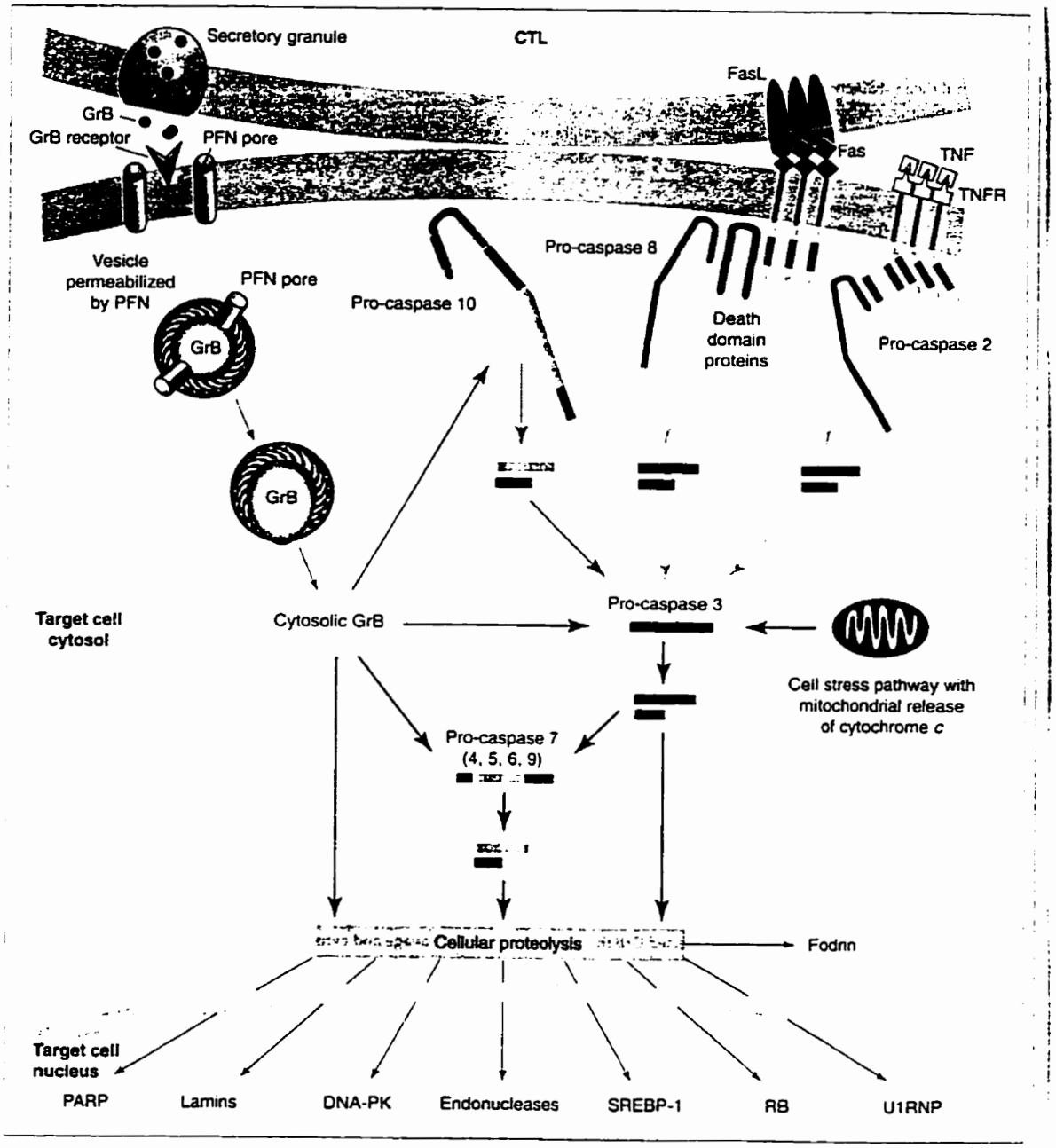
the protease exist on the cellular surface. These help to internalize the molecules which later progress through an endocytic pathway that currently awaits complete characterization. Perforin appears to be required inside the cell, in order for granzyme B to be released from the vesicle, into the cytosol, where it can initiate the program for cell death.

The process by which granzyme B initiates programmed cell death involves the cleavage of peptide bonds within the family of Interleukin-1β-converting enzyme (ICE)/Ced-3-like proteases (caspases). These enzymes become activated when they are cleaved at specific aspartic acid residues and form heterodimeric structures capable of executing the apoptotic program of cell death. Ten caspases have been identified to date, and they appear to become activated in a hierarchical order, similar to other proteolytic cascades. The order in which they act has not been completely elucidated, and the specific caspases activated in the early stages of the cascade seem to depend on the mechanism by which apoptosis is triggered (117). Figure 1 summarizes what is currently known about the events occurring in a cell undergoing apoptosis (117).

One interesting functional property of granzyme B is its ability to act at more than one step in the pathway, activating pro-caspase 10, or the "less-preferred" substrates, caspases 3,6,8 and 9 (117). This flexibility may have evolved because some viruses contain proteins that can inactivate caspases (120;121). By targeting caspases at later stages in the cascade, granzyme B may be able to overcome the effects of these viral inhibitors, thereby maintaining the protective effect that apoptotic cell death confers on the host. There is also some evidence that granzyme B can enter a cell and initiate apoptosis without assistance from perforin. This could be particularly advantageous to the host because activated CTL, which may not always be juxtaposed to a virally infected cell, are reported to secrete granzymes constitutively (117).

Figure 1.1 Cell surface interactions and the intracellular cell death pathway involved in apoptosis. Delivery of granzyme B to the cytosol, transmembrane signaling through Fas, and transmembrane signaling through TNFR are three ways in which programmed cell death may be initiated. Once inside the cell, granzyme B activates Pro-caspase 10 that can cleave Pro-caspase 3. Alternatively, it can activate Pro-caspase 3 directly. In contrast, Fas and TNF can only activate Pro-caspase 3 indirectly through activation of the upstream Pro-caspases 2 and/or 8. Cellular stressors such as mitochondrial damage can also result in the direct cleavage of Pro-caspase 3. The activated form of this enzyme then activates other downstream caspases in the pathway. Proteases enclosed in parentheses may become activated directly by granzyme B, which also has the ability to directly process other cellular proteins, thereby ensuring death to the target cell. *Abbreviations:* CTL, cytotoxic T lymphocyte; DNA-PK, DNA-dependent protein kinase; FasL, Fas ligand; GrB, granzyme B; PARP, poly (ADP-ribose) polymerase; PFN, perforin; RB, retinoblastoma protein; SREB-1, sterol regulatory element binding protein 1; TNF, tumor necrosis factor; TNFR, TNF receptor family; U1RNP; U1 small nuclear ribonucleoprotein.

Taken from Froelich, C.J., Dixit, V.M., and Yang, X. Lymphocyte granule-mediated apoptosis: matters of viral mimicry and deadly proteases (117).


Secretory granule
CTL
FasL
GrB
GrB receptor
PFN pore
Fas
TNF
TNFR
Vesicle permeabilized by PFN
PFN pore
Pro-caspase 8
Pro-caspase 10
Death domain proteins
Pro-caspase 2
GrB
GrB
Target cell cytosol
Cytosolic GrB
Pro-caspase 3
Cell stress pathway with mitochondrial release of cytochrome c
Pro-caspase 7 (4, 5, 6, 9)
Cellular proteolysis
Fodrin
Target cell nucleus
PARP
Lamins
DNA-PK
Endonucleases
SREBP-1
RB
U1RNP

Perforin, also known as cytolysin, can also mediate necrosis of target cells. This 70 kDa protein, expressed in CTL and NK cells, shows homology to the complement membrane attack complex. Following release into the intercellular space between the effector and target cell, approximately 20 perforin molecules polymerize in a Ca^{2+}-dependent manner to form a functional aqueous pore in the target cell membrane that can accommodate proteins up to 500,000 MW (110).

An important observation demonstrating a role for granule-mediated apoptosis in acute GVHD was the finding that survival is significantly prolonged in recipients of grafts from granzyme B-deficient donors (122). Similarly, it has been shown that perforin plays a role in the rapidity with which mortality occurs in mice with acute GVHD (123).

1.4.2.3 Tumor necrosis factor.

Cytotoxic granules are but one way in which cell death is initiated. The cytokine known as TNF can also induce cell death that may be either apoptotic or necrotic in nature. When this occurs, trimeric TNF binds to the 55 kDa receptor, TNFR1. This receptor contains an 80 amino acid sequence known as the "death domain" at its carboxyl end, which deletion studies have shown to be critical in the mechanism of apoptotic cell death. It is encoded by a gene belonging to a family of more than 12 genes. This family includes Fas, which also contains the death domain (124). A number of biochemical events are initiated after TNFα or TNFβ interact with TNFR1. One induces the production of oxygen radicals that contribute to the development of membrane and DNA damage. Another activates protein kinases which, through a complex series of biochemical events, stimulate the transcription of several protective genes such as those encoding superoxide dismutase, ferritin, and some heat shock proteins (115). Apoptosis can also be initiated by TNF-TNFR1 interactions, but probably requires the presence of another protein known as TRADD which is associated with

the death domain in TNFR1 (125). One noteworthy observation concerning TRADD is that it too may be targeted by viruses. This was demonstrated in a study showing that CrmA, a protein produced by cowpox virus, inhibits both caspase activation, and TRADD-mediated apoptosis (126).

Initial evidence indicating a relationship between TNF and the development of tissue damage in acute GVHD comes primarily from a study performed by Piguet and colleagues. They showed that administration of anti-TNFα to mice with acute GVHD diminishes the development of cutaneous and intestinal lesions and improves survival (127).

1.4.2.4 Fas and Fas ligand.

The third cell surface interaction known to trigger programmed cell death is the binding of FasL to Fas antigen, also known as APO-1 or CD95. The former is present on effector cells, whereas the latter is present on the surface of target cells. Fas antigen is a 45 kDa protein belonging to the TNFR/nerve growth factor receptor family of molecules. It is expressed not only on tumor cells and some non-lymphoid cells, but also on lymphocytes, where it is upregulated upon activation (128). Fas ligand is expressed by CTL, NK cells, and by cytotoxic, $CD4^+$ Th1 cells (129-131). In the case of CTL, recognition of the peptide-MHC complexes induce expression of FasL on the cell surface, which in turn binds to Fas on the target cell surface (132). Once engaged, Fas activates caspases 2 and/or 8, which then activate the more downstream enzymes, ultimately leading to death of the cell (117). The type of apoptosis that is initiated by a FasL/Fas interaction is Ca^{2+}-independent (128).

Recent studies have shown that both perforin-dependent and Fas/FasL-mediated mechanisms of cytotoxicity play a role in the development of acute GVHD (123;133;134). In a recent study, Levy and colleagues investigated the role of cell-mediated cytotoxicity in acute GVH reactions directed against non-H-2 antigens. In these experiments, they determined

how the outcome of a GVH reaction would be affected if the T cells in the graft came from *gld* mice which express functionally defective Fas ligand molecules, or if they came from donors that were perforin-deficient. They found that the type of GVHD observed when *gld* donors were used was characterized by cachexia, but not the severe hepatic and cutaneous lesions associated with acute GVHD. In contrast, the use of perforin-deficient donors produced a form of GVHD characterized by wasting, postural changes, alopecia, dermatitis, and cholangitis. The onset of GVHD-associated mortality in this model was, however, significantly delayed. These results suggest that both Fas-mediated and perforin-dependent mechanisms of cell death are involved in the development of acute GVHD. In particular, Fas/FasL interactions may be responsible for the skin and liver damage seen in acute GVHD, but not the cachexia. Perforin, on the other hand, is probably involved in processes that contribute to the rapid onset of GVHD-associated mortality (135). Some possible mechanisms that have been postulated to expedite the onset of mortality during acute GVHD are: the development of intestinal lesions which exacerbate the effector phase of the reaction eventually leading to sepsis; amplification of the GVH reaction by the increased destruction of host cells and the subsequent release of host antigen and/or inflammatory mediators; and, increased production of IFN-γ which may be involved in the process by which tissue damage occurs (136). Support for the latter mechanism comes from an experiment showing that IFN-γ can increase Fas expression on keratinocytes *in vitro* (111). A very recent report also indicated that expression of Fas-L can be increased on NK cells in bone marrow following exposure to either LPS or mannuronan (129).

1.4.3 Graft-versus-leukemia effect.

One benefit of an ongoing GVH reaction is an anti-leukemic, or graft-versus-leukemia (GVL) effect, in which effector cells in the graft eliminate either residual or re-

emerging neoplastic cells in the host. Clinicians have hoped for some time that it would be possible to separate the GVH and GVL reactions, but it appears that the two are "multicompartmental, dynamic, and highly interactive" (137). They have, however, identified two types of effector cells that mediate GVL. The first recognizes antigen expressed only by leukemic cells, and the second recognizes alloantigen present on both normal and malignant cells.

One promising approach to increasing the GVL effect without causing a similar increase in the GVH reaction has come from murine studies. Using an MHC-matched Secondary Disease model of acute GVHD, it was shown that injection of 10^7 bone marrow cells, followed 21 days later by the injection of 30 x 10^6 spleen cells, resulted in a low incidence of GVHD and a 96%, long-term survival rate. It was further demonstrated that the donor cells were able to mount an antileukemic reaction *in vivo* three weeks after the second infusion, and that complete donor T cell chimerism was achieved. In contrast, it was found that the mice succumbed to acute GVHD if the spleen cells were given concomitantly with the BM cells, or if they were given earlier than three weeks post-BMT. Similar results were also seen using MHC-mismatched donors and recipients (138). It was hypothesized that Thy-1^+/$CD3^+$/$CD4^-$/$CD8^-$ suppressor cells may develop following infusion of the bone marrow, and that these may decrease the ability of the spleen cells to cause GVHD. In summary, it is postulated that delayed leukocyte infusion may represent one of the best methods for harnessing the GVL reaction to benefit patients with leukemia (137).

1.5 Immune deficiency and suppression.

Individuals with acute GVHD experience immune deficiency that is severe and prolonged. It overlays the immunosuppressive effects of the cytotoxic therapies used to treat patients undergoing BMT, and occurs in murine F_1-hybrid recipients of parental strain

lymphoid grafts when no pre-conditioning regimen is used. Experiments performed using a murine F_1-hybrid model of acute GVHD have shown that a decrease in both mitogen and plaque-forming cell responses can be seen as early as 2-3 days post-induction. These responses reached their lowest levels by day 7-8 of the reaction, and usually did not recover (139). Currently, there are at least three different types of immunosuppression known to occur during acute GVH reactions, and it is thought that the overlap between these events is sufficient to sustain the observed reduction in immune responsiveness. This first involves a marked decrease in the number and responsiveness of host-reactive T cells due to anergy or clonal deletion, the second is due to the effects of cells that can actively suppress of T and B cell function, and the third results from diminished or delayed lymphopoiesis and thymic injury in the host (139;140).

1.5.1 Deletion and anergy of host-reactive T cells.

During the early stages of an acute GVH reaction, donor T cells respond to host antigen and proliferate. Shortly thereafter, their numbers decline rapidly, a phenomenon associated with programmed cell death. For example, when acute GVH reactions are induced in recipients that share the same MHC haplotype as the donors, but express a different mls antigen (mls^a), the mls^a-reactive donor T cells expressing T cell receptor (TCR) Vβ6 proliferate until they comprise up to 60% of the total number of $CD4^+$ T cells present in the host. Their numbers decrease thereafter until the host dies. Similar results have been seen using MHC-matched strain combinations (141-144). It has also been shown, using a B6→BDF_1-hybrid model of acute GVHD, that donor T cell numbers are markedly reduced in mice that survive 6 weeks post-BMT, and that the remaining cells respond poorly to mitogens, phorbol myristate acetate and calcium ionophore (145). Still other experiments have shown that the T cells present in mice surviving 3 to 6 months post-induction are mostly

mature cells derived from the graft. These cells are incapable of generating CTL activity directed against host target cells, but can mount a cytotoxic response against other allogeneic targets. The addition of interleukin (IL)-2 was not found to improve this responsiveness, suggesting that a lack of T cell help was not the cause. Furthermore, lymphoid cells from these recipients were unable to reduce the responsiveness of normal donor cells to host alloantigen in co-cultures, suggesting that suppressor cells were not responsible for this effect. The development of the type of host-specific tolerance observed in these experiments is therefore consistent with the idea that donor lymphocytes either become anergic to host antigen, or undergo clonal deletion (146;147).

1.5.2 Suppressor cells.

The existence of suppressor cells in mice with acute GVHD has been demonstrated in experiments showing that T or B cell responses in normal donor or host populations can be reduced *in vitro* by co-culturing them with cells from mice undergoing a GVH reaction. The types of cells capable of active suppression in GVH mice have been identified as $CD8^+$ T cells, macrophages, and natural suppressor cells (44;148;149).

In one study, $CD8^+$ cells from the spleens of mice with acute GVHD could inhibit the ability of cytotoxic host cells to kill allogeneic target cells (148). However, another series of experiments showed that the $CD4^+$ cells present during acute GVH reactions have suppressor function, but that it is non-specific (150). In a human study, it was also shown that a suppressor subset of $CD8^+$ cells expressing CD57 expands post-BMT and can persist for more than three years in the peripheral blood (151).

Macrophages have also been shown to mediate active immune suppression during the first 5-10 days of an acute GVH reaction. This effect is associated with an increase in the number of macrophages present in the spleen such that they make up over 30% of all the

mononuclear cells. Studies performed by Lapp and colleagues showed that this type of suppression is mediated, at least in part, by production of prostaglandin E (PGE) by macrophages in the spleen, lymph nodes and thymus. This was evidenced by a partial reduction in immunosuppression following treatment with indomethacin during the early stages of the reaction. Other experiments showed that removal PGE-producing cells was only successful in reducing the level of immunosuppression if performed during the first two weeks of the reaction and not later, suggesting that the effect is transient (152). Another study performed more recently by a different group of investigators showed firstly that the suppressor function exhibited by splenic macrophages from GVH mice is increased if they are stimulated with LPS, and secondly, that this is dependent on the presence of $CD8^+$ T cells (153). The former observation is probably associated with the ability of endotoxin to induce the release of TNFα from activated macrophages, particular those "primed" with IFN-γ (154). In experiments performed by Sheehan and colleagues, it was shown that anti-TNFα could reduce the level of T cell suppression in spleen cell cultures derived from mice with acute GVHD, suggesting that this cytokine has immunosuppressive properties (155). Macrophage-derived nitric oxide (NO) can also be immunosuppressive in GVH mice, since it has been shown that Concanavalin A (Con A) responsiveness increases in spleen cell cultures treated with an NO inhibitor (156).

Natural suppressor (NS) cells seen during the first few weeks post-induction represent the third type of suppressor cell present during acute GVH reactions (139). They fail to express CD4, CD8, macrophage markers, or surface immunoglobulin (Ig), and act in a non-MHC-restricted manner. Normally present in the adult bone marrow, NS cells may also be detected in the spleen following injury to the lymphoid tissue (157). These cells are IL-2- and IFN-γ-responsive, and have the ability to reduce mitogen responsiveness (158).

It is important to note that much of the suppressor function associated with these cell types is probably not due to their direct attack on other cells. Instead, it appears to be associated with their ability to produce cytokines. For example, the addition of anti-IFN-γ to spleen cell cultures from GVH mice has been found to improve both Con A and LPS responsiveness *in vitro*, which is consistent with the observation that *in vivo* levels of this cytokine rise during the early stages of a GVH reaction when immunosuppression develops (159;160). Similarly, anti-transforming growth factor (TGF)-β was found to restore B cell responsiveness in spleen cell cultures from GVH mice. Interestingly, these observations, made by Huchet and colleagues, also indicate that these two cytokines are ineffective at suppressing T and B cell responses in the form that they are secreted in during the GVH reaction. It has therefore been postulated that IFN-γ and TGF-β must first be converted to an active form by another cell present in the culture. These investigators suggested that NS cells may perform this function (159).

1.5.3 Maturational defects.

The major factor contributing to long term immunodeficiency in GVH mice is delayed lymphocytic recovery. In bone marrow transplant recipients, it has been found that both the number and activity of stem cells is significantly reduced in patients with GVHD, compared to those who do not develop the disease (161). It appears that the generation of NK, B, and T cells is more affected by the GVH reaction than that of other lineages, since peripheral blood counts of neutrophils and platelets recover much more quickly (144;161). One exception to this observation is the $CD8^+$ T cell subset, which can recover more quickly in patients with acute GVHD than in those who do not develop the disease, and can remain persistently high in patients with chronic GVHD (162-164).

Cytokines are believed to be partially responsible for diminished B cell production during GVHD. Using a B10.D2→BALB/C model of acute GVHD, Garvy and colleagues observed a reduction in both the number of IL-7-induced colony forming units (CFU), and the number of pro-B cells. This effect was mediated by IFN-γ, which may have directly inhibited the ability of the B cells progenitors to proliferate (144). Alternatively, this cytokine may have stimulated the release of IL-1 from macrophages, which could lead to a reduction in the ability of the marrow to generate pre-T and B cells (165).

The delay in T cell recovery observed in individuals with GVHD is largely attributable to thymic injury. This is evidenced by murine studies showing that the host thymus plays a critical role in T cell reconstitution following BMT, even if the donor is syngeneic (166). Other work has shown that the thymus becomes atrophied during acute GVH reactions, and incurs extensive damage to both its epithelial and lymphoid components. Experiments performed by Seemayer and colleagues using an F_1-hybrid model showed that thymic involution could be seen as early as 6 days post-induction. Severe "thymic dysplasia" was apparent, as evidenced by a loss of demarcation at the cortical-medullary junction, the disappearance of Hassall's corpuscles, and damage to the medullary epithelial cells. Two mechanisms were found to be responsible for this injury. The first affected only the cortex, was mild in severity, non-specific, and probably stress-related. The second affected both the medulla and cortex and resulted in a moderate-to-severe lesion with lymphocytic infiltration. The latter type of injury appeared to be a consequence of the ongoing GVH reaction, and was thought to result from a cytolytic attack on the medullary epithelial cells by NK and T cells (139;167;168). Based on these findings, it is not surprising that thymic selection can be disrupted during GVHD. This is supported by results from experiments performed by Desbarats and colleagues who showed that negative selection is disrupted during GVHD. More specifically, it was shown that that there is a marked reduction in class II expression in

the thymus (169). This, and two other studies showed that TCR Vβ-expressing clones reactive with host Mls antigen are not deleted during chronic GVHD (170;171). Based on these observations, it has been hypothesized that thymic injury allows host-reactive T cells to mature and could contribute to the development of the autoimmune disorders associated with chronic GVHD (172). However, recovery of the thymus has also been seen in some long-term survivors of GVHD, and is associated with the reappearance of medullary thymocytes, epithelial cells and Hassall's corpuscles (173-175).

1.6 Pathogenetic mechanism.

The pathogenetic mechanism responsible for the development of "stimulatory" GVHD occurs in two stages. The first involves the activation of donor-derived, alloreactive T helper cells that produce Th1 cytokines. The second is characterized by inflammation, tissue injury, severe immunosuppression and cachexia, and develops when cytokines, cytotoxic effector cells and LPS collaborate to produce an acute, lethal form of GVHD.

1.6.1 The Th1/Th2 paradigm.

$CD4^+$ T cell clones differentiate into two distinct immunoregulatory subsets *in vitro* (176). The first secretes IL-2, IFN-γ, TNFα and TNFβ, and is referred to as Th1. The second secretes IL-4, IL-5, IL-6, IL-10, IL-13, and some TNFα, and is referred to as Th2. Both subsets produce IL-3 and granulocyte/macrophage colony stimulating factor (GM-CSF) (177). A third subset, referred to as Th0, is reported to produce IL-2, IL-4, and IFN-γ, and is likely the precursor of $CD4^+$ Th1 and Th2 cells (178). Recent evidence also indicates that this dichotomy is not restricted to $CD4^+$ T cells, as $CD8^+$ T cells can also become polarized to produce cytokine profiles similar to Th1 and Th2 cells (179;180). Experiments performed both *in vitro* and *in vivo* have shown that IFN-γ inhibits the proliferation of Th2 clones. In

contrast, IL-10 inhibits cytokine production in Th1 clones by acting on antigen presenting cells (181-186).

Polarization of T helper cells to either a Th1 or Th2 phenotype determines, respectively, whether a cell-mediated or humoral immune response will develop (187). The importance of this phenomenon has been confirmed by *in vivo* experiments showing that the Th1/Th2 balance plays an important role in determining how effectively an animal can resist infection or tumor challenge (188-191). The conditions that promote the commitment of naive T cells to either the Th1 or Th2 phenotype are not completely understood, but evidence suggests that the environment in which antigenic stimulation occurs may be one factor involved in this process. For example, cytokines present during antigenic stimulation can determine whether a Th1 or Th2 response predominates (192-194). This is evidenced by studies showing that IL-12 mediates the commitment of antigen-specific precursor cells to the Th1 phenotype, whereas IL-4 promotes the development of a cells with a Th2 phenotype (188;192-196). Similarly, the type of antigen presenting cell involved in initiating the response appears to influence the phenotype of Th cells that predominates. For example, it has been shown that optimal proliferation of cells producing Th1 and Th2 cytokines occurs when antigen is presented by adherent APC and B cells, respectively (197).

A consequence of a Th1-mediated immune response is the activation of $CD8^+$ CTL. This occurs when $CD4^+$ T cells produce IL-2, which is required by most CTL in order to produce an effective cytotoxic response (198). Once activated, CTL can also release significant quantities of IFN-γ which can initiate inflammatory responses and other events that play a role in the effector phase of a Th1-mediated immune response (199;200). Interestingly, results from other *in vitro* studies have shown that precursor CTL can also be stimulated by Th2 cytokines to produce IL-4 (201). This observation is supported by results from an *in vivo* study in which it was shown that the ability of CTL to produce Th1 cytokines

and control a viral infection is mitigated during a Th-2-mediated response to schistosomiasis infection (202).

Although it is clear that cell-mediated and humoral immunity represent two different arms of the immune system, it is likely that a continuum exists between Th1 and Th2-mediated responses, such that both Th subsets participate in any particular antigen-specific immune response (203). For example, the IFN-γ released during a Th1-mediated response appears to prime macrophages to release several inflammatory mediators such as TNFα, IL-1, IL-6 and NO (172). Evidence suggests that regulation of this response is probably achieved when Th2 cytokines such as IL-4 and IL-10 downregulate the production of these inflammatory mediators by macrophages (204-208).

1.6.2 Acute GVHD results from a Th1 response.

There is now considerable evidence that acute GVH reactions are the result of a Th1-mediated immune response. Although many cytokines are involved in the pathogenesis of this disease, the following discussion is limited to those that are most important in the development of a Th1 response.

1.6.2.1 Interleukin-2.

The T helper response occurring in the alloreactive phase of an acute GVH reaction is characteristically Th1, and involves the production of significant amounts of IL-2 in the very early stages of the reaction (209;210). This cytokine is a 14-17 kDa glycoprotein produced by $CD4^+$ T cells, and to a lesser extent, $CD8^+$ T cells. It is synthesized in response to the binding of TCR to MHC-peptide antigen (177;211;212). The engagement of costimulatory molecules such as B7 and CD28 facilitate this process, as antigen binding in the absence of these signals results in little or no IL-2 production and a state of T cell anergy (213;214).

Interleukin-2 exerts its effects by binding to a high-affinity, multimeric IL-2 receptor (IL-2R) belonging to the type 1 cytokine receptor family which includes receptors for IL-3, IL-4, IL-5, IL-6 and IL-7 (212;215;216). The IL-2R chains, referred to as α, β and γ, must be present to assemble the high affinity form of the receptor. However, a lower affinity form, comprised of only the β and γ chains, is also known to exist (212).

When T cells are stimulated with IL-2, they respond by undergoing rapid mitosis. Natural killer cells respond by increasing both their cytolytic activity and rate of mitosis, but require higher concentrations IL-2 since they express only the low affinity IL-2R (217). Other cellular functions stimulated by IL-2 include the promotion of IFN-γ production by T and NK cells and the enhancement of growth and antibody production by B cells (212).

The importance of IL-2 in the development of acute GVHD is evidenced by experiments in which neutralization of IL-2 decreased the severity of the disease (218). Further support comes from clinical data showing that treatment with anti-IL-2 receptor antibody attenuates GVHD in some patients (219).

1.6.2.2 Interleukin-12.

This cytokine is a 75 kD heterodimeric molecule made up of a constitutively expressed, 35 kDa subunit, and an inducible 40 kDa subunit (220). Although many cells express the 35 kDa chain, evidence suggests that it is not secreted unless associated with the p40 chain (221;222). Cells known to produce the bioactive, p75 form of the cytokine are dendritic cells (DC), macrophages and neutrophils. Synthesis occurs in DC and macrophages through interaction with activated T cells, which is followed by costimulation with CD40 ligand. Macrophages are also reported to produce IL-12 when exposed to bacteria, LPS or intracellular parasites either *in vitro* or *in vivo* (223;224). Minor sources of IL-12 include

keratinocytes, Langerhans cells and resting B cells. The latter cells do, however, produce larger quantities of IL-12 following transformation with EBV (225).

The IL-12 receptor is expressed on activated T and NK cells, but not on monocytes or B cells (226). It consists of β1 and β2 chains, both of which bind IL-12 independently and with low affinity. Transfection studies indicate that expression of both subunits can produce a high affinity IL-12 receptor on the cellular surface. Both Th1 and Th2 cells express the β1 chain whereas the β2 chain is only expressed by Th1 cells, making them much more responsive to IL-12 (225).

Interleukin-12 acts on naive T cells in synergy with IL-2 to induce IFN-γ production, thereby promoting a Th1 phenotype and the development of a cell-mediated immune response (227-230). Its effects on NK cells include the promotion of growth, cytotoxic activity and IFN-γ production (231-235). Production of other cytokines such as TNFα, GM-CSF, macrophage colony stimulating factor (M-CSF), IL-3, IL-8 and IL-2 can also be induced when IL-12 stimulates NK and T cells (236).

Because of its powerful ability to promote a Th1-mediated immune response, it has been hypothesized that an antagonist for IL-12 also exists *in vivo*. The IL-12 p40 subunit may perform this function since it has no biological activity when bound to the IL-12R, and may be produced in quantities that far exceed the functional IL-12 heterodimer, particularly following stimulation with LPS (237).

Studies on the role of IL-12 in acute GVHD have demonstrated that chronic GVH reactions can be manipulated to follow an acute course if mice are given injections of IL-12 during the early phase of the reaction (238). Similarly, it was shown that neutralization of IL-12 during the induction phase of an acute GVH reaction can shift the cytokine profile towards a Th2-like response, thereby protecting mice against mortality. This finding is particularly important since treatment with anti-IL-12 antibody did not result in the development of

chronic GVHD, which would preclude it as a potential clinical therapy (239). Another study investigating IL-12 expression during acute GVHD showed that mRNA for both IL-12 p40 and IFN-γ were present in the thymus, salivary gland and lung during the second week of the reaction, suggesting that these cytokines are produced locally in target tissues (240). Because endotoxin was observed in the liver and spleens of GVH mice (241), and their macrophages were primed to produce NO in response to LPS *in vitro*, (242) the following mechanism was proposed for the development of target tissue injury: Endotoxin stimulates macrophages to produce IL-12 which induces local IFN-γ production. This cytokine primes macrophages to produce effector molecules such as NO and TNFα that have cytotoxic effects on local tissues. Interestingly, these investigators also suggested that NK cells were the source of IFN-γ, since T cell responses are known to be severely suppressed at this stage of the reaction (241).

1.6.2.3 Interferon-γ.

Interferon-γ is a 15 kDa glycoprotein belonging to a family of proteins involved in host defense against viral infections. Interferons are divided into two categories, referred to as types I and II. The first includes both IFN-α and IFN-β, and the second includes only IFN-γ. Type II IFN is unrelated to type I IFN either genetically, or with respect to its protein structure. It is therefore not surprising that significant functional differences also exist between these two types of IFN. Firstly, IFN-γ has a 10-100 fold lower level of antiviral activity than IFN-α/β and, secondly, it is 100-10,000 times more potent as an immunomodulator (99).

Interferon-γ is produced by CD4 or CD8-expressing, Th1 cells, some Th0 cells and NK cells. Stimuli known to induce its biosynthesis in T cells include T cell mitogens, pharmacological stimuli, and the binding of the TCR to MHC-peptide complexes, anti-TCR

or anti-CD3 antibodies (99). Interleukin-12 can induce IFN-γ production in both T and NK cells, whereas IL-10 can indirectly inhibit the ability of T cells to produce this cytokine. Other inducers of IFN-γ production in NK cells include mitogens and bacteria or their products (243). Recent evidence also indicates that IFN-γ release by NK cells may be triggered by cross-linking of either NK1.1, or CD45 (244;245). It is now known that IFN-γ protein can appear in the extracellular environment within 8-12 hours of transcription, and can persist *in vitro* long after the stimulus has been removed (99). Its presence *in vivo* appears to be more short-lived, as it can rarely be detected in circulation. It is hypothesized that this is probably due to its ability to bind rapidly to the IFN-γ receptor expressed on most cell types (246). Alternatively, it may be due to the presence of anti-IFN-γ natural antibodies (247).

The IFN-γ receptor is comprised of an α chain expressed at moderate levels on many cell types, and a β chain which is expressed at very low levels, but may be upregulated on some cells in response to certain stimuli (248). In 1995, two separate studies indicated that $CD4^+$ T cells differ in their ability to respond to IFN-γ. In particular, unresponsiveness was found to occur in Th1 cells due to IFN-γ-dependent downregulation of the IFN-γ receptor β chain. Expression of the β chain was seen in IFN-γ-responsive Th2 cells, but was also down-regulated after the cells were exposed to the receptor ligand. In contrast to that which was seen in T cells, decreased expression of the β chain was not seen on some fibroblast cell lines following exposure to IFN-γ. From this, it was concluded that IFN-γ regulates expression of it own receptor on certain cell types (248-250).

Interferon-γ plays an indispensable role in regulating the development of $CD4^+$ T cells so that a cell-mediated immune response prevails. In particular, it inhibits the proliferation of the $CD4^+$ Th2 subset known to produce IL-10, a potent inhibitor of IFN-γ

production (251;252) . A study performed using IFN-γ gene knockout mice has also shown that IFN-γ may suppress the response of cytotoxic T cells to allogeneic targets (253).

In addition to its role in the early stages of an immune response, IFN-γ also functions to activate and regulate macrophage functions (254). Firstly, it is involved in the differentiation of myeloid precursors into mature monocytes. Secondly, it promotes antigen presentation by increasing the expression of class I and II MHC antigens, the enzymes involved in antigen processing, and ICAM-1, which assists in the interaction between macrophages and T cells (253;255-257). Thirdly, IFN-γ activates cytolytic activity in macrophages which protects the host against intracellular and extracellular parasites, as well as neoplasms (258-260). This event is associated not only with increased production of reactive oxygen intermediates, reactive nitrogen intermediates, and TNF-α, but also with an increase in FcγRI expression which is known to mediate antibody-dependent cellular cytotoxicity (259;261).

The role of IFN-γ during an inflammatory response is largely related to its ability to increase TNF-α production. This occurs when macrophages are exposed to IFN-γ and LPS concomitantly, or sequentially (262;263). Studies have shown that IFN-γ can cause an increase in the rate of TNF-α transcription and have suggested that it also increases the stability of TNFα transcripts (262;264). Other work has demonstrated that this cytokine can increase expression of TNFR1 and TNFR2 on a number of cell types (265;266). A role for IFN-γ in septic shock was demonstrated in a murine study showing that injection of IFN-γ 18 hr prior to injection of endotoxin significantly increased mortality, whereas pre-treatment with anti-IFN-γ antibody had a protective effect (263). These results are further supported by the observation that IFN-γ receptor-deficient mice are resistant to endotoxic shock (267). During an acute inflammatory response, IFN-γ can also up-regulate the expression of

adhesion molecules on the vascular endothelium. For example, it has been shown that IFN-γ can increase expression of ICAM-1 on HUVEC cells *in vitro*, and that this effect is enhanced if the cells are cultured in the presence of both IFN-γ and TNF-α. Similar synergistic effects of these cytokines were demonstrated by studies showing that IFN-γ and TNFα could increase ELAM-1 expression, which may be important in prolonging the recruitment of lymphocytes from circulation (256;268).

Evidence for involvement of IFN-γ in acute GVHD comes primarily from a study in which it was shown that treatment with anti-IFN-γ prevented much of the intestinal injury associated with the later stages of the disease (269). In two other studies, it was shown indirectly, that IFN-γ is required for the induction of an acute GVH reaction. In the first, mature donor T cells were incubated with both IL-4 and irradiated recipient spleen cells prior to induction of the GVH reaction. Unlike untreated T cells from the same donors, these polarized Th2 cells failed to induce acute, lethal GVHD. Instead, their use abrogated mitogen-induced IFN-γ production and LPS-induced TNFα release in recipient spleens (270). In a similar study, Th2 donor cells were prepared by injecting donor mice with either a combination of IL-2 and IL-4, or a high dose of IL-2 prior to inducing the GVH reaction. The $CD4^+$ T cells isolated from these donors secreted higher levels of IL-4 and IL-10, and lower levels of IL-2 and IFN-γ, compared to similar untreated cells. When given grafts containing the polarized Th2 donor cells, allogeneic recipients that would normally have developed acute, lethal GVHD showed reduced levels of $CD8^+$ T cell engraftment, *in vivo* suppression of IFN-γ mRNA, *in vivo* elevation of IL-4 mRNA, and lower levels of LPS-induced TNFα release (271).

It was already mentioned that IFN-γ mRNA is detectable in target tissues during acute GVHD, and that this may contribute to the development of lesions by priming macrophages.

However, it is also important to note that these affects may also be influenced by IFN-γ's ability to up-regulate the expression of class I and II molecules, thereby making these tissues more vulnerable to attack (99).

1.6.2.4 TNFα

Tumor necrosis factor-α and TNF-β have similar functions but are encoded by different genes. The former is a product of activated macrophages, and is usually produced in much larger quantities than the latter, which is derived mostly from T cells (217). Although activated macrophages are the major source of TNF-α, other cells are also known to produce it. These include $CD4^+$ Th1 and Th2 cells, NK cells, mast cells, polymorphonuclear cells, eosinophils, astrocytes, Langerhans cells and Kupffer cells (203;262;272-274).

One of the most important functions of TNF-α is to promote inflammation. This ability makes it similar to interleukin-1, and the two cytokines often work synergistically. However, TNF-α has other unique properties including the ability to lyse tumor cells and mediate cachexia (217).

Tumor necrosis factor-α is a 26 kDa nonglycosylated transmembrane protein that can remain bound to the surface of the cell as a cytotoxic effector molecule, or can be cleaved to form a 17 kDa secreted protein if the cell is stimulated with LPS. The 17 kDa subunits can then unite to form a biologically active, trimeric structure (275) that is relatively unstable, with a serum half-life of only 6 to 20 minutes (276). Although lipopolysaccharide is the primary inducing factor for TNF-α production, it is well known that macrophages primed with IFN-γ will produce much larger quantities of TNF-α following stimulation with LPS. However, other stimuli including pathogens, complement fixation products, immune complexes and other cytokines can also induce TNF-α production in macrophages (154;274;277).

Tumor necrosis factor receptor-1 and TNFR2 bind both TNFα and TNF-β, and have molecular weights of 55 and 75 kDa, respectively (124;278). The first is expressed on most cells types, whereas the second is present on cells of the liver, muscle, fat, intestines, lung and hematopoietic organs (274;279). Reports indicate that the murine forms of these receptors probably have distinct functions, with TNFR1 signaling cytotoxicity and TNFR2 signaling proliferation of primary thymocytes and T cells (280). Binding studies have shown that TNFR2 has a higher affinity for TNF than TNFR1, and TNFR2 antagonists can inhibit TNFR1 activity when TNF is present at low concentrations. The following mechanism for TNF binding has therefore been proposed: At high concentrations, TNF binds to TNFR1 and TNFR2, signaling both cytotoxicity and proliferation. At low concentrations, TNF binds preferentially to TNFR2 and signals cellular proliferation. When this occurs, TNF becomes more accessible to TNFR1 and can induce cytotoxicity. Whether the latter effect results simply from an increase in the local TNF concentration, or whether TNFR2 actually presents the cytokine to TNFR1 is not known (124;278). Another finding regarding the kinetics of TNF binding suggests that the cytokine must bind to both TNFR1 and TNFR2 in order to be lethal *in vivo*. This study showed that murine TNF-α, which binds to both receptors, was lethal in mice when injected at sufficiently high concentrations. In contrast, human TNF-α, which binds exclusively to TNFR1 in mice, was not lethal when injected at similar concentrations (281). It has also been reported that the extracellular portions of TNFR1 and TNFR2 can be released following proteolytic cleavage, making them detectable in serum and urine as soluble proteins (282;283).

The biological activity of TNF-α *in vivo* seems to depend on the concentration at which it is present. At low concentrations of approximately 10^{-9} M, it acts on a local level to increase the expression of adhesion molecules on vascular endothelium, to stimulate the production of proinflammatory cytokines such as IL-1, IL-6, IL-8 and TNFα itself, and to

provide costimulation for T- and B-cell activation. Low concentrations of this cytokine can also promote migration of inflammatory cells, proliferation of fibroblasts, expression of MHC molecules and cytotoxic activity. If high TNF-α levels in excess of 10^{-7} M occur, vasodilatation, a reduction in tissue perfusion, hypoglycemia, and intravascular thrombosis can occur. High concentrations of this cytokine can also induce a syndrome consistent with that seen during an episode of endotoxemia. These include the development of fever, shock, tissue injury and the production of acute phase proteins by the liver. Prolonged TNFα release can result in metabolic changes in muscle and fat tissue resulting in a severe wasting syndrome known as cachexia, as well as immunodeficiency. The latter effect is thought to result from the ability of TNFα to interfere with stem cell division (217).

As mentioned previously, TNF-α has been shown to act as an effector molecule in the development of skin and gut lesions during acute GVHD. The demonstration that anti-TNF-α prevents the development of these lesions and markedly reduces mortality indicates that this cytokine plays a role in the pathogenesis of the disease (127). Consistent with these findings is the observation that TNF-α levels increase in the sera of bone marrow transplant recipients before, or at the time they developed interstitial pneumonitis or severe acute GVHD (284;285). Recently, a study was performed using an acute model of GVHD in which some recipients carried a mutation in the gene encoding TNFR1. Results indicated that the onset of mortality was significantly delayed in the TNFR1 KO recipients if low doses of donor lymphocytes were used to induce the reaction. However, if high numbers of donor spleen cells were injected, the reaction in TNFR1 knockout recipients followed a course very similar to that seen in the wild-type recipients (286).

1.6.3 Role of non-MHC-restricted cytotoxic cells.

1.6.3.1 Natural killer and natural killer-like cells.

Natural killer cells were originally described as cells that could kill tumorous or virally-infected target cells without previous sensitization (287). Today, we know them as large granular lymphocytes (LGL) that express FcγRIII (CD16) and CD56 in humans or FcγRIIA and NK1.1 in mice. They do not express CD3, or the α, β, γ or δ chains of the T cell receptor (TCR). Other markers present on the surface of murine NK cells include Thy-1 and $ASGM_1$ (288). Unlike cytotoxic T cells, NK cells can lyse targets lacking expression of either class I or II MHC molecules, a term commonly referred to as "non-MHC-restricted cytotoxicity". However, some T cells, often referred to as "NK-like", can also lyse target cells in a manner that is TCR-independent and does not require the presence of MHC antigen on the target cell (289).

When viewed by transmission electron microscopy, LGL can be seen to contain round nuclei with condensed chromatin and prominent nucleoli. Numerous organelles and lysosomes are present within the cytoplasm. These lysosomes contain granules composed of phospholipids, proteoglycans, granzymes and perforin (290-292). Characteristic structures known as parallel tubular arrays can also be seen adjacent to the granules, but their function remains elusive (293). Although collectively referred to as LGL, it is important to note that some cells with NK activity are neither large nor granular. Whether these features represent differences in the activation status of different NK cells or whether the population is morphologically heterogeneous is not known (290;294).

Natural killer cells usually survive for no more than a few weeks *in vivo*, and are derived from the bone marrow (295). When appropriately stimulated, their numbers and pattern of distribution change due either to increased production by the marrow, or possibly to increased proliferation of mature cells (296). Evidence that NK cells originate in the bone

marrow comes from experiments demonstrating that treatment with strontium 89, known to have a high affinity for bone, results in a decrease in the level of splenic NK activity, but does not affect CTL- or macrophage-mediated cytotoxicity (19). In another experiment, mice with a low, baseline level of NK activity were irradiated and reconstituted with bone marrow from another strain with a high baseline level of NK cell-mediated cytotoxicity. The recipients developed levels of NK activity consistent with that seen in the donor strain, suggesting that these cells can develop normally in an allogeneic recipient (297). Furthermore, it has also been shown that NK cell function is impaired in mice with an altered bone marrow microenvironment, such as that seen in osteopetrotic mice (298). Other evidence that the differentiation of NK cells is not thymus-dependent comes from studies showing that these cells develop normally in nude mice and in humans with DiGeorge's syndrome, and that they appear in the human fetus before the thymus is fully developed (289;299;300).

Because they do not rearrange TCR genes, NK cells also develop normally in severe combined immunodeficiency (SCID) and RAG-2-deficient mice. However, results from several studies support the hypothesis that NK and T cells are developmentally related. One shows that the ζ chain of CD3 can be seen in association with CD16 (FcγRIIIA) on the surface of human NK cells (301). CD2 is also expressed on 80% of all NK cells and can mediate cytotoxicity. The CD8α chain is present on approximately 30% of all NK cells, but is expressed at a considerably lower density than that seen on T cells. CD7 is also expressed on both cell types (302-305). Other support for the idea that NK and T cells may originate from a common precursor comes from experiments in which it was shown that $CD7^+/CD3^-/CD4^-/CD8^-$ (triple negative; TN) human thymocytes could give rise to $\alpha\beta TCR^+$, $\gamma\delta TCR^+$, and a small number of $CD3^-/CD16^+$ NK-like clones (306). Furthermore, TN cells expressing

CD16 could generate NK cells either *in vivo* or *in vitro* when removed from the thymic microenvironment (307).

Once NK cells mature, they can comprise up to 15% of all peripheral blood lymphocytes in a normal individual. The organ with the highest percentage of mature NK cells is the spleen, but they also appear in the liver, lung and intestinal mucosa, as well as in the decidua during early human pregnancy (288;296;308).

Natural killer cell-mediated cytotoxicity involves the binding of effector to target, followed by the exocytosis of lytic granules and either apoptotic or necrotic death of the target cell (292). One molecule known to activate the cytotoxic mechanism in mice is FcγRIIα. It is expressed on murine NK cells and is homologous to the human NK cell surface molecule, CD16. FcγRIIA recognizes the Fc portion of IgG on the surface of target cells, and mediates antibody-dependent cell-mediated cytotoxicity (288). Also implicated in triggering this cytotoxic mechanism are adhesion molecules, and other surface molecules such as those capable of mediating cytotoxicity in the absence of antibody (31;309).

As mentioned earlier in this review, the Ly49 family of molecules present on murine NK cells are receptors for self, class I MHC antigens. Once engaged, these receptors deliver an inhibitory signal to the NK cell, preventing lysis of the class I-expressing target cell. However, Ly49 is not expressed on all murine NK cells, and it has therefore been hypothesized that homologes to Ly49 may perform a similar role (31). Furthermore, single amino acid substitutions in the α1/α2 peptide-binding domain of class I molecules can abrogate the delivery of an inhibitory signal to an NK effector cell, indicating that this groove participates in the process (310).

Another important function of NK cells is cytokine production. When appropriately stimulated, these cells can secrete IFN-γ, GM-CSF, M-CSF, TNFα, IL-3 and IL-8 (288;311-313). Stimulation may be mediated by other cytokines such as IL-2, IL-12, TNFα, IFN-α

and IL-1. It may also occur following the the interaction of FcγR or other undefined receptors with the ligands present on the surface of NK-sensitive target cells (288;314-316). The ability to produce cytokines and to lyse certain target cells without previous sensitization makes NK cells a first line of defense against a number of threats including viruses, bacteria, parasites and tumors.

Support for the idea that NK cells are important in antiviral immunity is provided by clinical data indicating that patients experiencing recurrent viral infections are often deficient in NK cell function, and by other experiments showing that depletion of NK cells increases the level of virus-induced hepatitis *in vivo* (317). Still other *in vivo* studies have shown that early NK cell responses to viruses are associated with both an increase in the size of the NK population, and a higher level of activation. This response peaks 3 days post-infection, is associated with a decrease in viral titre, and is followed by a strong antigen-specific T-helper and CTL response 7-9 days post-infection (296;318). Other *in vitro* studies have shown that NK cells lyse virally infected targets, and that this ability is dependent, at least in part, on activation by IFN-α (316).

Evidence for NK cell involvement in resistance to intracellular bacterial infections was demonstrated in a study showing that SCID mice were partially able to control infection with *Listeria monocytogenes*, even though they lacked T and B cells. The macrophages in these mice showed increased expression of class II MHC molecules and increased tumoricidal activity, responses that were blocked by pre-treating the mice with either anti-IFN-γ, or anti-ASGM$_1$, a murine marker known to be expressed on NK cells, and some T cells. Overall, these results suggest that NK cells produce IFN-γ which activates antibacterial activity in macrophages, thereby leading to control of the infection (319). Consistent with this finding is the observation that NK-derived IFN-γ is important in macrophage-mediated resistance to infection with the intracellular parasite, *Toxoplasma gondii* (320). Another series of

experiments studying the role of NK cells in *T. gondii* infection showed that these cells compensate for the absence of CTL-mediated resistance in class I-deficient mice by increasing their numbers, and the level of IFN-γ production (321). Still other experiments have shown that NK cells are involved in early defense against murine infection with *Leishmania major*, and that they are the source of IFN-γ that promotes the commitment of $CD4^+$ T cells to a Th1 phenotype in this model (322;323). Taken together, these results suggest that NK cells respond early during infection by producing IFN-γ. This, in turn, promotes the development of a Th1-mediated immune response, thereby activating antibacterial or antiparasitic responses in macrophages.

How NK cells become activated to respond to infection is associated with IL-12, a cytokine capable of inducing IFN-γ production, cytotoxic activity, and proliferation in T and NK cells (231;324-327). However, a number of studies have also indicated that TNFα is partially responsible for inducing IFN-γ production in NK cells (183;319;320). Furthermore, a study investigating the role of NK cells and/or $NK1.1^+$ T cells in a murine model of human immediate hypersensitivity showed that *in vivo* depletion of these cells did not produce different antibody and cytokine profiles, compared to those observed in undepleted mice stimulated with the same exogenous antigen (328). The latter study underscores the complexity of mechanisms involved in immune regulation, particularly those dependent on NK cells.

Early support for the idea that NK cells may be involved in the development of acute GVHD came from studies showing that patients with low NK activity prior to BMT had a lower incidence of GVHD than those with higher pre-transplantation levels (329). Similar results from murine studies showed that the moderate-to-severe lesions seen in acute GVHD occurred only in mice experiencing high splenic levels of NK activity during the early stages of the reaction (330). Work from our laboratory has also shown that another population of

cells with a broader lytic spectrum than conventional NK cells also exists in F_1-hybrid mice with acute GVHD. These cells, referred to as NK-like, can kill both NK-sensitive (YAC-1), and NK-resistant (P815 and BW1100) target cells by a mechanism that does not require expression of self-MHC on the target cell, or a sensitization period. The phenotype of these cells is NK1.1$^+$, Thy-1$^{+/-}$, ASGM$_1^+$, Lyt-2$^-$, L3T4$^-$, and they can be detected in the spleen and lymph nodes of mice with acute GVHD, but not in normal, unstimulated control mice (209).

While these earlier experiments showed that NK activity increases during acute GVH reactions, more definitive studies were required to demonstrate that they participate in the pathogenetic mechanism of the disease. One of the first models used to address this question employed mice deficient in NK cell-mediated cytotoxicity due to a genetic defect known as the Beige (*bg*) mutation. Results indicated that mice were protected against GVHD-associated mortality when *bg/bg* donors were used to induce GVH reactions, suggesting that donor-derived NK cells are involved in the pathogenetic mechanism (331). It was later shown that pre-treatment of donor mice with anti-ASGM$_1$ antiserum abrogated the development of histopathological lesions and severe immunosuppression in recipient mice. However, this treatment was only effective if an "induced" population of ASGM$_1^+$ cells was also removed from donor mice by pre-treating them with recipient lymphoid cells prior to the depletion procedure (332). Experiments performed in our laboratory showed similar results when anti-NK1.1 mAb was used to deplete the graft of NK cells *in vitro* by complement-mediated lysis. In these experiments, protection against mortality and the severe wasting syndrome associated with acute GVHD was only seen if donor mice were pre-treated with the IFN-inducer, polyinosinic polycytidylic acid (poly I:C), 18 hr before the grafts were harvested and depleted of NK1.1$^+$ cells (333).

As there is now considerable evidence that NK cells participate in the pathogenesis of this disease, the search for a better understanding of their precise role in the mechanism of acute GVHD is underway. It is possible that they are cytotoxic effector cells capable of mediating the development of histopathological lesions, or alternatively, that they are involved in immunoregulation, particularly in promoting a Th1, cell-mediated immune response.

1.6.3.2 γδ T cells.

Discovered in the mid 1980's, these cells represent a lineage of T cells distinct from the $\alpha\beta TCR^+$ subset (334). They are present in the peripheral blood, spleen and lymph nodes, and epithelia of several organs and appear to participate in defense against microorganisms. During ontogeny, the genes for the γδTCR rearrange before those of the αβTCR. However, it is clear from studies employing transgenic mice expressing rearranged γ and δ genes, that cells failing to successfully rearrange genes for the γδTCR are not the progenitors for αβTCR-expressing cells (335-337). Instead, it is the activity of a γ gene silencer that facilitates commitment of a progenitor cell to the αβT cell lineage (338).

Subsets of γδT cells expressing certain Vγ genes are present at high levels in different tissues. For example, the monospecific Vγ5 and Vγ6 subsets are the first to differentiate in the fetal thymus and exclusively express the Vδ1 chain. The former subset can be detected in the epidermis, whereas the latter is prominent in the mucosa of the uterus, vagina and tongue in mature animals, but can also be seen in the liver and lung. The Vγ1, Vγ4 and Vγ7 subsets have a more extensive antigenic repertoire since the γ chains associate with a number of different δ chains and both express a high level of junctional diversity. The Vγ1 subset consists of both a thymus-dependent and -independent component, and is present in the spleen, intestine and skin. It responds to autoantigen, and mycobacterial HSP 60 in a non-

MHC-restricted fashion. Also present in the intestine is the Vγ7 subset. Differentiation of these cells is thymus-independent, and they are known to express the CD8α chain. The subset expressing Vγ4 is present in the peripheral blood, lymph nodes, spleen, adult thymus, lung and mammary gland (334;339). It is now known that these distinct subsets arise from at least three different progenitor cells. One requires a fetal thymus and develops into the Vγ5 and Vγ6 subsets, another has the ability to differentiate into to the Vγ4 and Vγ1 subsets in an adult thymus, and another can generate the Vγ7 subset extrathymically (340-343). Analysis of non-productive gene rearrangements in the Vγ4 and Vγ5 subsets has also indicated that these cells preferentially rearrange these genes, suggesting that the progenitor cell subsets may "target" certain rearrangements of variable region genes (344-346).

Recently, it has been possible to study the function of γδT cells by using mice with mutations in the genes required to produce functional αβTCR and γδTCR. Infection with *Listeria, Leishmania, Mycobacterium, Plasmodium* and *Salmonella* has been studied in these gene KO mice (347-352). Results from these experiments were similar in that the absence of both subsets led to a more severe infection in all cases, and the absence of only one subset reduced the level of resistance somewhat. However, in most cases, mice lacking γδT cells did better than those lacking αβT cells, but were still immunocompromised. It is clear from these studies that γδT cells are involved in maintaining immunocompetence, and that they can function in the absence of αβT cells.

The number of γδT cells has been found to increase before an αβT cell-mediated response can be observed during a bacterial infection, or infection with a virulent strain of Sendai virus. It is therefore likely that they are part of the first line of defense against certain pathogens (347-349;353-355). However, other studies have shown that cells expressing γδTCR mRNA do not appear in inflammatory lesions until the later stages of infection with

influenza or a less virulent strain of Sendai virus (356;357). These findings therefore suggest that γδT cells respond differently to different types of pathogens. Other experiments using mice infected with either *Leishmania* and *Listeria* showed that inflammatory lesions were larger in the absence of γδT cells, suggesting that γδT cells may play a regulatory role in this type of response (348;350). Other functions attributed to γδT cells include the ability to produce growth factors, to regulate both the growth of epithelial cells and the function of αβT cells, to promote isotype switching in B cells, and possibly, to mediate resistance to tumors (358-362).

Several antigens recognized by γδT cells have been identified. These include alloantigen, CD1, HSP-60, staphylococci enterotoxin, and small non-peptide, phosphate components of mycobacterial extracts (363-368). Very recently, three different experimental approaches have shown that antigen recognition by γδT cells is probably very different from that of αβ T cells, and does not require antigen processing. The first involved I-E^k, which binds a variety of peptides and most superantigens, and is recognized by the γδT cell clone, LBK5. Results from this experiment showed that the ability of I-E^k to activate LBK5 did not require antigen processing, as defects in this pathway had no effect on the response. Similarly, it did not depend on the type of antigen presenting cell (APC) used, but only on the density of I-E^k on the surface. In fact, the responder cells could be stimulated by I-E^k bound to plastic, without an APC present. The absence of invariant chains, and the use of different I-E^k-binding peptides was also found to be ineffective at abrogating the ability of I-E^k to activate LBK5. After performing several point mutations in the α and β chains of I-E^k molecule, it was found that disruption of amino acid 79 in the α chain prevented recognition by LBK5. None of the residues required for recognition by αβTCR$^+$ cells were found to be important in recognition by LBK5 (369). The second approach to studying antigen

recognition by γδT cells involved the use of the T10 and T22, two murine, non-classical, class I MHC molecules that are recognized by the γδT cell clone G8. Results from these experiments were very similar to the I-E^k/LBK5 system, in that antigen processing was not required for recognition of T10 and T22 by G8. In fact, it appears that T10 and T22 do not bind peptide at all (369). The third approach involved the γδTCR$^+$ clone TgI4.4 which recognizes the HSV type I transmembrane glycoprotein, gI. Results indicated that this molecule is recognized without processing or presentation by other molecules (370). Although these findings are convincing, it is important to note that they contradict observations made using other systems. In particular, three independent studies have shown firstly, that recognition of a synthetic co-polymer by γδT cells requires the presence of Qa-1^b on the cell surface, secondly, that tetanus toxin recognition requires expression of HLA-DRw53, and thirdly, that recognition of tumor-specific Ig requires the presence of mitochondrial HSP, grp75 (371-373).

Like the αβTCR, the γδTCR must associate with CD3 in order to be expressed on the cellular surface. Cross-linking of these receptors must also be achieved for activation to occur, indicating that the antigen must be multivalent. Costimulation is also required, as γδ T cells require at least two signals to become fully activated. One interaction known to be important in this process is CD28/B7 (374).

Overall, γδT cells appear to have the ability to recognize intact proteins to which αβT cells cannot respond. In addition, studies have suggested that their CDR3 length distributions may be more like immunolglobulins with respect to antigen recognition properties, and that these cells may be able to recognize a variety of antigens due to the high level of sequence variability in the CDR3 region of the TCR (375). Although their biological function remains puzzling, it is possible that they respond directly to pathogens, damaged tissues, or to T and

B cells, allowing an immune response to be initiated without antigen processing or the involvement of APC (374).

The first suggestion of γδTCR$^+$ cells may be important in the pathogenesis of acute GVHD came from studies of αβ- and γδ TCR$^+$ lymphocytes in histologic sections of skin from normal subjects and from patients suffering from acute GVHD. The proportion of γδTCR$^+$ cells in patients with acute GVHD was low, but still considerably higher than that seen in the normal subjects (376). In another study investigating the distribution of γδ T cells in the liver, intestine, and major lymphoid organs following BMT showed that the number of γδT cells in proportion to the total number of CD3$^+$ cells did not differ from that found in normal tissues, suggesting that there was no clear role for γδTCR$^+$ cells in the pathogenesis of GVHD (377). Still another study found that there was an increase in the relative proportion of γδT cells in the population of CD3$^+$ lymphocytes in the peripheral blood of bone marrow transplant recipients, and that this increase correlated with the development of acute GVHD (378). In a more recent murine study, acute GVH reactions were induced using transgenic donor mice with a T cell population comprised almost exclusively of γδT cells from the clone G8. These reactions were as lethal and severe as those seen when normal donor mice were used, even though they were induced using grafts devoid of αβT cells (379).

1.6.4. Role of endotoxin.

Endotoxin is the lipid A domain of lipopolysaccharide (LPS). It makes up the outer monolayer of the membrane on most gram-negative bacteria, and has profound effects on eukaryotic cells, particularly those of the immune system. These effects include the activation of both macrophages and the complement cascade, as well as the induction of endotoxic shock. The effects of endotoxin are mediated largely by macrophage-derived

cytokines such as TNFα and IL-1β which are released when LPS complexes with a 60 kDa glycoprotein known as LPS binding protein (LBP). Synthesis of the latter molecule occurs in the liver and becomes upregulated during an acute phase response. LBP serves to opsonize gram negative bacteria or LPS-coated erythrocytes by binding to the lipid A moiety of LPS. Macrophage activation occurs when LPS-LBP complexes bind to CD14, a 55 kDa protein present on the surface of the macrophages. The importance of the LPS-LBP interaction is evidenced by experiments showing that LPS cannot independently activate macrophages without first binding to LBP (380).

Triggering the secretion of macrophage-derived cytokines is central to the role played by endotoxin during an acute GVH reaction. It is well established that IFN-γ can prime macrophages for LPS-induced activation. This effect results in an increased level of sensitivity to endotoxin, such that quantities of LPS insufficient to induce secretion of TNFα in unprimed macrophages cause significant TNFα release in IFN-γ-primed macrophages (154;381). Although type I IFN can produce a similar priming effect, it does so to a much lesser extent than IFN-γ (261;382). Studies of macrophage activation during acute GVHD have indicated that these cells are primed during the reaction. This has been demonstrated in experiments showing that doses of endotoxin that are sublethal in normal mice produce a rapid, fatal reaction when injected into mice with acute GVHD. The cytotoxic activity directed against TNFα-sensitive and NO-sensitive target cells by macrophages isolated from GVH mice is also higher *in vitro*, following stimulation with LPS (383). Support for the idea that IFN-γ is responsible for this priming effect comes from a study demonstrating that treatment with anti-IFN-γ antibody can prevent augmented bactericidal activity during GVH reactions (384).

Gram negative bacteria are normally present in the gut, but are confined to the intestinal lumen by the mucosa. Although it has been reported that a small number of gram-

negative organisms can be translocated across the epithelium in the gut, these are rapidly phagocytosed by the large number of macrophages present in the gastrointestinal tract, liver, and mesenteric lymph nodes (385;386). However, if these macrophages are primed, the insignificant amounts of endotoxin that are normally present in the lamina propria could trigger the release of TNFα and NO in amounts sufficient to cause epithelial injury (383;387;388). In the case of NO, it is postulated that the regeneration of epithelial cells may be mitigated by the cytostatic effects of reactive nitrogen intermediates (241). In GVHD-associated intestinal damage, cytotoxic effector cells have also been implicated in the process, in addition to macrophage-mediated effects. For example, it has been demonstrated that effector cells involved in the development of epithelial cell injury during acute GVHD are $ASGM_1^+$, donor-derived cells with NK activity and some allospecificity (241). Experiments from our laboratory have confirmed that NK-like cells are activated during acute GVH reactions, and that these cells may also be important in the pathogenesis of the disease (209;333).

If gram negative organisms are able to cross the intestinal epithelium through lesions produced during acute GVHD, they will provide a source of endotoxin which, if present in circulation at high enough levels, will lead to the development of septic shock. Lapp and colleagues have postulated that endotoxin present in the gut flora of GVH mice reaches the capillaries and intestinal lymphatics. From here it is transported to the liver where it would normally be detoxified and released into the bile, which makes its way back to the intestine. However, during an acute GVH reaction, the liver's ability to detoxify LPS is overwhelmed by the large amounts of LPS present, and hepatic injury is incurred. Consequently, active LPS escapes into systemic circulation via the portal vein and triggers the release of proinflammatory cytokines from primed macrophages throughout the body. These cytokines then mediate cachexia and the development of endotoxic shock (241). Indeed, it has been

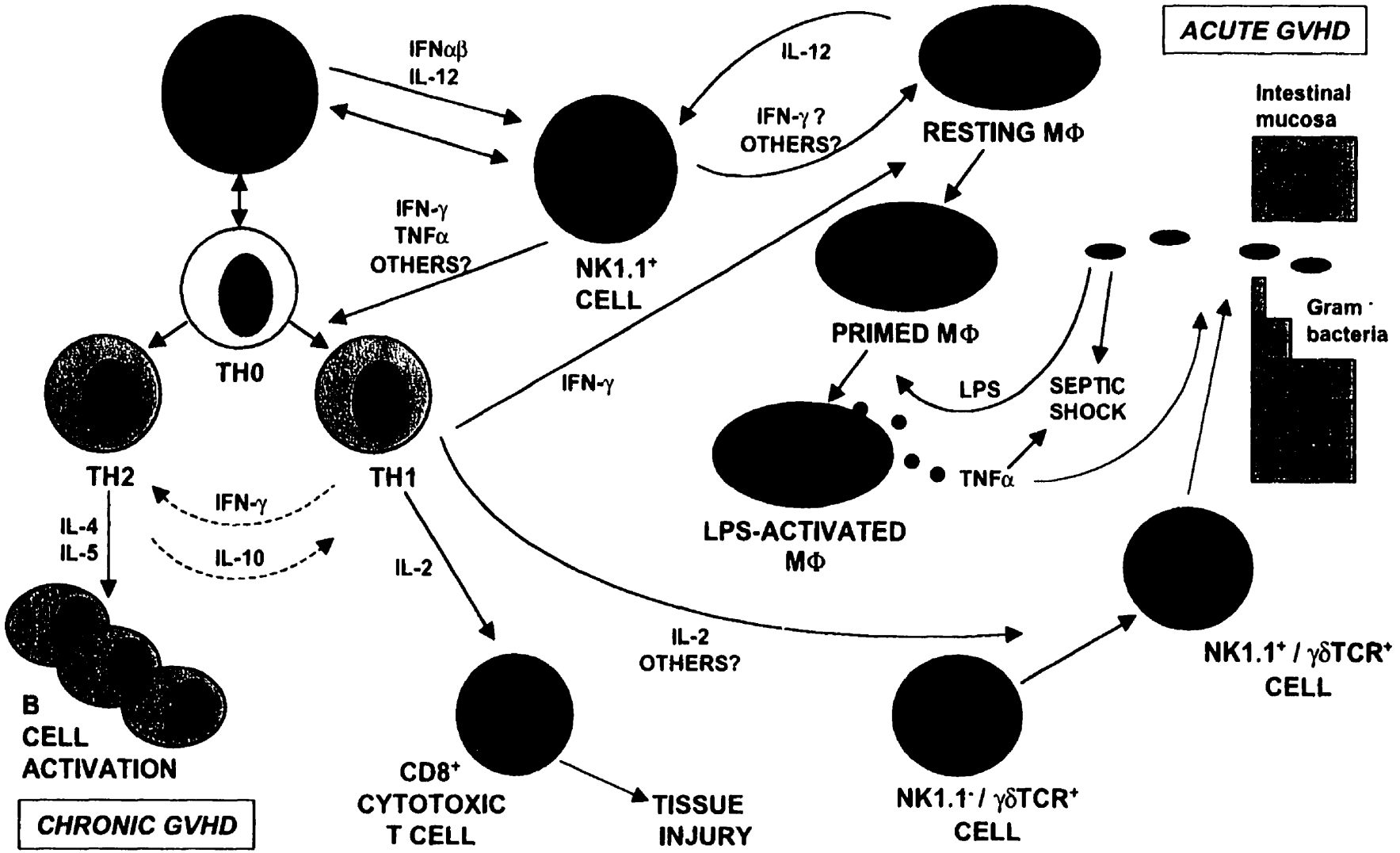
shown that death occurs only a few hours after endotoxin enters systemic circulation in an animal with primed macrophages (383).

1.7 Hypothetical model of acute GVHD.

Figure 1.2 is a diagrammatic summary of the cells and cytokines thought to be involved in the pathogenesis of acute GVHD. The exact mechanism by which a GVH reaction is initiated is unknown. However, one early event probably involves presentation of alloantigen to a Th0 cell by an APC. This cell then has the ability to differentiate into a T helper cell that secretes either Th1 or Th2 cytokines. It is thought that the presence of IFN-γ during the very early stages of the reaction promotes commitment to a Th1-mediated immune response, thereby setting the stage for an acute GVH reaction. A possible source of this cytokine is the NK1.1$^+$ cellular population, which forms an integral part of the natural immune system, capable of launching a very early response against foreign antigen. How NK1.1$^+$ cells may be stimulated to produce IFN-γ is unclear, but it may involve activation by IFN-$\alpha\beta$ and/or IL-12. In the absence of IFN-γ, the reaction is thought to follow a Th2-mediated pathway, resulting in the development of chronic GVHD.

It has been hypothesized that the IFN-γ produced by the ongoing Th1 response primes macrophages to secrete TNFα and other mediators upon stimulation with LPS. This event is believed to be responsible for the development of endotoxemic shock, which is invariably fatal. The source of LPS is likely gram negative bacteria, which invade the host through intestinal lesions. How these lesions initially develop is not entirely clear, but we postulate that donor-derived, NK1.1$^+$, $\gamma\delta$T cells, activated by IL-2 and/or other cytokines, may be responsible. The TNFα released by activated macrophages may also contribute to the intestinal injury observed. In addition, CTL activated by the reaction have been implicated in the mechanism of tissue injury associated with acute GVHD.

Figure 1.2 Diagram illustrating the possible roles of different immunoregulatory and cytotoxic cells in the pathogenesis of acute GVHD. Also shown are the cytokines that may be involved, as well as the effects of LPS. Solid arrows indicate activatory effects, dotted arrows indicate inhibitory effects, and red arrows indicate effects that mediate tissue injury. The source of unidentified cytokines, referred to as "OTHERS?" is not known.


ACUTE GVHD
IFNαβ
IL-12
IL-12
IFN-γ ?
OTHERS?
RESTING MΦ
Intestinal
mucosa
IFN-γ
TNFα
OTHERS?
NK1.1+
CELL
PRIMED MΦ
Gram -
bacteria
TH0
IFN-γ
LPS
SEPTIC
SHOCK
TH2
TH1
TNFα
LPS-ACTIVATED
MΦ
IFN-γ
IL-4
IL-5
IL-10
IL-2
IL-2
OTHERS?
NK1.1+ / γδTCR+
CELL
B
CELL
ACTIVATION
CD8+
CYTOTOXIC
T CELL
TISSUE
INJURY
NK1.1+ / γδTCR+
CELL
CHRONIC GVHD

1.8 Current strategies for prevention.

Because of its ability to reduce the risk of GVHD, T cell depletion of the graft is still being used as a method of prevention (389). More than 800 patients worldwide received T cell-depleted bone marrow transplants between 1981 and 1986, confirming the expectation that this approach is associated with a reduced level of engraftment and a decrease in the GVL effect. Nonetheless, efforts are still being made to determine the mechanisms that contribute to successful engraftment and the GVL reaction, so that these problems may be overcome (68).

In vivo treatment with immunosuppressive drugs such as methotrexate, cyclosporine, corticosteriods, or antithymocyte immunoglobulins are also being used post-transplantation for the prevention and control of GVHD. Unfortunately, acute GVHD still occurs in 10-50% of patients receiving allogeneic grafts from HLA-matched siblings, even if these drugs are used (390).

Another approach involves treatment with antagonists specific for inflammatory cytokines. Included in this category are corticosteriods, pentoxifyline, anti-TNFα mAb, and IL-1 receptor antagonists (IL-1ra) (391). Results with broad-spectrum cytokine inhibitors such as corticosteroids indicate that serious side effects are associated with the use of these drugs, some of which reduce the patient's ability to control opportunistic infections (391). The use of anti-TNFα was found to be less problematic, as it did not increase the incidence of infection or interfere with hematopoiesis. In two independent studies, it was found that up to 80% of patients with acute GVHD given anti-TNFα antibodies showed considerable improvement in skin and gut-associated symptoms, and some showed a positive response in the liver (392;393). However, these effects were temporary, as a relapse was seen when the treatment was withdrawn. In another clinical trial, patients with steroid-resistant GVHD were treated with IL-1ra, an anti-inflammatory molecule produced by monocytes *in vivo* and

capable of blocking the activities of IL-1α and IL-1β. In this study, 92% showed improvement in intestinal GVHD by a least one stage, 55% experienced complete resolution of diarrhea, 57% showed improvement in cutaneous GVHD, and 21% experienced complete resolution of their rash, and 63% showed an overall reduction in GVHD by at least one stage. There were no serious side effects associated with this therapy except for augmented transaminase levels in a small number of patients. Unfortunately, the responses were also transient, as many of these individuals relapsed when the treatment was discontinued (394). Pentoxifylline, formerly used for treatment of arteriosclerosis, has recently been shown to inhibit TNFα transcription. However, studies evaluating the effect of this drug on the development of acute GVHD have been inconclusive . Interleukin-10, an anti-inflammatory cytokine with pleiotropic effects has been suggested as a possible candidate for GVHD therapy, but results from experimental models have also been inconsistent (395). In summary, it appears that inhibition of individual proinflammatory cytokines can ameliorate some GVHD-associated symptoms, but long-term resolution of the disease will require a treatment that controls the immune response on a greater level (395).

One promising method for preventing GVHD involves using purified $CD34^+$ stem and progenitor cell grafts for hematological reconstitution following myeloablative therapy. These cells may be isolated from the bone marrow, peripheral blood, or from cord blood, and have the ability to mediate "prompt, sustained, multilineage engraftment". This method is now used for autologous transplants in patients with solid tumors, but is not appropriate for patients with cancers expressing CD34, which includes many of the acute leukemias. In cases of allogeneic BMT involving related donors and recipients, it has been found that the problems associated with using lymphocyte-depleted grafts can be overcome if these grafts are supplemented with purified $CD34^+$ stem cells. Patients receiving such grafts have a low incidence of GVHD and other BMT-associated complications. Similar results were seen

when this type of graft was used to reconstitute unrelated, HLA-mismatched recipients, but intense immunosuppressive therapy was still required post-transplantation. It is currently not known if the use of undepleted grafts enriched for CD34 expression will provide similar results (396).

Another recent approach to controlling GVH reactions involves transducing donor lymphocytes in the graft with a herpes simplex virus thymidine kinase suicide gene. Preliminary trials indicate that recipients of these genetically engineered grafts who develop GVHD can be effectively treated with ganciclovir, which has the ability to eliminate the transduced cells (397). Experiments performed using a murine model also indicate that recipients of grafts containing this suicide gene become tolerant of host alloantigen if ganciclovir therapy is given for the first 7 days post-BMT (398).

1.9 Rationale and summary of results.

From the literature, it is clear that acute GVHD is a serious complication of allogeneic BMT in humans. However, the GVH reaction is also a complex immunological phenomenon that may share common mechanisms with other pathological conditions such as those associated with solid organ transplantation, infection, immunodeficiency and malignancy. The study of experimental GVHD is therefore important not only in the identification of potential strategies for prevention, but also in furthering our understanding of this immune response.

Experiments performed previously in our laboratory focussed on the role of non-MHC-restricted cells in the pathogenesis of this disease. Using a murine F_1-hybrid model, it was shown that removing NK1.1$^+$ cells from the graft protects recipients against the mortality and wasting syndrome associated with acute GVHD. However, because the NK1.1$^+$ population is very diverse, we wished to determine which NK1.1$^+$ cells are the relevant

effector cells and where they might be involved in the pathogenetic mechanism of this disease. We hypothesized that NK1.1$^+$ cells with NK-like function may be particularly important. This was supported by the observation that these cells appeared during the development of an acute GVH reaction, but could not be detected in normal, control mice. Previous results had also shown that NK-like cells are derived mainly from the donor, unlike conventional NK cells, which originate mostly from the host. Indirect evidence suggested that these cells may be γδT cells. We therefore performed experiments to determine whether NK1.1 and γδTCR are coexpressed on cells in the spleens of GVH mice, whether γδT cells mediate GVHD-associated NK-like cytotoxic activity, and whether they are involved in the pathogenesis of acute GVHD. All three of these questions were answered affirmatively. However, recipients of grafts from γδT cell-depleted donors were only partially protected against mortality. Unsure of whether the lack of complete protection resulted from the persistence of donor-derived γδT cells, or the limited usefulness of γδT cell graft depletion in the prevention of acute GVHD, we then used grafts from TCRδ KO donor mice to induce GVH reactions. Surprisingly, this approach provided no protection against mortality, and resulted in exceedingly high levels of IFN-γ, a cytokine which we later showed to be associated with the acceleration of GVHD-associated mortality. We postulated that the high levels of IFN-γ diminished any protection that may have been provided by removing donor-derived γδT cells from the reaction.

Experiments performed in the second part of this project were designed to determine whether NK1.1$^+$ cells are involved in promoting an early commitment to the Th1 response that underlies the development of acute GVHD. This hypothesis was supported by the observation that NK1.1$^+$ cells are capable of IFN-γ production, and have been implicated in the development of Th1-mediated immune responses associated with other diseases. The idea that NK1.1$^+$ cells play an immunoregulatory role in the development of acute GVH reactions

represented an alternative to our long-held hypothesis that NK1.1$^+$ cells in the graft act as effector cells mediating GVHD-associated tissue injury. Our findings indicated that the Th1 response, as measured by IFN-γ production and LPS-induced TNFα release, is markedly reduced in recipients of NK1.1-depleted grafts. We therefore induced GVH reactions using IFN-γ *gko* donor mice to determine whether the removal of IFN-γ alone could produce a similar effect. Recipients of these grafts experienced a more protracted form of GVHD characterized by large cellular infiltrates and pathological changes in the liver, lung, skin and kidney. Skin and eye lesions resembing the sicca/Sjogrens syndrome associated with chronic GVHD were also observed. This outcome sharply contrasted that which was seen when NK1.1-depleted grafts from wild-type donors were used, and suggested that the protection conferred by NK1.1 graft depletion results from factors other than, or in addition to, abrogated IFN-γ production. This was further supported by preliminary data showing that recipients of IFN-γ *gko* grafts do not develop these pathological changes or a high level of mortality if the grafts are depleted of NK1.1$^+$ cells.

Another important finding in this study was the observation that intestinal lesions and high levels of serum LPS develop in recipients of NK1.1-depleted grafts from wild-type donors. This too was surprising, since we had previously hypothesized that NK1.1$^+$ cells in the graft mediate GVHD-associated gut injury. We hypothesize that this damage may be mediated by γδT cells that acquire NK1.1 expression and NK-like cytotoxicity under the influence of the various cytokines produced during the GVH reaction.

The results from these experiments have helped to elucidate the functions performed by some of the donor-derived, NK1.1$^+$ cells involved in the pathogenesis of acute GVHD. In doing so, they have provided a foundation for future experiments that could be designed to determine, more precisely, how NK1.1 graft depletion protects recipients from acute GVHD, since it appears to involve more than abrogated IFN-γ production. A closer look at the role

played by $\alpha\beta TCR^+$ NK1 T cells in the development of a Th1-mediated acute GVH reaction would be particularly useful. Other experiments designed to determine whether $\gamma\delta$T cells contribute to the intestinal pathology associated with this disease are also warranted, since this is a critical event in the development of GVHD-associated endotoxemic shock.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice.

2.1.1 Wild-type mice.

We obtained C57BL/6 (female, H-2^b) and BDF_1-hybrid (female, H-$2^{b/d}$) mice from Charles River Laboratories (Wilmington, MA). These were used to induce GVH reactions when they reached 13-16 weeks of age. C3H (male, H-2^k) and BALB/C (female, H-2^d) mice were also obtained from Charles River Laboratories (Wilmington, MA) and bred in our animal care facility to produce (C3H x BALB/C)F_1-hybrid mice (male and female, H-$2^{k/d}$). These mice were used at 8-12 weeks of age to produce anti-NK1.1 ascites fluid.

2.1.2 Genetic knockout mice.

Male and female mice carrying a mutation in the constant region of the TCRδ gene segment (Cδ), and having a C57BL/6J genetic background, were obtained from Jackson Laboratories (Bar Harbor, MN) and bred in our animal care facility. The offspring were housed in the same facility until they reached 13-16 weeks of age and used to induce GVH reactions in age- and sex-matched BDF_1-hybrid mice, also obtained from Jackson Laboratories. Male and female C57BL/6J-*Ifg*^{tm1ts} mice carrying a mutation in the gene encoding IFN-γ were obtained from Jackson Laboratories and bred in our facility. The offspring were housed in filter-topped, sterilized cages and received sterilized food and water. These mice were used at 13-16 weeks of age to induce GVH reactions in age- and sex-matched BDF_1-hybrid mice, also obtained from Jackson Laboratories.

2.2 Cell lines.

The murine hybridoma PK136 (H-$2^{k/d}$) and the Moloney murine virus-induced lymphoma YAC-1 (H-2^a) were obtained from American Type Culture Collection (ATCC,

Rockville, MD). The mouse T cell lymphoma, BW5147/M1100.129.237 (BW1100) was a gift from Dr. P. Marrack, Denver, CO. Cell lines were maintained in complete RPMI 1640 culture medium (Gibco, Grand Island, NY) containing penicillin-streptomycin (5000 IU/ml) and glutamine (200 mM). This was supplemented with 10% fetal calf serum (FCS).

2.3 Antibodies, antisera and complement.

Anti-NK1.1 mAb directed against the strain-specific (H-2^b) NK cell surface marker, NK1.1, and produced by the hybridoma PK136, was generated as ascites and used at a dilution of 1/20. For biotinylation, anti-NK1.1 ascites fluid was purified on protein A (Sigma Chemical Co., St. Louis, MO) to a concentration of 5 mg/ml and conjugated according the method outlined by Vector Laboratories (Burlingame, CA). Anti-NK1.1-biotin was used at a concentration of 0.5 μg/10^6 cells. Streptavidin (SA)-phycoerythrin (PE; Sigma) was used at a concentration of 10^6 cells/5μl. Mouse IgG (15-20 mg/ml; Jackson ImmunoReseach Laboratories, West Grove, PA) was used at a concentration of 0.5 μg/10^6 cells. Anti-γδTCR mAb (GL3; 1 mg/ml; PharMingen, San Diego, CA) and anti-αβTCR mAb (H57-597; 1 mg/ml; PharMingen) were each used at a dilution of 1/25. Purified hamster IgG (0.5 mg/ml; PharMingen) was used at a dilution of 1/12.5. Affinity-purified rabbit anti-hamster IgG (2.4 mg/ml; Jackson) was used at a dilution of 1/50. Affinity-purified, fluorescein isothiocyanate (FITC)-conjugated, rabbit anti-hamster IgG (H+L, 1.4 mg/ml; Jackson) was used at a dilution of 1/50. Anti-$ASGM_1$ rabbit antiserum (Wako Fine Chemicals, Dallas, TX) was reconstituted in 1 ml of ddH_2O and used at a dilution of 1/100. Lyophilized anti-Thy 1.2 ascites fluid (Cedarlane, Hornby, ON) was reconstituted in 0.5 ml of ddH_2O and used at a dilution of 1/50. FITC-conjugated mouse anti-H-$2D^d$ (34-5-8S; Cedarlane), PE-conjugated rat anti-

CD4 (CT-CD4; Cedarlane), PE-conjugated rat anti-CD8 (CT-CD8$_a$; Cedarlane), FITC-conjugated mouse IgG2a (UPC-10; Caltag Laboratories, Burlingame, CA) and PE-conjugated rat IgG2a (LO-DNP-16; Caltag) were all used at a concentration of 10 μg/ml). Lyophilized Low-Tox-M rabbit complement (Cedarlane) was reconstituted in 1 ml of ddH_2O and used at a dilution of 1/9. For *in vivo* depletion of γδTCR$^+$ cells, donor mice were injected with 450 μl of lyophilized GL3 ascites fluid (Cedarlane) that had been reconstituted in ddH_2O to a concentration of 0.5 mg/ml. For *in vivo* depletion of NK1.1$^+$ cells, donors were injected with 200 μl of PK136 ascites fluid containing 5 mg/ml of protein.

2.4 Induction of GVH reactions.

To prepare the graft, spleens and lymph nodes (cervical, axial and inguinal) were harvested from C57BL/6 donor mice that had been sacrificed by CO_2 asphyxiation. These organs were pooled, placed in 60 x 15 mm petri dishes containing HBSS, and transferred onto a #60 stainless steel screen, where they were minced with scissors. A single cell suspension was produced by pressing the tissue through the screen into a 60 x 15 mm petri dish containing HBSS. This was washed by centrifugation for 10 min at 300 x *g*, in 15 ml conical bottom centrifuge tubes that had been filled with approximately 7.5 ml HBSS before the suspension was added. One 15 ml tube was used for each donor mouse sacrificed, and only half the number of tubes were used for the second wash. The graft was then passaged through cotton gauze and adjusted to a concentration of 200 x 10^6 cells/ml in HBSS. Each BDF_1-hybrid mouse received an i.v. injection of 60 x 10^6 lymphoid cells suspended in 0.3 ml of HBSS. Grafts were delivered into the tail vein.

2.5 *In vitro* depletion of NK1.1$^+$ cells from the graft.

In order to deplete NK1.1-expressing cells from the graft, it was first necessary to prepare ascites fluid containing anti-NK1.1 mAb from the hybridoma PK136. This fluid was then tested for its ability to deplete NK and NK-like activity in effector cells pooled from the spleens and lymph nodes of C57BL/6 donor mice that had, or had not, been stimulated with the interferon inducer, poly I:C; Sigma, 18 hr prior to sacrifice. A cytotoxicity assay was used to determine the dilution of PK136 ascites fluid that could effectively deplete this activity by complement-mediated lysis. This dilution was then used to deplete NK and NK-like cells from the graft *in vitro*. In these experiments, the terms "NK activity" and "NK-like activity" refer to the ability of effector cells to lyse NK-sensitive target cells such as YAC-1, and NK-resistant target cells such as BW1100, respectively (209;399).

2.5.1 Preparation of anti-NK1.1 ascites fluid.

One week following i.p. injection of 1.0 ml of pristane (Sigma), C3H x BALB/C were given an injection of 2 x 10^6 PK136 hybridoma cells suspended in 0.5 ml of HBSS. Mice were sacrificed two to three weeks later, and ascites fluid containing $IgG2_a$ mAb directed against NK1.1 was collected from the peritoneal cavity. Clots were removed by centrifugation for 10 min at 300 x *g* and the fluid was stored at -70°.

2.5.2 Cell-mediated cytotoxicity assay.

2.5.2.1 Isolation of effector cells from spleens and lymph nodes.

Spleens and lymph nodes were harvested from C57BL/6 mice that were either untreated, or injected i.p., 18 hr prior to sacrifice, with 100 μg of poly I:C dissolved in 100 μl of HBSS. Organs were pooled, placed in HBSS and pressed through a #60

stainless steel screen to obtain a single cell suspension. Cells were washed in HBSS, centrifuged at 350 *x g* for 10 min and resuspended in complete RPMI 1640 supplemented with sodium pyruvate (100 mM) and 5% FCS. Adherent cells were removed by passing the cells through nylon wool columns affixed with 22 gauge needles. Each column was prepared by gently packing 60 mg of nylon wool (Polysciences Inc., Warrington, PA) into the barrel of a 10 cc polypropylene syringe (Becton Dickinson, Franklin Lakes, NJ) Red cells were removed by centrifugation on Lympholyte-M (Cedarlane) at 350 x *g* for 25 min. Cells were then recovered from the interface, washed twice in supplemented RPMI 1640, pelletted, and stored overnight at 4° C.

2.5.2.2 Treatment of effector cells with antibody and complement.

Effector cells were incubated in anti-NK1.1 ascites fluid that had been diluted 1 in 10, 1 in 20, or 1 in 30 in Cytotoxicity Medium (Cedarlane, Hornby, ON). They were then incubated in Low-Tox-M rabbit complement that had been diluted 1 in 9 in Cytotoxicity Medium. Both incubations were performed for 1 hr at a concentration of 0.5 ml/10^7 cells, but the first was performed on ice and the second at 37° C. Controls consisted of effector cells that were either untreated, or treated with complement only. The level of YAC-1 and BW1100-directed lysis remaining after the depletion was measured in a 4 hr ^{51}Cr release assay. Using this method, it was found that a 1 in 20 dilution of PK136 ascites fluid could deplete virtually all of the NK and NK-like activity in the effector cell population.

2.5.2.3 Preparation of target cells.

YAC-1 and BW1100 target cells were prepared by labeling with $Na_2{}^{51}CrO_4$ (^{51}Cr; Amersham, Oakville, ON) for 1 hr, at 37°C, at a concentration of 50 μCi/10^6 cells. Each

cell pellet was then washed 3 times in complete RPMI supplemented with sodium pyruvate (100 mM) and 5% FCS, and resuspended to a final concentration of 1 x 10^5 cells/ml.

2.5.2.4 Four hr ^{51}Cr release assay.

Nylon wool-nonadherent effector cells were prepared as previously described. One hundred µl of the effector cell suspension were added, in triplicate, to the wells of a 96 well, V-bottom microtitre plate, in numbers sufficient to achieve four effector to target (E:T) ratios ranging from 100:1 to 6.25:1. One hundred microlitres of the target cell suspension was then added to each well containing effector cells. The plates were incubated at 37°C for 4 hr in humidified air containing 5% CO_2. Following centrifugation at 350 x g, 100 µl of supernatant was harvested from each well and counted for 2 min in an LKB gamma counter (LKB, Rockville, MD). The level of spontaneous ^{51} Cr release was determined by analyzing supernatants collected from cultures containing 100 µl of target cells and 100 µl of culture medium without effector cells. The level of maximum ^{51}Cr release was determined by analyzing cell suspensions from cultures containing 100 µl of target cells and 100 µl of culture medium and without effector cells. In this case, samples were collected by thoroughly mixing the culture and removing 100 µl of the suspension. The mean number of counts/min (cpm) was determined for each sample and the percentage of lysis was calculated as follows:

$$\% \text{ lysis} = \frac{\text{Cpm (experimental)} - \text{cpm (spontaneous)}}{\text{cpm (maximum)} - \text{cpm (spontaneous)}} \times 100$$

The mean percentage of lysis and standard error was determined for each triplicate. Dose-response curves were then generated from these values at the specific E:T ratios used in the assay, and lytic units (LU) per 10^7 effector cells were calculated using exponential fit as described by Pross *et al* (400). One LU was defined as the number of effector cells required to achieve 10% cytotoxicity.

2.5.3 Depletion of NK1.1$^+$ cells from the graft by complement-mediated lysis.

Grafts of pooled lymph node and spleen cells were prepared as previously described from C57BL/6 donor mice that either had, or had not been injected with 100 μg of poly I:C i.p., 18 hr prior to sacrifice. These cells were then subjected to two consecutive rounds of depletion with PK136 ascites fluid followed by complement. During the first round, cells were incubated for 1 hr on ice with PK136 ascites fluid that had been diluted 1 in 20 in Cytotoxicity Medium, and then with Low-Tox-M rabbit complement for 1 hr at 37°C. Both incubations were performed at a concentration of 10^7 cells/0.5 ml, and a wash was performed following each incubation period. During the second round, cells were incubated with PK136 ascites fluid and then with complement, at a concentration of 2 x 10^7 cells/0.5 ml. All other conditions were the same as those used in the first round of depletion. Fifty ml conical bottom centrifuge tubes were used throughout the procedure, and centrifugation was performed for 10 min at 300 x *g*. The suspension was then passaged through cotton gauze to remove debris, and the number of viable, non-red cells was determined using a trypan blue exclusion assay. The cellular concentration was adjusted to 2 x 10^8 cells/ml and the graft was injected i.v. into recipient mice at a dose of 60 x 10^6 cells/ mouse. The levels of YAC-1 and BW1100-directed lysis in grafts that either had, or had not been depleted of NK1.1$^+$ cells were determined using a 4 hr ^{51}Cr release assay.

2.6 *In vivo* depletion of γδTCR$^+$ or NK1.1$^+$ cells from the graft.

2.6.1 Treatment of donor mice.

Four days before sacrifice, C57BL/6 donor mice were injected i.p. with 450 μl of GL3 ascites fluid to eliminate γδT cells from the graft. The γδTCR-depleted grafts were used to induce GVH reactions in BDF_1-hybrid recipients.

The *in vivo* NK1.1$^+$ depletion procedure was performed as follows: Donors were first injected, i.p., with 100 μg of poly I:C dissolved in 100 μl of HBSS. Eighteen hours later, they received a. 200 μl injection of PK136 ascites fluid, i.p. These mice were sacrificed four days later and used, as previously described, to induce GVH reactions in recipient mice.

2.6.2 Testing the effectiveness of *in vivo* depletion.

The percentages of γδTCR$^+$ cells in grafts from untreated and GL3-treated donors were compared using flow cytometry. Cells were prepared for the analysis by removing red cells by centrifugation on Lympholyte-M at 350 x *g*, washing the suspension twice in PBS/1% bovine serum albumin (BSA), resuspending the pellet to 20 x 10^6 cells/ml in PBS/BSA, and incubating the suspension for 5 min with purified rat anti-mouse CD32/CD16 (Fc_γRII/III receptor) mAb (Fc Block; Cedarlane) at a concentration of 1μg/10^6 cells. Cells were then incubated with either anti-γδTCR mAb (2μg/10^6 cells; Cedarlane), or hamster IgG mAb (2μg/10^6 cells; Cedarlane) for one hr on ice, washed twice in PBS/BSA, and incubated with goat-anti-hamster IgG-FITC (Cedarlane) at a concentration of 3.5 μg/10^6 cells for 40 min. Before proceeding to the flow cytometry analysis, cells were washed twice in PBS/BSA.

A cytotoxicity assay was used to show that *in vivo* treatment of donors with anti-NK1.1 mAb eliminated all NK activity in the graft. This was accomplished by comparing the level of YAC-1-directed lysis in grafts from untreated donors, donors stimulated with poly I:C but not injected with PK136 ascites fluid, and donors stimulated with poly I:C and then injected with PK136 ascites fluid.

2.7 Monitoring GVH reactions.

2.7.1 Weight loss and mortality.

Recipient mice were monitored daily for signs of morbidity beginning in the second week post-induction. These signs included a hunched posture, anorexia, inactivity, and a level of weight loss exceeding 20%. Those that had succumbed to the reaction were removed from the cage and those that appeared moribund were weighed and sacrificed. This process was repeated until mortality reached 100%, or the experiment was terminated. The mean body weights of recipient mice were determined periodically throughout the reaction by including not only the weights of surviving mice, but also the last recorded weights for any mice that had already died (phantom weights).

2.7.2 Splenomegaly.

The pattern of splenomegaly was determined by calculating the spleen index (SI) in recipient mice on several days post-induction. This was accomplished by determining the body and spleen weights for control, BDF_1-hybrid mice that had not received grafts, and BDF_1-hybrid recipient mice. The SI for a particular day was calculated for each mouse using the following equation:

$$\text{Spleen index} = \frac{\text{spleen weight (experimental)} \div \text{body weight (experimental)}}{\text{spleen weight (control)} \div \text{body weight (control)}}$$

2.8 Preparation of anti-NK1.1-biotin.

2.8.1 Purification of anti-NK1.1 mAb from PK136 ascites fluid using Protein A.

Ascites fluid was filtered through cotton gauze, diluted 1 in 4 in Tris buffer (pH 8.6), filtered a second time using a 25 mm cellulose acetate syringe filter (Canlab Scientific Products, Mississauga, ON) and then applied to a protein A (Sigma) column that had been equilibrated with 10 ml of Tris buffer. The optical density (OD) of the eluted solution at a wavelength of 280 nm was monitored using an LKB Bromma 2138 Uvicord S spectrophotometer. Tris buffer was slowly added to the column until all unbound protein was eluted. When the OD of the effluent returned to baseline, glycine-Cl (pH 2.4) was added to the column and $IgG2_a$ was eluted into a tube containing neutralizing buffer (0.5 M phosphate, pH 7.7). The pH of the effluent was immediately adjusted to 7.0. Purified antibody was dialyzed against PBS for 12 hr, frozen to -70° C and lyophilized. A portion of the mAb was biotinylated and the remainder was reconstituted in ddH_20 to 5 μg/ml, diluted 1 in 10^3 in PBS/0.1% NaN_3, and stored at -70° C.

2.8.2 Conjugation of anti-NK1.1 mAb to biotin.

For biotinylation, anti-NK1.1 mAb was diluted in $NaHCO_3$ to a concentration of 5 mg/ml and combined with a volume of N-hydroxy-succinimidobiotin (Sigma)/dimethyl sulfoxide (40 mg/ml; Fischer Scientific Co., Fairlawn, NJ) equal to 1/10 the weight of

the protein. The reaction was allowed to proceed for 2 hr at 25°C, after which it was stopped by the addition of 10 mg of glycine. Unreacted biotin was removed by dialysis against PBS/0.1% NaN_3. A column of agarose-avidin D (Vector Laboratories, Burlingame, CA) was assembled by placing 1 ml of matrix in a Pasteur pipette and washing it with PBS until the OD_{280} was equal to that of PBS. A 1.0 ml aliquot of the antibody-biotin solution was then applied to the column, which was subsequently washed with 1 ml of PBS. The OD_{280} of the 2 ml effluent (solution A) and of a 1 in 2 dilution of the antibody solution (solution B) were then determined. The percentage of biotin-reacted antibody (which in this case is the percentage of protein that binds to agarose avidin D) was calculated as follows:

$$\% \text{ biotinylation} = \frac{OD_{280\ (\text{solution B})} - OD_{280\ (\text{solution A})}}{OD_{280\ (\text{solution B})}} \times 100$$

Using this method, it was found that 95% of the anti-NK1.1 mAb had reacted with biotin.

2.9 Isolation of NK1.1$^+$ cells using a magnetic cell separator (MACS).

2.9.1 Labeling spleen cells for enrichment.

Nonadherent, red cell-free spleen cells were prepared as described in the effector cell isolation protocol. They were then washed twice in cold PBS (4°C) and resuspended in 500 µl of cold PBS to which biotinylated anti-NK1.1 mAb had been added at a concentration of 2 x 10^6 cells/µg. A 15 min incubation on ice followed and cells were washed in PBS containing 500 mM EDTA. The pellet was then resuspended to a concentration of 1.1 x 10^5 cells/µl in PBS/EDTA and incubated for 15 min with streptavidin (SA)-conjugated MACS microbeads (10^6 cells/ µl; Miltenyi Biotech, Germany) and for an additional 5 min with SA-phycoerythrin (SA-PE; Becton Dickinson,

ON) at a concentration of 2 x 10^6 cells/μl. Cells were washed in PBS/EDTA supplemented with 1% bovine serum albumin (BSA) prior to the separation procedure.

2.9.2 Isolation of NK1.1$^+$ cells on the MACS.

The spleen cell suspension labeled with anti-NK1.1-biotin, SA-MACS microbeads, and SA-PE was slowly added to a size A2 MACS column (Miltenyi) that had been affixed with a 22 gauge needle and positioned within the magnetic field of a MACS magnetic cell separator (Miltenyi). Labeled (NK1.1$^+$) cells adhered to the matrix while unlabelled (NK1.1$^-$) cells were eluted. The remaining NK1.1$^-$ cells were washed from the column by replacing the 22 gauge needle with a 21 gauge needle and adding 2 column volumes of PBS/EDTA/BSA. In order to free weakly adherent cells from the matrix, the column was removed from the magnetic field and filled from the bottom with PBS/EDTA/BSA. When the column was returned to the magnetic field, strongly adherent cells bound to the matrix while weakly adherent cells were eluted through a 22 gauge needle. To ensure that all weakly bound cells had been removed from the column, the 22 gauge needle was replaced with a 21 gauge needle and the column was washed again with 2 volumes of PBS/EDTA/BSA. The bound NK1.1$^+$ cells were finally eluted from the column by removing it from the magnetic field and flushing it with approximately 10 ml of PBS/EDTA/BSA.

2.9.3 Determining the effectiveness of the MACS enrichment.

Two approaches were used to determine the effectiveness of the MACS separation technique. The first involved measuring the cytotoxic activity directed against NK-sensitive, YAC-1 target cells and NK-resistant, BW1100 target cells in the precolumn, bound and pass-through cell fractions. This was accomplished using a 4 hr

^{51}Cr release assay. We also used this method to determine whether the labeling procedure had influenced the level of cytotoxic activity in the precolumn fraction by comparing YAC-1-directed lysis in effector cells with, and without the addition of anti-NK1.1-biotin, SA-microbeads and SA-PE. The second approach involved using an Epics 753 fluorescence-activated cell sorter (Coulter, Hialeah, FL) fitted with a 76 μm quartz flow cell. In these experiments, a sample of spleen cells from the precolumn fraction was labeled for detection of $NK1.1^+$ cells using the same protocol as that used to label $NK1.1^+$ cells for enrichment on the MACS column. Cell samples from the bound and pass-through fractions were analyzed immediately following enrichment, since they had already been labeled for flow cytometry analysis prior to the separation procedure. By analyzing forward angle light scatter (FALS) and side angle light scatter (SALS), the size and granularity of the splenocytes in each cellular fraction was determined. Two subpopulations of lymphocytes were observed: one consisting of LGL, and the other consisting of small agranular lymphocytes (SAL). By gating on each subpopulation individually, it was possible to determine the percentage of $NK1.1^+$ LGL and SAL in the pre-column, bound and pass-through fractions. For these experiments, laser excitation was at 488 nm and the PE fluorescence emissions were detected through a 575 nm bandpass filter.

2.10 Immunophenotypic characterization of NK-like activity in mice with acute GVHD.

These experiments were performed on days 7 or 8 post-induction when the level of NK-like activity is maximal in GVH mice. Effector cells were prepared from either the spleens or lymph nodes of GVH mice as previously described, resuspended in Cytotoxicity Medium to a concentration of 10^7 cells/0.5 ml and incubated for 1 hr, on

ice, with anti-Thy 1.2, anti-ASGM$_1$, anti-NK1.1, anti-γδTCR, anti-αβTCR, or hamster IgG. Cells were then washed in ice cold HBSS, centrifuged at 300 x *g* for 10 min, and resuspended to a concentration of 10^7 cells/0.5 ml in Low-Tox-M rabbit complement. A 1 hr incubation at 37° C followed, and cells were washed again in cold HBSS. Because anti-γδTCR mAb and anti-αβTCR mAb do not fix complement, we employed indirect complement mediated lysis. This was accomplished by performing a second incubation with rabbit anti-hamster IgG before the complement fixation procedure. The level of YAC-1 and BW1100-directed lysis remaining after the depletion was assayed by ^{51}Cr release.

2.11 Detection of NK1.1$^+$/γδTCR$^+$ spleen cells in mice with acute GVHD.

To facilitate the detection of NK1.1$^+$/γδTCR$^+$ cells, spleen cells from GVH mice expressing NK1.1 were enriched using the MACS. Flow cytometry was then used to determine the percentages of cells expressing NK1.1, γδTCR, or both. These were compared with those seen in suspensions of unenriched spleen cells. Analyses were performed by gating on LGL and SAL subpopulations separately.

Nylon wool non-adherent, red cell-free spleen cells were prepared using the method described previously for splenic effector cells. These were then incubated with either anti-NK1.1-biotin followed by SA-PE or with anti-γδTCR mAb followed by rabbit-anti-hamster IgG-FITC. To reduce the level of non-specific binding, all samples to be labeled with anti-γδTCR mAb were preincubated consecutively with hamster IgG and unconjugated rabbit anti-hamster IgG. All incubations were performed for 1 hr on ice in 100 μl of PBS containing 1% BSA and the appropriate labeling reagent. All samples were washed once with PBS/BSA following each incubation period. To determine the percentage of cells coexpressing NK1.1 and γδTCR, cells were incubated with anti-

NK1.1-biotin and anti-γδTCR mAb simultaneously, washed, and then incubated with SA-PE and rabbit anti-hamster IgG-FITC simultaneously.

Because cells were labeled with anti-NK1.1-biotin and SA-PE before the MACS separation procedure was performed, the percentage of NK1.1$^+$ cells in this fraction could be determined immediately after eluting bound cells from the column. A one-colour analysis of γδTCR$^+$ cells in the bound fraction could not be performed. To determine the percentage of NK1.1$^+$/γδTCR$^+$ cells, a two-colour analysis was performed following labeling of γδTCR with specific mAb and rabbit anti-hamster IgG-FITC as described for unenriched spleen cells.

Unenriched and NK1.1-enriched spleen cells were analyzed by flow cytometry as described previously. However, the FITC and PE fluorescence emissions were split with a 550 dichroic filter and detected through 525 and 575 nm bandpass filters, respectively. Electronic compensation for spectral overlap was defined and verified with cell samples labeled with only FITC or PE. Forward vs right angle light scatter histograms were set up to define bit map gates for LGL and SAL, with acquisition based on 5000 gated events. Subsequent analyses of the data were performed using Coulter Elite workstation software.

2.12 Measurement of IFN-γ and IL-10 in spleen cell bulk cultures.

Spleens were harvested asceptically in HBSS from recipient mice on days several days post-transplantation. A cell suspension was prepared and washed in complete RPMI 1640 culture medium supplemented with 5% FCS and Hepes (10 mM). Cells were counted, adjusted to a concentration of 15 x 10^6 cells/ml in 5% RPMI 1640 with Hepes and serially diluted to give final concentrations of 7.5, 3.75 and 1.875 x 10^6 cells/ml. Two milliliters of suspension at each cell concentration were added to wells of a 24-well

flat bottom culture plate and incubated at 37°C in 5% CO_2. Three hundred microliters of supernatant were removed from each well at 24, 48 and 72 h, frozen at 4°C, and later assayed for the presence of IFN-γ and IL-10 using enzyme-linked immunosorbent assays (ELISA). The viability of the cultures over the collection period was verified by daily inspection of the wells by phase contrast microscopy. A sandwich ELISA using an affinity-purified preparation of the anti-IFN-γ, rat anti-mouse mAb, XMG, and the affinity-purified, biotinylated rat anti-mouse, anti-IFN-γ antibody, R4-6A2 (ATCC) in combination with streptavidin-alkaline phosphatase was carried out as described elsewhere (401). Internal standards consisting of IFN-γ-containing, Con A stimulated mouse spleen cell supernatants, calibrated against WHO-NIAID international reference reagent Gg02-901-533 (provided by Dr. C. Laughlin, NIAID, NIH), were included in each assay. Each supernatant was evaluated in duplicate using four two-fold serial dilutions with values within the linear portion of the standard curve. The lower limit of detection was 0.2 U/ml of IFN-γ and amounts were quantified at >0.5 U/ml. Standard error was $<10\%$ in most experiments. Two purified anti-IL-10 mAb were used in the IL-10 ELISA, namely SXC1 and biotinylated SXC2. Hybridomas were initially provided by Dr. T. Mosmann (University of Alberta, Edmonton, AB). Each plate contained a two-fold serial dilution of standard rIL-10. The lower limit of detection was 0.2 U/ml and the amounts were quantified at >0.5 U/ml.

2.13 Measurement of systemic LPS-induced TNFα release.

On several days post-transplantation recipient mice were injected i.v. with 10 μg LPS (Sigma). LPS was prepared as stock solution in PBS to obtain a concentration of 1 mg/ml, stored at -70°C, and diluted 1 in 20 in PBS for injection. Ninety minutes after

injection the mice were bled from the tail. This interval had been determined to be optimal for LPS release in pilot experiments in which normal mice were injected first with 0.1 mg poly I:C *i.p.* to prime macrophages for LPS-induced TNFα release. Blood samples were allowed to clot overnight at 4°C and centrifuged at 9000 x *g* for 5 min. The serum was harvested and stored at -70°C. The ELISA used to measure TNFα in the serum samples was performed in Costar high-binding EIA 96 well flat-bottom plates coated with murine anti-TNFα mAb (clone MP6-XT22; Pharmingen), diluted to 4 μg/ml in 0.1 M $NaC0_3$, pH 8.2. After an overnight incubation at 4°C, the wells were washed twice with PBS/Tween 20 (PBS/T_{20}). Blocking was performed at 25°C for 2 hr using PBS/3% BSA followed by two washes with PBS/T_{20}. The assay was standardized with recombinant murine TNFα (10 μg/ml, R & D Systems, Minneapolis, MN) diluted to 4 ng/ml in PBS/3% BSA. Doubling dilutions of serum samples and standard were performed in PBS/3% BSA, starting at 1:1. Each well received 100 μl of standard, serum sample, or dilution buffer. After an overnight incubation at 4°C, the wells were washed 4 times with PBS/T_{20}. Each well was then incubated (45 min at 25°C) with 100μl of biotinylated rabbit anti-mouse TNFα polyclonal Ab (0.5 mg/ml; Pharmingen) diluted to 4 μg/ml in PBS/3% BSA and washed 6 times in PBS/T_{20}. This was followed by an incubation (30 min at 25°C) with 100 μl of avidin-peroxidase conjugate (2 mg/ml; Sigma) diluted 1:2000 in PBS/3% BSA followed by 8 washes in PBS/T_{20}. Substrate was prepared by dissolving ABTS (Sigma) in 0.1 M citric acid, pH 4.5, to a concentration of 300 μg/ml, and then adding 30% H_2O_2 at a concentration of 10 μl per 11 ml, immediately before use. One hundred microlitres of substrate was added to each well and plates were incubated for 30 min at 25°C with gentle agitation. Optical densities were read at 405 nm on an EL308 Bio Tek Instruments microplate reader (Mandel Scientific, Rockwood, ON).

Although the lower level of detection was 60 pg/ml, measurements were taken only in the linear portion of the curve. Standard errors were usually less than 10%.

2.14 Histopathologic analysis by light and electron microscopy.

Mice were euthanized by CO_2 asphyxiation. Samples of skin, lung, liver, spleen, lymph node, salivary gland, and kidney were collected, fixed in 10% neutral buffered formalin for 24 hr, machine processed through graded alcohol, and embedded in paraffin. Sections with a 4 μm thickness were cut and stained with hematoxylin and eosin. Samples of kidney were also taken for electron microscopy. This tissue was fixed in 2% buffered gluteraldehyde for 2 hr, rinsed in phosphate buffer, post-fixed in buffered osmic acid for 2 hr and stained for 20 min in 2% aqueous uranyl acetate. After dehydration in graded ethanol, the tissue was embedded in Spurr. Ultra thin sections were cut, stained with lead citrate for 5 min and examined using a Phillips EM 201 electron microscope.

2.15 Detection of donor cells in recipient mice by flow cytometry.

Flow cytometry was used to detect the percentage of donor-derived cells in the spleens of recipient mice. The proportion of these cells that expressed either CD4 or CD8 was also determined. We used anti- H-2D^d to detect host cells in the H-2^b → H-2$^{b/d}$ strain combination employed in these experiments. Lymphocytes in the flow histograms that did not express this marker were therefore deemed to be of donor origin. This indirect method of detecting donor-derived, parental-strain cells in F_1-hybrid hosts has been used by other investigators (402). Recipients of grafts from both wild-type and IFN-γ gene knockout (*gko)* donors were assayed on days 4, 8 and 15. In the latter group, we also performed an analysis on day 40. To demonstrate the specificity of H-2D^d labeling, an analysis of spleen cells from C57BL/6 and BDF$_1$-hybrid mice was

performed. Between 95 and 100% of the cells were H-2D^{d+} when BDF$_1$-hybrids were used, whereas these cells were undectable when C57BL/6 mice were used.

Since we were interested primarily in the engraftment of T cells, spleen cell suspensions were passaged through nylon wool columns to remove adherent cells. Red cells were also removed by centrifugation on a Lympholyte-M gradient. Details of these methods are described in a previous publication (399). To perform flow cytometry analyses, cells were incubated in PBS/1%BSA containing 10 μg/ml of antibody. Co-expression of H-2D^d and CD4 or CD8 was determined by co-incubating cells with FITC-conjugated mouse anti-H-2D^d and of either PE-conjugated rat anti-CD4 or PE-conjugated rat anti-CD8. All incubations were performed for 30 min on ice using 0.5 x 10^6 cells suspended in 100 μl of reagent that had been diluted in PBS/BSA. The cells were washed in PBS/1% BSA and resuspended in saline containing 2% paraformaldehyde. FITC-conjugated mouse IgG2a and PE-conjugated rat IgG2a (LO-DNP-16; Caltag) were used as isotype controls. Two-colour flow cytometry analyses were performed as described previously, except that acquisition was based on 6000 gated events.

2.16 Comparing the percentage of CD4$^+$ and CD8$^+$ cells in grafts from wild-type and IFN-γ *gko* mice.

Flow cytometry was used to verify that wild-type and IFN-γ *gko* grafts contained equal numbers of CD4$^+$ and CD8$^+$ cells. The protocols used to perform this analysis were identical to those described above, with the following exceptions: grafts consisted of pooled lymph node and spleen cells, and nylon wool purification was omitted.

2.17 Donor/host origin of NK and NK-like cytotoxic activity.

The rationale for the method used to detect the relative contribution of donor and host cells to NK and NK-like cytotoxicity observed in recipient mice is similar to that used in the engraftment experiment described earlier. We used anti-H-2D^d and complement to deplete host-derived cytotoxic activity directed against YAC-1 and BW1100 target cells. The activity remaining after depletion was deemed to be of donor origin. Because NK and NK-like activity were maximal on different days in the two recipient groups, we only assayed for the donor/host origin of this activity on the days on which it was greatest. NK activity (YAC-1-directed lysis) was maximal on day 4 in both recipient groups, whereas NK-like activity (BW1100-directed lysis) was highest on day 8 in recipients of wild-type grafts and on day 4 in recipients of IFN-γ *gko* grafts. Nylon-wood non-adherent, red cell free effector cells were incubated at a concentration of 2 x 10^7 cells/ml, for 1 hr, on ice, with anti-H-2D^d (34-5-8S; Cedarlane), diluted 1 in 40. This was followed by an incubation at a concentration of 2 x10^7 cells/ml, for 1 hr, at 37 °C, with lyophilized Low-Tox-M Rabbit Complement (Cedarlane). Negative and positive controls consisted of effector cells incubated with either complement only or anti-ASGM$_1$ rabbit antiserum (Wako Chemicals, Dallas, TX) followed by complement. (403)

2.18 Limulus Amoebocyte Lysate (LAL) Assay for Endotoxin.

Recipients were anaesthetized with ether and exsanguinated by cardiac puncture. All subsequent procedures were performed under sterile conditions, using pyrogen-free LAL reagent water (LRW; Associates of Cape Cod, Woods Hole, MA). Instruments and glassware were baked for 2 hr at 180°C and tested for the presence of endotoxin by preparing samples of sera from control mice and measuring the level of LPS present.

Blood was allowed to clot overnight at 4°C, and then frozen at -70°C until the assay date. After thawing, serum samples were diluted 1:1 in LRW and boiled for 2 minutes. Endotoxin was measured using an LAL Pyrochrome assay (Associates of Cape Cod) kit in which LPS, present in the sample or standard, initiates a cascade of enzymatic activity that terminates in the cleavage of a chromogenic substrate. This produces p-nitroaniline (pNA) which is yellow and absorbs at 405 nm. We chose to use the LAL kit containing a diazo-coupling modification in which pNA is further reacted with nitrite in HCl and then with N-(1-Naphthyl)-ethylenediamine to form a magenta derivative that absorbs at 540-550 nm. This was done so that our results would not be influenced by the yellow-colored components present in the sera. In preliminary experiments, we found that endotoxin levels could be accurately determined for sera diluted at least 1 in 4 in LRW. To perform the LAL assay, serial dilutions of either control standard endotoxin (CSE) or serum samples were performed in LRW. Fifty microlitres of diluted serum, diluted CSE or LRW and 50 ml of Pyrochrome reagent were then placed in the wells of a pyrogen-free, 96 well, Pyroplate™ (Associates of Cape Cod). This was placed on a plate shaker for 30 seconds and incubated for 30 minutes at 37°C. Diazo-coupling reagents were then added and the OD_{550} was determined for each well.

CHAPTER 3

POSITIVE SELECTION OF NK1.1$^+$ CELLS ON A MAGNETIC CELL SEPARATOR (MACS)*

* This work by C.A. Ellison and J.G. Gartner has been published in the Journal of Immunological Methods 1995; 186:233-243.

3.1 Abstract.

Natural killer cells are known to play a role in the pathogenesis of acute GVHD and earlier results from our laboratory suggested that NK-like cells may also be involved. Both of these cellular populations express the strain specific (H-2^b) cell surface marker, NK1.1, in the C57BL/6→BDF_1-hybrid model of acute GVHD. Because we were particularly interested in determining the precise phenotype of the NK-like cells seen in GVH mice, it was necessary to develop a specific and efficient method for purifying large numbers of NK1.1^+ cells. Described here is a method for positively selecting these cells from normal and poly I:C-stimulated C57BL/6 mice using a magnetic cell separation technique known as MACS. The results show that the cytotoxic activity directed against YAC-1 and BW1100 target cells were increased 5-and 2-fold, respectively. Flow cytometry analysis of poly I:C-stimulated MACS-enriched, NK1.1^+ spleen cells revealed the presence of two subpopulations: one consisting of LGL, and another consisting of SAL. Following enrichment, the percentage of NK1.1^+ LGL increased from 69 to 91% and the percentage of NK1.1^+ SAL increased from 33 to 73%. These results demonstrate the effectiveness of the MACS technique for purifying large numbers of NK1.1^+ cells for both flow cytometric and functional analyses.

3.2 Introduction.

One of the earliest methods to isolate and enrich NK cells was based on cell density employed discontinuous Percoll gradients (404). With the availability of a variety of monoclonal antibodies, lectins, and other reagents for the selection of cell populations, it became possible to enrich NK cell activity by removing irrelevant cell types (405). More direct approaches have used positive selection of cells expressing cell surface molecules such as FcγR (CD16). Other surface markers present on both NK cells and other lymphoid

cells include $ASGM_1$, Qa-2, Qa-4, Qa-5, Thy-1 and Lyt-1 (406-408). Since these markers are present some non-NK cells, methods for selecting NK cells based on the expression of these markers are useful only for the enrichment of these cells, and not for the isolation of them *per se*.

In 1977, Glimcher and colleagues identified NK1.1, a cell surface marker present on NK cells in $H\text{-}2^b$ mice (409). Although immunogenetic studies have shown that this marker defines murine NK cells (408), NK1.1 is also present on a small number of T cells (399;410). It has therefore been suggested that this marker is associated more with NK function than with a specific cell type (411). The development of an anti-NK1.1 mAb (412) has made it possible to separate $NK1.1^+$ from $NK1.1^-$ cells by FACS (413).

At the time this work was done, a magnetic cell separation technique known as MACS had been used to isolate and enrich several human cell types including lymphocytes (414-416), thymocytes (417), hematopoietic stem cells (418), eosinophils (419), follicular dendritic cells (420), megakayocytes (421) and NK cells (422;423). However, there were only a few reports in which this technique had been used to isolate and enrich murine cells. These included the depletion of $Thy\text{-}1^+$ cells (424), the isolation of Ig^+ cells (424), the depletion of $CD4^+$ and $CD8^+$ cells (411), and the isolation of T cells with class I MHC mutants (425). To our knowledge, this technique had never been used for the positive selection of murine NK cells, nor had an analysis of cytotoxic function following positive selection with the MACS technique ever been investigated. The purpose of our study was therefore to determine the extent to which this method was appropriate for the isolation and enrichment of murine NK cells, and whether there was preservation of NK function in the positively selected fraction.

We also wished to demonstrate that this technique could be used to isolate cells with NK-like function. These cells have the capacity to lyse targets such as P815, which

are resistant to conventional NK cell lysis and are observed in mice that have been stimulated with interferon or interferon inducers such as poly I:C (426), and in mice wth acute GVHD (209). Because these cells exist only in low numbers in lymphoid organs, and produce only a low level of cytotoxicity, studies designed to characterize NK-like cells are difficult. Therefore, we wished to further determine whether the MACS technique could be used to isolate and enrich these cells for subsequent analysis of their phenotype and function. Our results showed that this method is very effective for the isolation of large numbers of NK1.1$^+$ spleen cells from both untreated, and poly I:C-stimulated mice.

3.3 Experimental design.

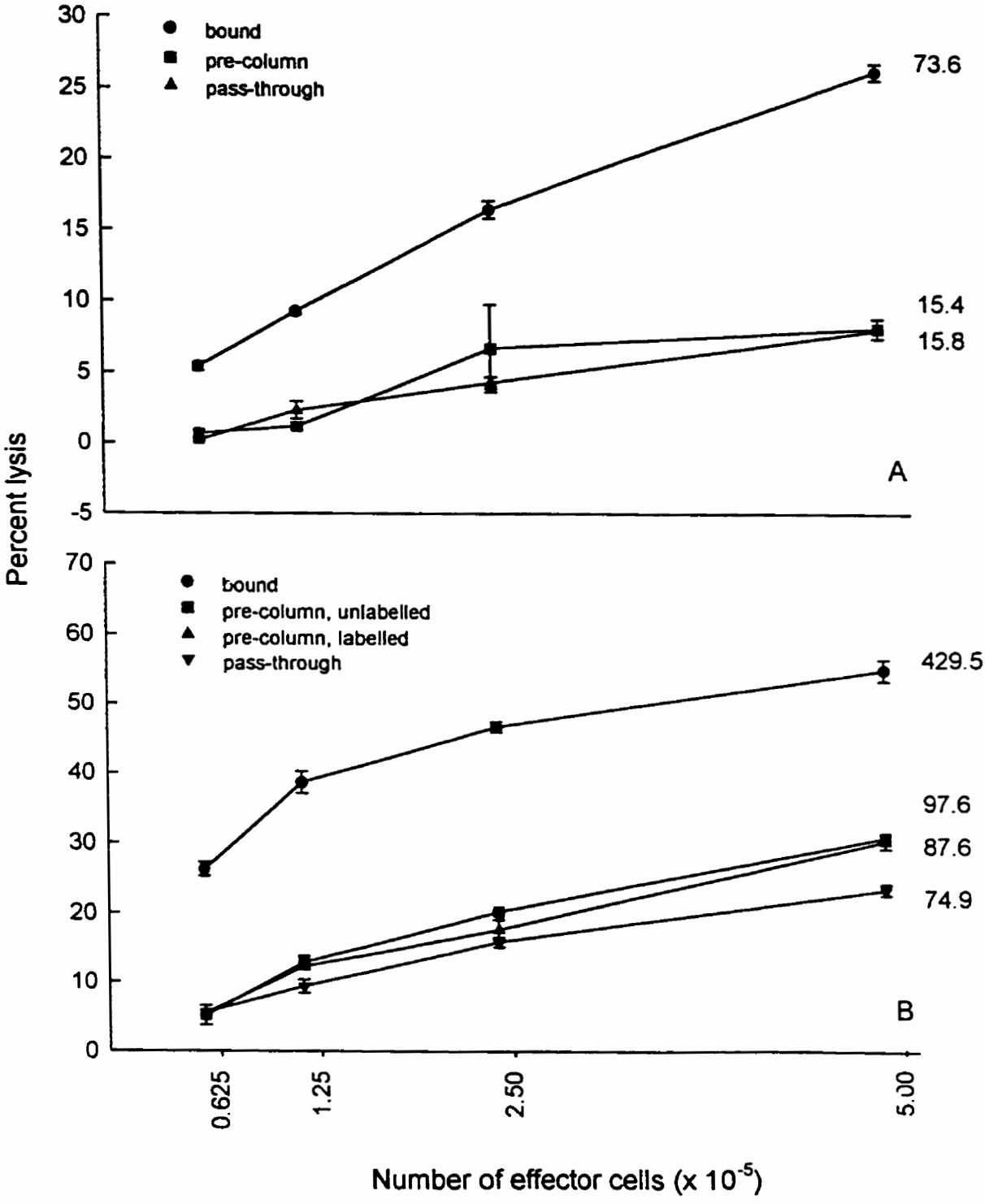
Spleens were harvested from normal, untreated C57BL/6 mice, and from C57BL/6 mice that had been injected i.p., 18 hr before sacrifice, with 100 µl of poly I:C dissolved in HBSS to a concentration of 1 mg/ml. A red cell-free, nylon wool-non-adherent cell suspension was prepared as described in the Materials and Methods, and labeled with anti-NK1.1-biotin, SA-conjugated MACS microbeads, and SA-PE. Cells expressing NK1.1$^+$ were enriched on the MACS, and the levels of YAC-1 and BW1100-directed lysis in the precolumn, bound, and pass-through cellular fractions were measured using a 4 hr ^{51}Cr release assay. The percentage of NK1.1$^+$ cells in the LGL and SAL subpopulations were also compared in the precolumn and bound fractions by flow cytometry.

3.4 Results.

3.4.1 Analyisis of cytotoxic activity following MACS enrichment.

Positive selection of NK1.1$^+$ cells from the spleens of normal, unstimulated mice using MACS is shown in Figure 3.1A. Cytotoxic activity directed against YAC-1 target

Figure 3.1 Graph showing NK cytotoxic activity directed against YAC-1 target cells by splenic effector cells harvested from untreated mice (A), or from mice stimulated with poly I:C (B). The cytotoxicity directed against targets in the bound, precolumn and pass-through fractions is compared following positive selection of NK1.1$^+$ cells on the MACS. The cytotoxic activity in the precolumn fraction with (labeled) and without (unlabeled) the addition of anti-NK1.1-biotin, SA microbeads and SA-PE is also compared in B. The error bars indicate the standard error of the mean percent lysis at each E:T ratio. The number beside each plot represents the LU per 10^7 effector cells calculated from the mean percent lysis at each E:T ratio.


30
25
20
15
10
5
0
-5
bound
pre-column
pass-through
73.6
15.4
15.8
A
70
60
50
40
30
20
10
0
bound
pre-column, unlabelled
pre-column, labelled
pass-through
429.5
97.6
87.6
74.9
B
Percent lysis
0.625
1.25
2.50
5.00
Number of effector cells (x 10-5)

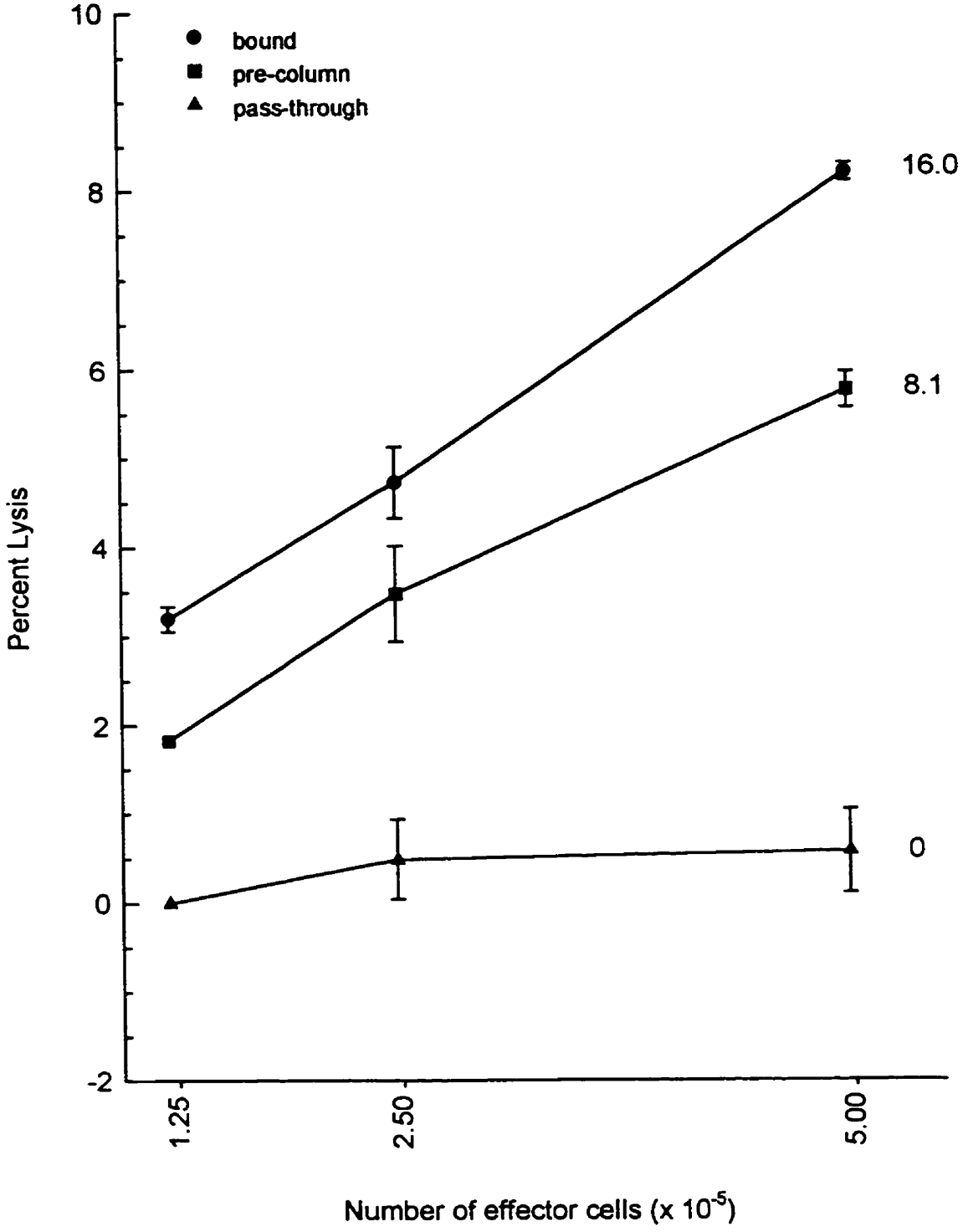
cells increased from 15.4 LU in the pre-column fraction to 73.6 LU in the bound fraction. We could still detect 15.8 LU in the pass-through fraction indicating that some NK cells had failed to adhere to the column, or that some NK1.1$^-$ cells may have the ability to lyse YAC-1 target cells. We consistently observed that the cytotoxic activity directed against NK-resistant, BW1100 target cells was virtually undetectable in spleen cells from normal mice (i.e. not greater than 5% lysis at an E:T ratio of 100:1), and that this activity could not be enriched using MACS (data not shown).

Figure 3.1B shows that cytotoxic activity directed against YAC-1 target cells by unlabeled, poly I:C-stimulated spleen cells in the pre-column fractions was 97.7 LU. This was markedly increased to 429.5 LU in the bound fraction. Again, some cytotoxic activity remained in the pass through fraction (74.9 LU).

The level of YAC-1-directed lysis was 10 LU lower than unlabeled cells in the precolumn fraction when cells were labeled with anti-NK1.1-biotin, SA-conjugated microbeads and SA-PE, indicating that the labeling procedure was not responsible for the increased level of cytotoxity seen in the bound fraction. This control is particularly important because stimulation of the NK1.1 molecule is known to activate NK cells in some circumstances (244;245). It is possible that the reduction in YAC-1-directed lysis seen following the labeling procedure resulted from inhibition of effector-target cell contact caused by the presence of these reagents on the effector cell surface.

Figure 3.2 shows that the NK-like activity directed against the NK-resistant target cell, BW1100 was increased nearly 2-fold from 8.1 LU in the precolumn fraction to 16.0 in the bound fraction.

Figure 3.2 Graph showing NK-like cytotoxic activity directed against BW1100 target cells by splenic effector cells harvested from mice stimulated with poly I:C. The levels of cytotoxicity seen in the pre-column, bound and pass-through cellular fractions are compared following positive selection of $NK1.1^+$ cells on the MACS. The error bars indicate the standard error of the mean percent lysis at each E:T ratio. The number beside each plot represents the LU per 10^7 effector cells calculated from the mean percent lysis at each E:T ratio.


10
8
6
4
2
0
-2
bound
pre-column
pass-through
16.0
8.1
0
Percent Lysis
1.25
2.50
5.00
Number of effector cells (x 10-5)

3.4.2 Analysis of MACS-enriched NK1.1$^+$ cells by flow cytometry.

We performed flow cytometry analyses to determine the extent to which NK1.1$^+$ cells were purified. The bit map in Figure 3.3 shows the two subpopulations of cells that were analyzed. Lines A and B delimit the SAL and LGL subpopulations, respectively. We first determined the percentage of cells that were NK1.1$^+$ in the precolumn fraction. It was found, firstly, that the percentage of cells expressing NK1.1 were 3-fold higher in poly I:C-stimulated mice (data not shown). Since poly I:C is a well known stimulator of NK cell activity and since our functional data indicated that there was an increase in the level of NK cytotoxicity in this group before enrichment, we were not surprised by this observation. In subsequent experiments, flow cytometry analyses were only performed on cells harvested from poly I:C-stimulated mice.

The histograms in Figure 3.4 show the percentage of NK1.1$^+$ LGL and SAL in the precolumn, bound and pass-through fractions. The value shown above the line in each histogram is the percentage of NK1.1$^+$ cells before correction for non-specific staining with SA-PE. To correct for this background, we subtracted the percentage of cells in the pre-column fraction labeled with SA-PE only, from the cells labeled with anti-NK1.1-biotin followed by SA-PE. We could not establish a separate value for non-specific binding of SA-PE in the bound and pass-through fractions, because this cellular fraction could not be generated without first incubating cells with anti-NK1.1-biotin. With this correction, we found that 68.6% LGL and 32.9% SAL were NK1.1$^+$ in the precolumn fraction. The ratio of LGL to SAL was 147:5000. In the bound fraction, 90.6% of the LGL and 73.4% of the SAL were NK1.1$^+$ and the ratio of LGL to SAL was 374:5000. Of the cells that passed through the column, 50.6% and 17.3% were LGL and SAL, respectively, and the ratio of LGL to SAL was 101:5000. We also observed that LGL stained more brightly than SAL.

Figure 3.3 Bit map showing forward versus 90° light scatter analysis of poly I:C-stimulated splenocytes in the bound cellular fraction. The gates shown delimit subpopulations of SAL (A) and LGL (B).

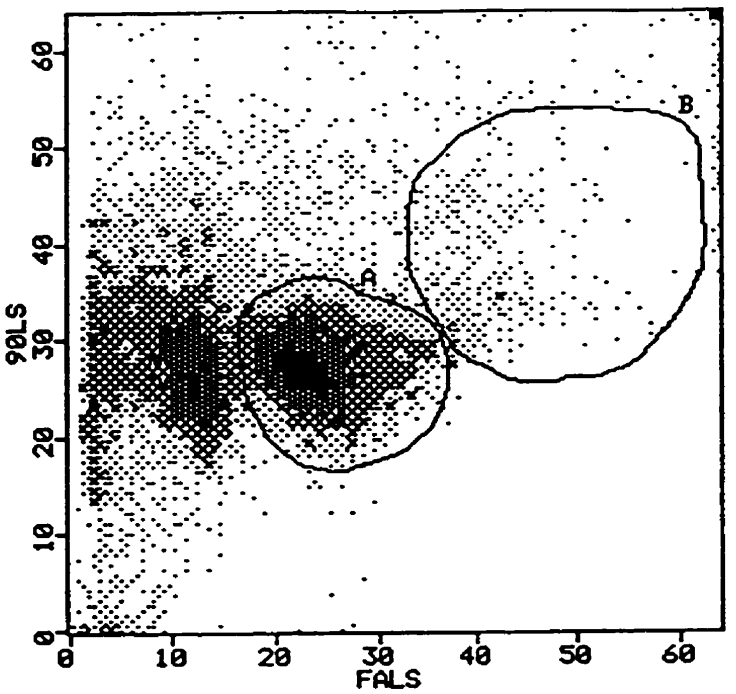
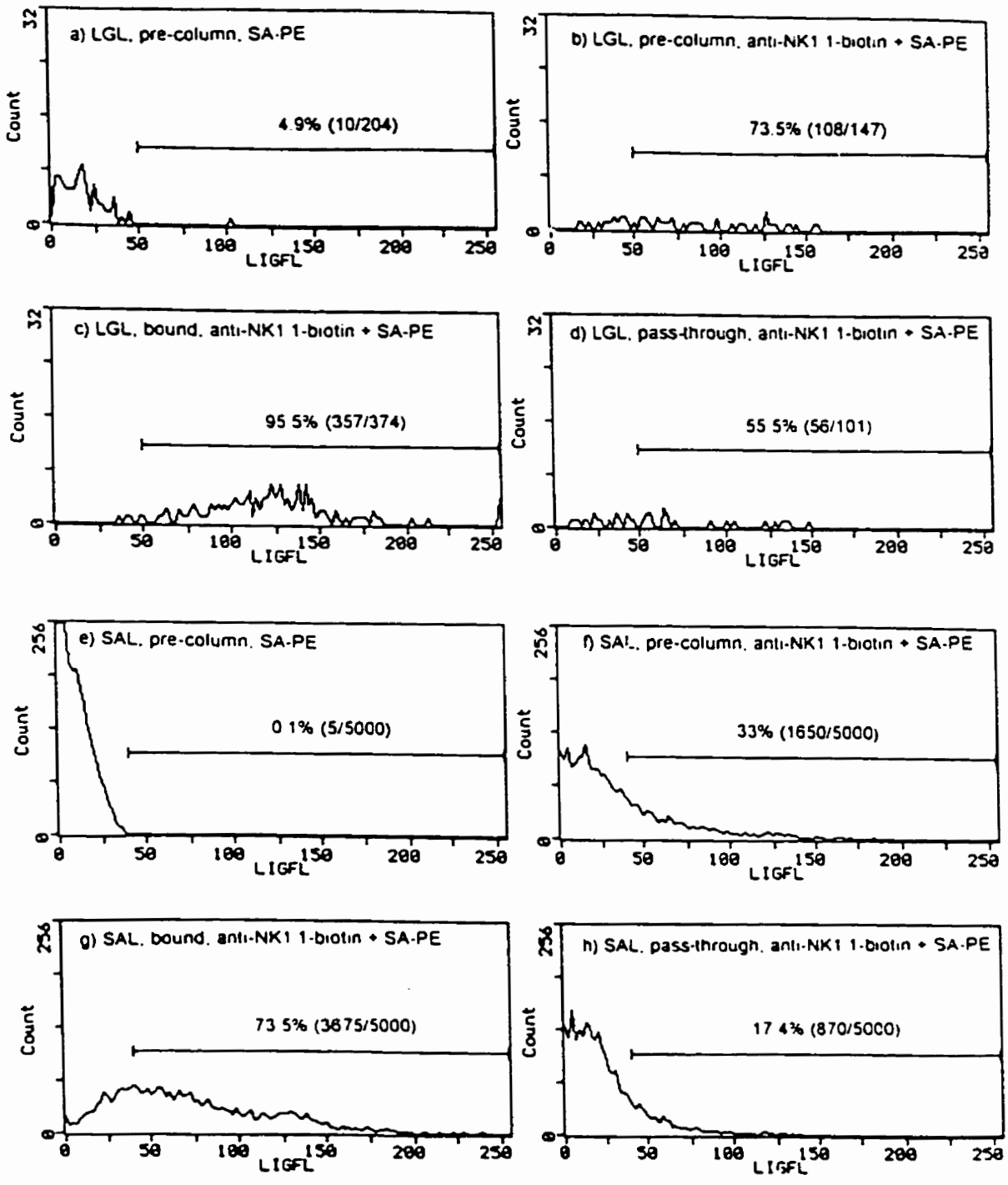

90LS
FALS
0
10
20
30
40
50
60
A
B

Figure 3.4 Histograms showing the results of flow cytometry experiments performed to determine the proportion of NK1.1$^+$ LGL and SAL present in the precolumn, bound and pass-through cellular fractions. In each histogram, the percentage of NK1.1$^+$ cells is shown. This is followed, in parenthesis, by the absolute number of NK1.1$^+$ cells detected, divided by the total number of LGL and SAL counted. The proportion of LGL labeled with SA-PE only in the precolumn fraction is shown in (a). The proportion of LGL labeled with anti-NK1.1-biotin and SA-PE in the pre-column, bound and pass-through fractions are shown in histograms (b), (c) and (d), respectively. The proportion of SAL labeled with SA-PE only in the pre-column fraction is shown in (e). The proportion of SAL labeled with anti-NK1.1-biotin and SA-PE in the precolumn, bound and pass-through fractions are shown in histograms (f), (g), and (h), respectively.


a) LGL, pre-column, SA-PE
4.9% (10/204)
b) LGL, pre-column, anti-NK1 1-biotin + SA-PE
73.5% (108/147)
c) LGL, bound, anti-NK1 1-biotin + SA-PE
95 5% (357/374)
d) LGL, pass-through, anti-NK1 1-biotin + SA-PE
55 5% (56/101)
e) SAL, pre-column, SA-PE
0 1% (5/5000)
f) SAL, pre-column, anti-NK1 1-biotin + SA-PE
33% (1650/5000)
g) SAL, bound, anti-NK1 1-biotin + SA-PE
73 5% (3675/5000)
h) SAL, pass-through, anti-NK1 1-biotin + SA-PE
17 4% (870/5000)
Count
LIGFL

3.5 Discussion.

The purpose of our study was to determine whether MACS could be used to isolate large numbers of NK1.1$^+$ cells without compromising cytolytic activity. Our results indicate that this is possible since the level of NK cytotoxic activity was increased 500% and the percentages of LGL and SAL increased 25% and 40%, respectively, using the separation protocol we established. This apparently disproportionate increase in cytolytic activity following enrichment may have resulted not only because positive selection increased the relative number of NK cells at each E:T ratio, but also because it reduced the number of non-NK cells that may have interfered with NK-mediated cytotoxicity. Although we isolated up to 50 x 10^6 NK1.1$^+$ cells in a single experiment, this method could, if necessary, be adapted to purify up to 100 x 10^6 positive cells at one time using a larger separation column.

When comparing NK1.1 expression in the LGL and SAL subpopulations, we found that the overall number of NK1.1$^+$ SAL was greater than that seen in the LGL subpopulation; however, the percentage of NK1.1$^+$ LGL was greater. This finding is consistent with previous studies from other laboratories showing that most NK cells are LGL, but agranular cells with NK function also exist (288). Our results indicate that not all NK1.1$^+$ cells are positively selected on the MACS since some are present in the pass-through fraction. We suggest that some of these cells could potentially have been recovered if this fraction had been passaged through the MACS column a second or third time.

Alternative approaches that we could have considered at the time this work was done include flow cytometry and other immunomagnetic cell separation systems that use beads with a larger diameter. However, we felt that neither of these technologies were as effective as the MACS technique because: cell separation by flow cytometry would have required several hours of sorting to obtain enough NK cells to conduct a cytotoxicity assay.

The MACS technique achieved the same result in a much shorter period of time and at a substantially lower cost. Other immunomagnetic cell separation systems employ fairly large-diameter beads (5 μm) that are known to interfere with both the optical properties of labeled cells as well as their viability in culture (414). The microbeads used with the MACS system have a diameter of only 100-150 nm, and results from these experiments, and those of others (423) indicate that they interfere only minimally with the ability of NK cells to function as cytotoxic effector cells. Furthermore, the small size of the beads does not significantly alter the optical properties of positively labeled cells and they can therefore be used in subsequent flow cytometry studies to ascertain the expression of other lymphocyte markers.

3.6 Appendix.

All experiments were performed by the candidate. Dr. E. S. Rector provided assistance with flow cytometry analyses.

CHAPTER 4

γδT CELLS IN THE PATHOBIOLOGY OF MURINE ACUTE GRAFT-VERSUS-HOST DISEASE*

*This work by C.A. Ellison, G.C. Macdonald, E.S. Rector and J.G. Gartner has been published in the Journal of Immunology, 1995; 155:4189-4198.

4.1 Abstract.

NK-like cytotoxicity in F_1-hybrid mice with acute GVHD is mediated by donor-derived $CD3^+/CD4^-/CD8^-/NK1.1^+/ASGM_1^+$ cells that can lyse both NK-sensitive, YAC-1 target cells and NK-resistant targets such as BW1100. Our objective was to determine whether $\gamma\delta TCR^+$ cells mediate this activity. We had shown previously that the NK-like cytotoxic activity seen in the spleens of mice with acute GVHD could be depleted by indirect complement-mediated lysis using an antibody against γδTCR (399). Here we show that NK-like cells in the lymph nodes can be similarly depleted using this antibody. When purified $NK1.1^+$ spleen cells that had been positively selected using the MACS were used as effector cells, we found that $\gamma\delta TCR^+$ cells mediated a substantial proportion of the NK-like cytotoxicity we observed. This suggested that a significant subpopulation of cells with NK-like activity are $\gamma\delta TCR^+/NK1.1^+$. Using flow cytometry, we further demonstrated that co-expression of NK1.1 and γδTCR occurs on a large proportion of large granular lymphocytes (LGL) in the spleens of GVH mice. However, fewer than 10% of small, agranular $NK1.1^-$ lymphocytes were found to express both of these markers. Coexpression of these two molecules in splenocytes from normal, control mice was undetectable. On the basis of these observations, we postulated that graft-derived LGL develop a $NK1.1^+/\gamma\delta TCR^+$ phenotype during the reaction, and that these cells play a role in the pathogenesis of acute GVHD. We therefore performed experiments to determine whether depletion of γδT cells from donor mice affected the outcome of lethal GVHD. Our results showed that there was a reduction in mortality in recipients of γδTCR-depleted grafts.

4.2 Introduction.

There is a growing body of evidence suggesting that non-MHC restricted killer cells may be involved in the pathogenesis of acute GVHD (329;331;332;427-429). A study

from our laboratory also showed, using an F_1-hybrid model, that this disease could be prevented if NK1.1$^+$ cells were depleted from the graft with antibody and complement. However, this treatment was only effective if the parental strain donors were injected with poly I:C 18 hr before the grafts were harvested (333). These experiments also showed that both donor-derived CTL activity directed against host MHC and conventional, host-derived, NK cell activity were preserved in recipients of the modified grafts, even though the lethal effects of the GVH reaction had been eliminated. This suggested that neither of these responses is associated with the wasting syndrome and high mortality associated with acute GVHD.

In two earlier studies, we showed that two populations of non-MHC-restricted cytotoxic cells were activated during acute, lethal GVH reactions. One consisted of conventional NK cells that could lyse NK-sensitive tumour target cells (YAC-1), and the other of cells that can lyse a much broader range of tumour cell targets, including those insensitive to lysis by conventional NK cells (BW1100, P815, K562 and DAUDI) (209;430). The latter donor-derived population, which were termed "NK-like", had an ASGM$_1^+$, CD8$^-$, CD4$^-$, NK1.1$^+$ phenotype (209;333). In an immunohistochemical study, Ferrara and colleagues observed the presence of large granular lymphocytes (LGL) in proximity to dead and dying epithelial cells in the skin, liver and colon of mice with acute GVHD (431). The surface markers of these cells closely resembled those present on the "NK-like" cells in our functional studies. Since it has been suggested that these LGL may be γδTCR$^+$, we postulated that NK-like cells we observed were, in fact, γδT cells (431). The purpose of these experiments was to provide evidence that this speculation was correct and to show that elimination of γδT cells from donor mice reduces the mortality associated with acute murine GVHD.

4.3 Experimental design.

Experiments performed previously in our laboratory had shown that NK-like cytotoxic activity developed both in the spleen and lymph nodes of mice with acute GVHD. They had also shown that these cells have a γδTCR$^+$ phenotype in the spleen. Because it was not known whether the NK-like cells in the lymph nodes were of a similar phenotype, our first objective was to determine whether these cells also expressed γδTCR. This was accomplished by determining whether any NK-like cytotoxic activity remained in effector cells isolated from the lymph nodes of GVH mice, after they had been depleted *in vitro* by indirect complement-mediated lysis using anti-γδTCR mAb. To confirm our previous observation that NK-like cells in GVH mice are NK1.1$^+$, we also depleted these effector cells by direct complement-mediated lysis using anti-NK1.1 mAb.

Because the level of NK-like cytotoxic activity is relatively low in mice with acute GVHD (not more than 15 LU_{10} cells), our second objective was to perform a purification procedure, thereby permitting a more definitive phenotypic analysis. We chose to use the MACS magnetic cell separation technique, and to select NK-like cells based on their expression of NK1.1. The NK-like cytotoxic activity remaining in NK1.1-enriched splenic effector cells after depletion with anti-γδTCR mAb or anti-αβTCR followed by rabbit anti-hamster IgG and complement was then measured in a 4 hr ^{51}Cr release assay. We also measured the level of NK-like cytotoxic activity remaining following depletion with anti-$ASGM_1$ and complement, since our previous work has shown that all NK-like cells also express this marker. We chose to limit our study to NK-like cells in the spleen because of the large number of cells required to perform each experiment.

Our third aim in this study was to demonstrate, using two-colour flow cytometry, that cells in the spleens of GVH mice coexpress NK1.1 and γδTCR. Analyses were performed before, and after enrichment of NK1.1$^+$ cells on the MACS.

Finally, to determine whether γδT cells were actually involved in the pathogenesis of acute GVHD, and were not simply an epi-phenomenon of the GVH reaction, we depleted donor mice *in vivo*, using anti-γδTCR mAb. Grafts from these donors were then used to induce GVH reactions.

4.4 Results.

4.4.1 Expression of γδTCR on NK like cells harvested from the lymph nodes of mice with acute GVH reactions.

In most of our experiments, "NK-like" activity, peaked in the spleen on day 7 of the reaction. Table 4.1 shows the mean level of cytotoxicity directed against BW1100 target cells in both the lymph nodes and spleen. Although the level of this activity was never as high as conventional NK activity, the results demonstrate that BW1100-directed lysis in GVH mice is significantly greater than that seen in control mice.

When lymph node cells were used as effectors, anti-γδTCR produced a marked reduction in the levels of BW1100 and YAC-1-directed lysis (Figure 4.1), suggesting that all GVH-induced, non-MHC restricted cytotoxic activity in this organ is mediated by NK-like cells. The fact that BW1100- and YAC-1-directed lysis could also be completely depleted with an antibody against NK1.1 provides some evidence that NK-like cells co-express γδTCR and NK1.1. However, because the yield of NK-like cells from lymph nodes of mice with GVHD was very low, we chose to limit subsequent investigations to the spleen.

4.4.2 Enrichment of splenic NK and NK-like cytotoxic activity using the MACS.

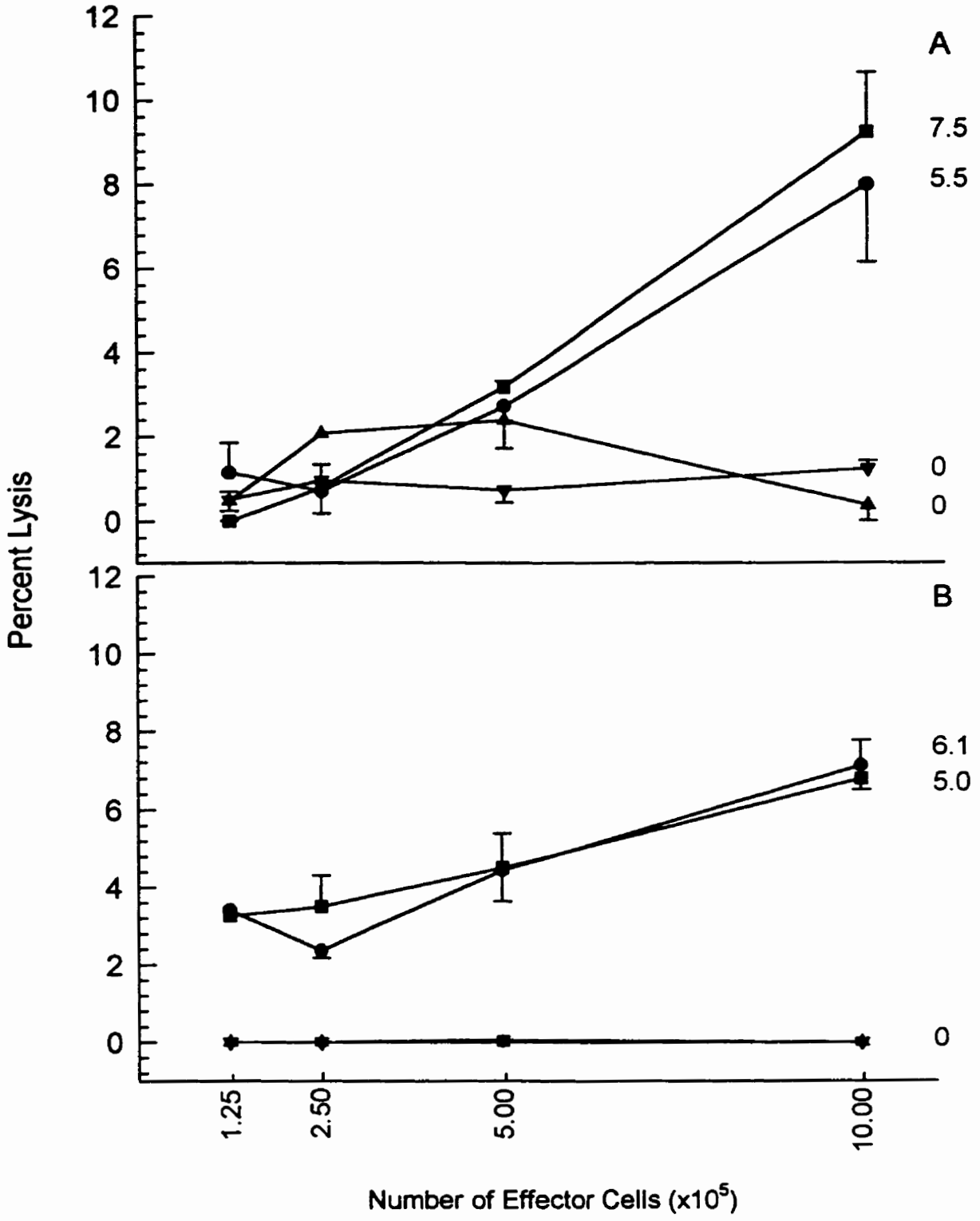
NK1.1$^+$ cells were positively selected from spleen cells harvested from normal controls and GVH mice on day 7 of the reaction. The NK1.1-enriched fraction

Table 4.1. Table showing the mean level of NK-like cytotoxic activity in the spleen and lymph nodes of control mice and mice with acute GVH disease on day 7 post induction.

Organ	Experimental Group	Number of Experiments	Mean $LU_{10}/10^7 \pm$ SEM	p -value[a]
Spleen	Control	6	1.38 ± 0.66	<0.01
	GVH	6	9.29 ± 2.23	
Lymph Nodes	Control	3	1.37 ± 1.11	<0.02
	GVH	3	10.93 ± 2.23	

[a]Students *t* test was used to analyse the statistical significance of the differences between the mean level of lytic activity in the two groups.

Figure 4.1 Graphs showing NK-like cytotoxic activity directed at BW1100 (A) and YAC-1 (B) target cells in lymph node cells from mice sacrificed on day 7 post-induction. The cytotoxicity directed against target cells by effector cells treated with complement only (●) is compared with that observed when effector cells were treated with rabbit anti-hamster IgG and C (■), anti-γδTCR mAb followed by rabbit anti-hamster IgG and C (▲) or anti-NK1.1 mAb and C (▼). The error bars indicate the standard error of the mean percent lysis for three replicate samples at each E:T ratio. The number beside each plot represents the LU_{10} calculated from the mean percent lysis at each E:T ratio. ND beside a plot indicates that an LU_{10} could not be calculated from the dose response curve.


Percent Lysis
12
10
8
6
4
2
0
A
7.5
5.5
0
0
B
6.1
5.0
0
1.25
2.50
5.00
10.00
Number of Effector Cells (x10^5)

demonstrated a level of BW1100-directed lysis that was much greater than that seen in the pre-enrichment fraction (Figure 4.2A). In this particular experiment, we were unable to calculate an accurate LU_{10} value from the dose-response curve for unenriched BW1100-directed lysis in GVH mice (Figure 4.2A), but the actual percent lysis values were clearly greater than those seen in unenriched spleen cells from control mice (Figure 4.2B). The increase in YAC-1-directed lysis in GVH mice was less marked (Figure 4.2C). In control mice, there was only a slight enrichment of NK-like activity (Figure 4.2B), but a fairly marked enrichment of YAC-1-directed lysis (Figure 4.2D). The reason why the levels of YAC-1-directed in the GVH mice are lower than those seen in the controls is not known. However, we have periodically observed a reduction in the level of this activity during the early stages of the GVH reaction. When we compared levels of lysis before and after labelling with biotinylated anti-NK1.1 mAb and SA-microbeads, we found that neither BW1100- nor YAC-1-directed lysis was enhanced (data not shown). This indicated that the increased lytic activity observed after enrichment was attributable to positive selection of effector cells and not to activation of the cells by antibody binding to a membrane receptor.

4.4.3 Immunophenotypic analysis of NK1.1$^+$ MACS-enriched spleen cells.

Immunophenotyping for expression of γδTCR and αßTCR on NK1.1$^+$ MACS-enriched spleen cells was done using negative selection with specific antibody and indirect complement-mediated lysis. Data in Figure 4.3 show that BW1100-directed lysis could be almost completely eliminated with anti-γδTCR, but was unaffected by anti-αßTCR. Because the number of effector cells harvested from the column in any given experiment was very limited, we restricted our efforts to examining the effects of these two antibodies on BW1100-directed lytic activity only, since BW1100 is a definitive target cell for NK-like activity.

Figure 4.2 Graphs showing NK-like and conventional NK cytotoxicity in spleen cells from GVH mice (A and C) and control mice (B and D) before (▲) and after (●) enrichment of $NK1.1^+$ cells using MACS. Splenic effector cells were prepared from mice on day 7 post-induction. The error bars indicate the standard error of the mean percent lysis for three replicate samples at each E:T ratio. The number beside each plot represents the LU_{10} calculated from the mean percent lysis at each E:T ratio. ND beside a plot indicates that an LU_{10} could not be calculated from the dose response curve.

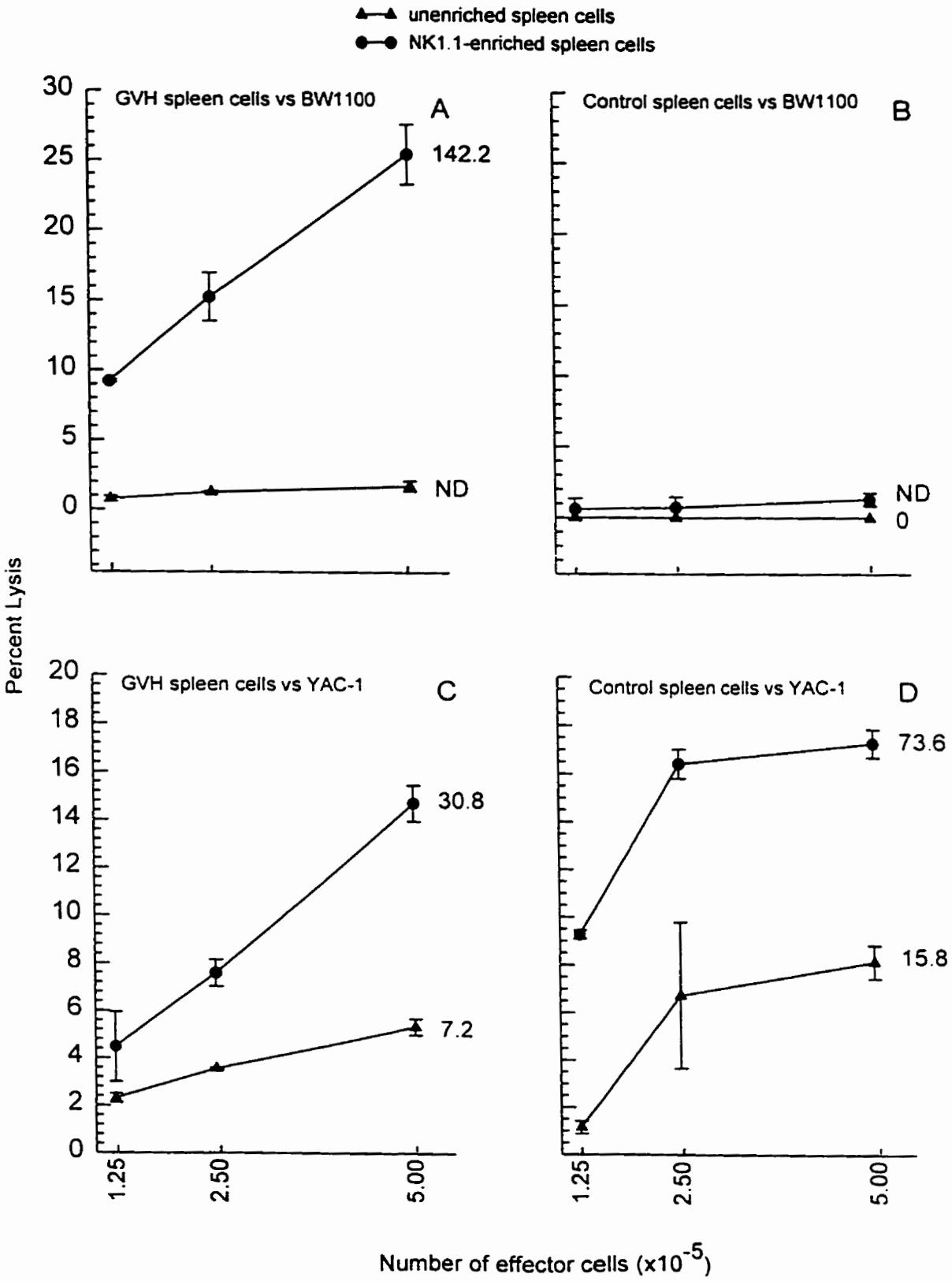
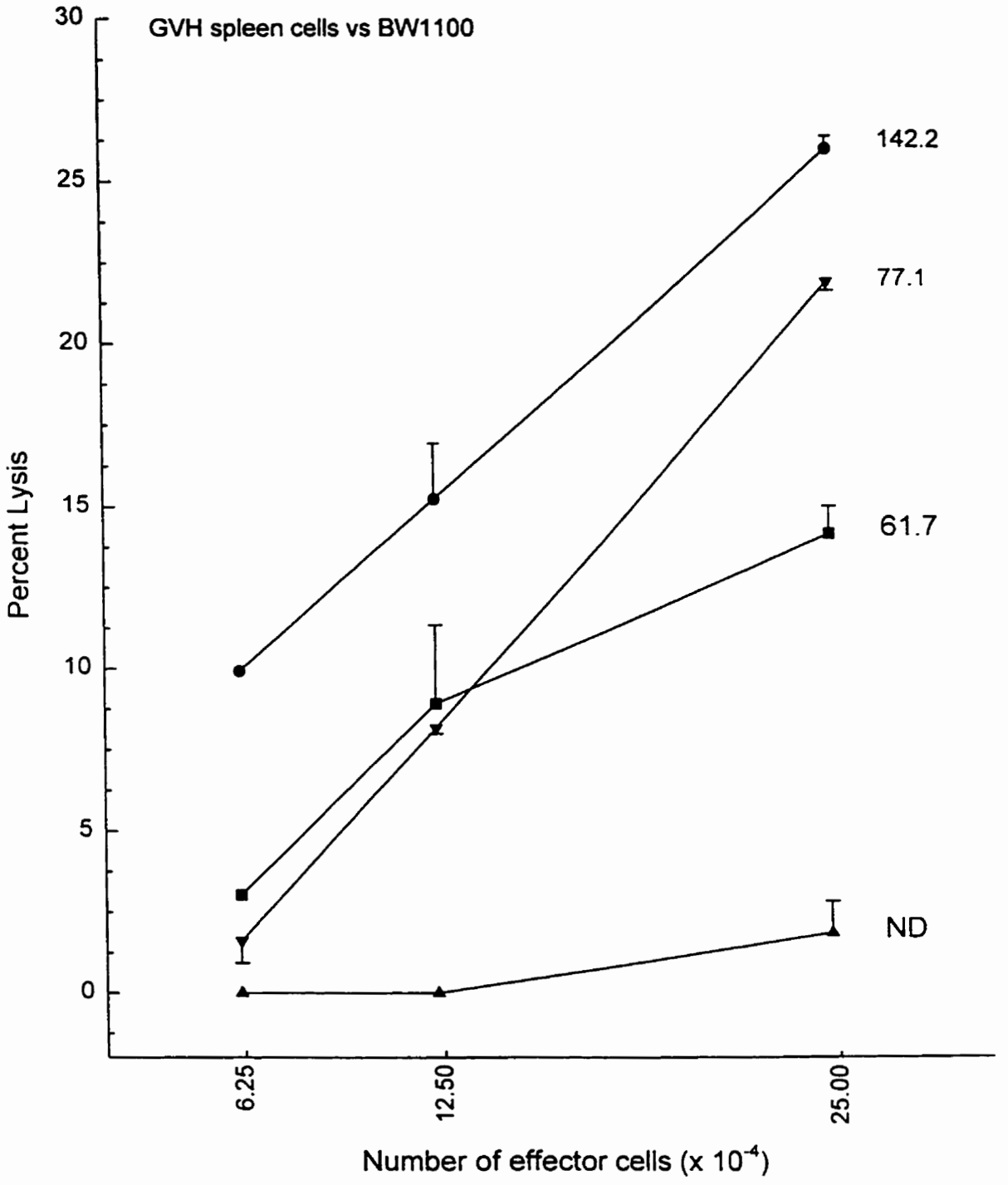

unenriched spleen cells
NK1.1-enriched spleen cells
GVH spleen cells vs BW1100
A
142.2
ND
Control spleen cells vs BW1100
B
ND
0
GVH spleen cells vs YAC-1
C
30.8
7.2
Control spleen cells vs YAC-1
D
73.6
15.8
Percent Lysis
Number of effector cells (x10-5)
1.25
2.50
5.00

Figure 4.3 Graph showing NK-like cytotoxic activity directed against BW1100 target cells (10^4 cells per well) by GVH spleen cells positively selected on the MACS for NK1.1 expression. Effector cells were prepared from mice on day 7 post-induction. The NK-like cytotoxic activity in the untreated NK1.1-enriched cell fraction (●) is compared with that observed when effector cells were treated with rabbit anti-hamster IgG and complement (C; ■), anti-γδTCR followed by rabbit anti-hamster IgG and C (▲), and anti-αßTCR followed by rabbit anti-hamster IgG and C (▼). The error bars indicate the standard error of the mean percent lysis for three replicate samples at each E:T ratio. The number beside each plot represents LU_{10} calculated from the mean percent lysis at each E:T ratio. ND beside a plot indicates that an LU_{10} could not be calculated from the dose response curve.


GVH spleen cells vs BW1100
30
25
20
15
10
5
0
Percent Lysis
142.2
77.1
61.7
ND
6.25
12.50
25.00
Number of effector cells (x 10⁻⁴)

4.4.4 Two-colour flow cytometry for co-expression of NK1.1 and γδTCR on spleen cells.

These analyses were performed on spleen cells harvested from GVH mice on day 7 post-induction. Cells were first analysed by forward and right angle light scatter to resolve two subpopulations of lymphocytes: LGL and SAL. The gates used to define these two subpopulations can be seen in the two bit maps shown in Figure 4.4. In the unenriched (pre-column) fraction (Figure 4.4.1), 35% of the cells were LGL and 65% were SAL. Following enrichment of NK1.1$^+$ cells, the proportion of LGL increased to 46% (Figure 4.4.2).

4.4.4.1 Analysis of unenriched spleen cells.

In each subpopulation we determined the percentage of cells that were NK1.1$^+$, the percentage that were γδTCR$^+$, and the percentage that expressed both markers. The pattern of analysis is shown for LGL in the histograms and accompanying table in Figure 4.5. We first determined that 95% of LGL were NK1.1$^+$ by subtracting the background staining depicted in histogram A from the percentage of cells labelled with anti-NK1.1-biotin and SA-PE (histogram B). The corresponding figure for SAL was 42% (analysis and data not shown). We next calculated the proportion of γδTCR$^+$ LGL by subtracting the background staining depicted in histogram C from the percentage of cells labelled with anti-γδTCR and rabbit anti-hamster IgG-FITC (histogram D). With this correction, we found that 56% of the LGL and were γδTCR$^+$. The corresponding figure for SAL was 14% (analysis and data not shown). Finally, we determined the proportion of LGL expressing both NK1.1 and γδTCR by subtracting the background labelling for FITC (histogram C) plus background labelling for PE (histogram A) from the percentage of LGL stained with both fluorochromes in histogram E. With this correction we found that both NK1.1 and γδTCR

Figure 4.4 Bit maps showing forward versus 90° light scatter analysis of spleen cells from GVH mice before (1) and after (2) MACS enrichment of NK1.1$^+$ cells. The gates shown delimit subpopulations of SAL (A and C) or LGL (B and D).

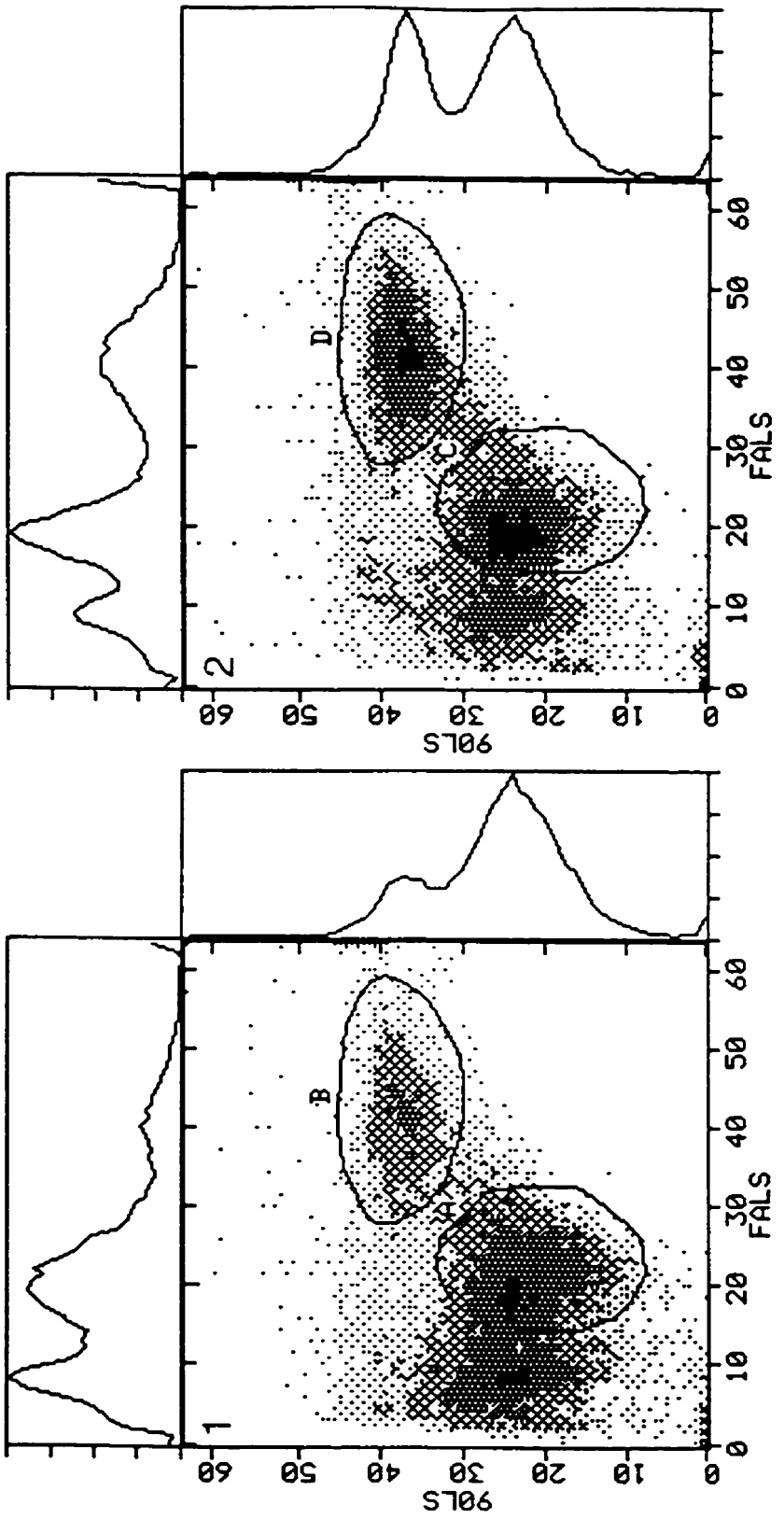
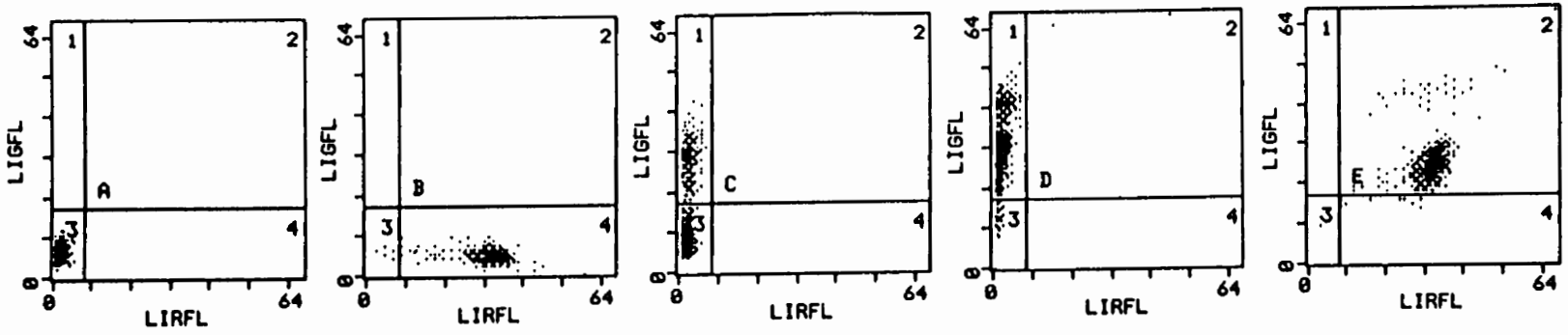

90LS
FALS
0
10
20
30
40
50
60
A
B
C
D
1
2

Figure 4.5 Five two-parameter histograms showing the pattern of analysis used to detect coexpression of NK1.1 and γδTCR on splenic LGL by two-colour flow cytometry. The intensities of green (FITC) and red (PE) fluorescence are shown on the vertical and horizontal axes, respectively. The table shows the sequence of incubations used to label the cells analysed in each histogram. To obtain the corrected percentage of NK1.1$^+$ cells, background staining for PE (histogram A) was subtracted from the raw percentage of cells labelled with anti-NK1.1-biotin and SA-PE (histogram B). To obtain the corrected percentage of γδTCR$^+$ cells, background staining for FITC (histogram C) was subtracted from the raw percentage of cells labelled with anti-γδTCR and rabbit anti-hamster IgG-FITC (histogram D). In histograms C and D, cells were preincubated with hamster IgG and rabbit anti-hamster IgG to control for the effects of non-specific binding. To obtain the corrected percentage of NK1.1$^+$/γδTCR$^+$ cells, background staining for both PE and FITC (histograms A and C) were subtracted from the raw percentage of cells labelled with both fluorochromes (histogram E). Five to 10 percent of the spleen cells from control mice were NK1.1$^+$, 0-5% were γδTCR$^+$, and cells coexpressing these two surface molecules were undetectable (data not shown).



Histogram	Incubation No.1	Incubation No. 2	Incubation No. 3	Incubation No. 4	% positive[a] (raw)	% positive[b] (corrected)
A	---	---	IgG-biotin	SA-PE	1.5	---
B	---	---	anti-NK1.1-biotin	SA-PE	96.6	95.1
C	hamster IgG	rabbit anti-hamster-IgG	---	rabbit anti-hamster-IgG-FITC	33.3	---
D	hamster IgG	rabbit anti-hamster-IgG	anti-TCRγδ	rabbit anti-hamster-IgG-FITC	89.5	56.2
E	hamster IgG	rabbit anti-hamster-IgG	anti-NK1.1-biotin + anti-TCRγδ	SA-PE + rabbit anti-hamster-IgG-FITC	91.3	56.5

were present on 56.5% of the LGL. Only 4% of SAL coexpressed both NK1.1 and γδTCR (analysis not shown). All the data from this experiment are summarised in Figure 4.7A. In control mice, 5-10% of spleen cells were NK1.1$^+$, 0-5% were γδTCR$^+$, and cells coexpressing these two surface molecules were undetectable (data not shown).

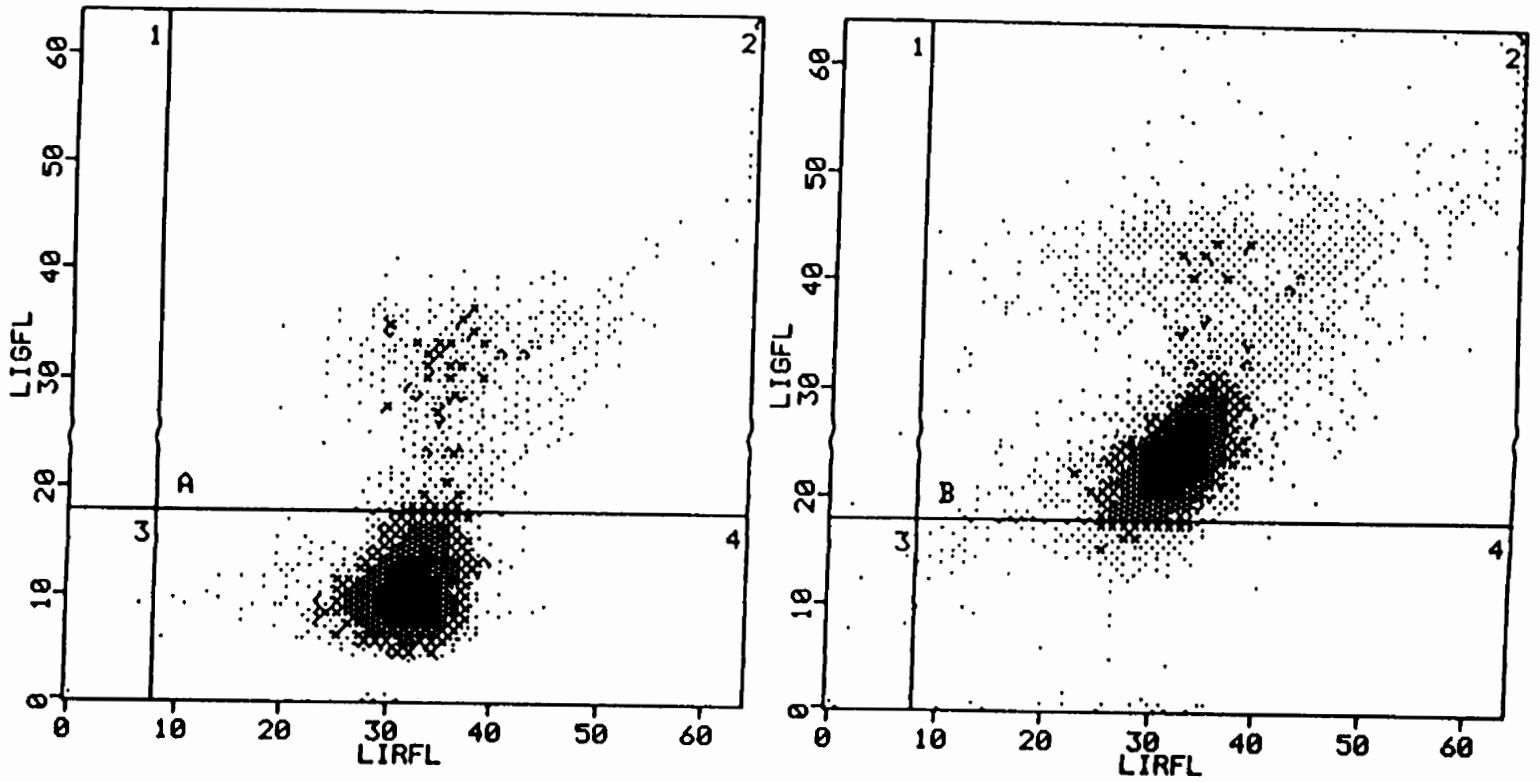
4.4.4.2 Analysis of NK1.1-enriched (bound) spleen cells.

An analysis similar to that conducted on unenriched spleen cells was performed using the MACS-enriched NK1.1$^+$ cells. Since these cells were incubated with SA-PE before enrichment on the column, all bound, NK1.1$^+$ cells were labeled with PE. In most experiments, enrichment was not absolute, and a very small percentage of bound cells (2-5% of LGL and 10-20% of SAL) were NK1.1$^-$ when analysed by flow cytometry. For the particular experiment from which data is shown (Figure 4.6), 96% of LGL and 83% of SAL in the bound fraction were NK1.1$^+$. The percentage of γδTCR$^+$ LGL was calculated by subtracting the background staining for FITC (Figure 4.6, histogram A) from the percentage of cells that was labeled with anti-γδTCR and rabbit anti-hamster IgG-FITC. With this correction, we found that 75.9% of LGL were NK1.1$^+$/γδTCR$^+$. The corresponding percentage for SAL was 6.0%. Data from this experiment are summarised in Figure 4.7B.

4.4.5 Comparison of the effect of depleting γδT cells or NK1.1$^+$ cells from donors on the mortality and weight loss associated with acute GVHD.

Results from the experiments using GL3-treated donors are shown in Figure 4.8A and 4.8B. Recipients of unmodified grafts began to die on day 18 post-transplantation. All mice in this group succumbed to the reaction by day 38. Mortality in the group that had received grafts from GL3-treated donors also began on day 18. However; by day 65, the

Figure 4.6 Two, two-parameter histograms showing the pattern of analysis used to detect expression of γδTCR on MACS-enriched, NK1.1$^+$, splenic LGL by two-colour flow cytometry. The intensities of green (FITC) and red (PE) fluorescence are shown on the vertical and horizontal axes, respectively. The table shows the sequence of incubations used to label the cells analysed in each histogram. Spleen cells were incubated with anti-NK1.1-biotin, SA-microbeads and SA-PE and then positively selected on MACS. Analysis of the NK1.1-enriched (bound) fraction revealed that 98% of the LGL were NK1.1$^+$ (data not shown). To obtain the corrected percentage of NK1.1$^+$/γδTCR$^+$ cells, the background labelling for FITC (histogram A) was subtracted from the raw percentage of cells labeled with both fluorochromes (histogram B).



Histogram	Incubation No.1	Incubation No. 2	Incubation No. 3	Incubation No. 4	% positive[a] (raw)	% positive[b] (corrected)
A	hamster IgG	rabbit anti-hamster-IgG	anti-NK1.1-biotin + ---	SA-PE + rabbit anti-hamster-IgG-FITC	20.0	---
B	hamster IgG	rabbit anti-hamster-IgG	anti-NK1.1-biotin + anti-TCRγδ	SA-PE + rabbit anti-hamster-IgG-FITC	95.9	75.9

Figure 4.7 Bar graphs comparing the percentage of NK1.1$^+$, γδTCR$^+$, and NK1.1$^+$/γδTCR$^+$ SAL (hatched bars), and LGL (solid bars) that were detected by two-colour flow cytometry. Analysis of unenriched (A) and NK1.1$^+$, MACS-enriched (B) spleen cells harvested from GVH mice on day 7 of the reaction are shown.

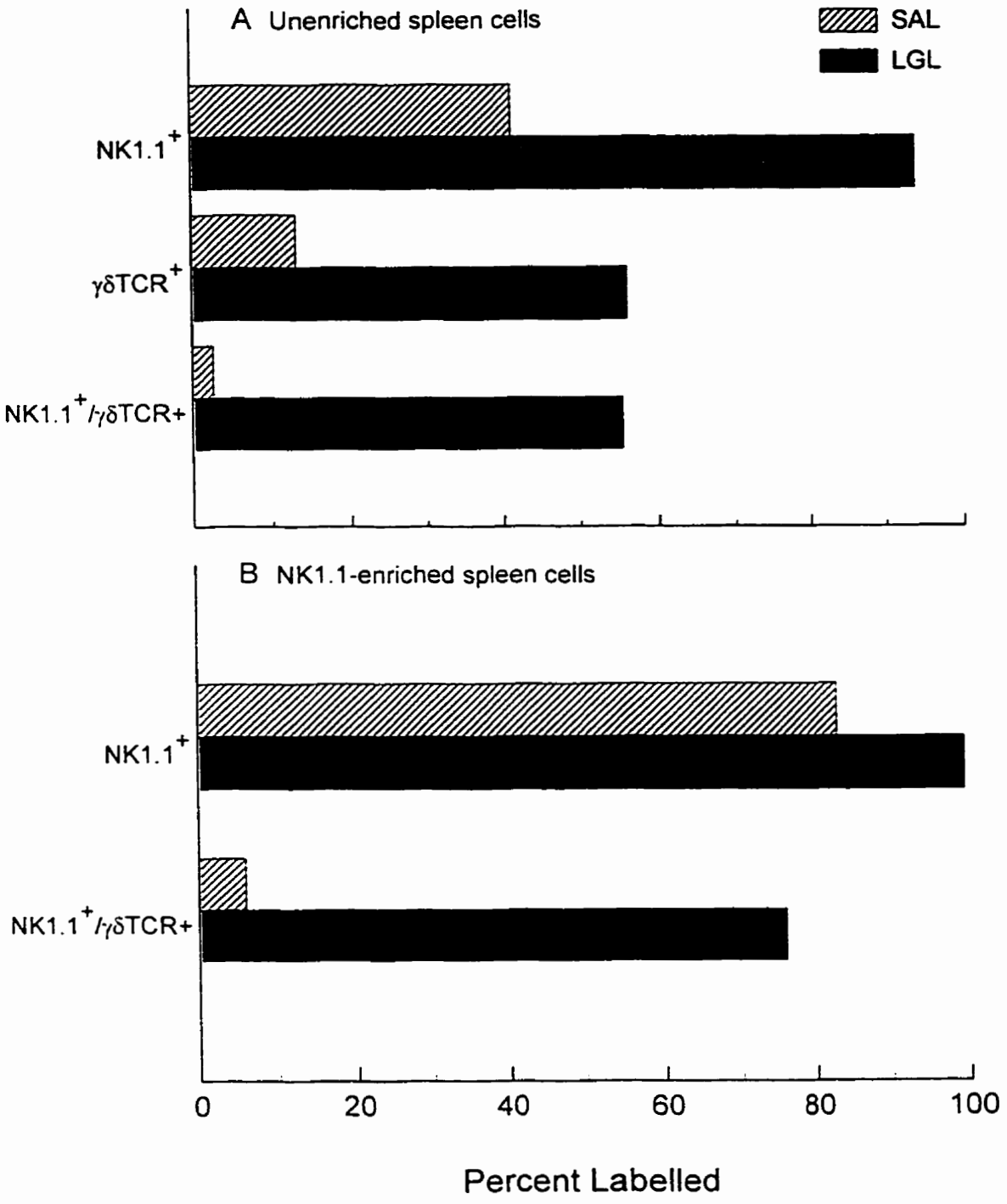
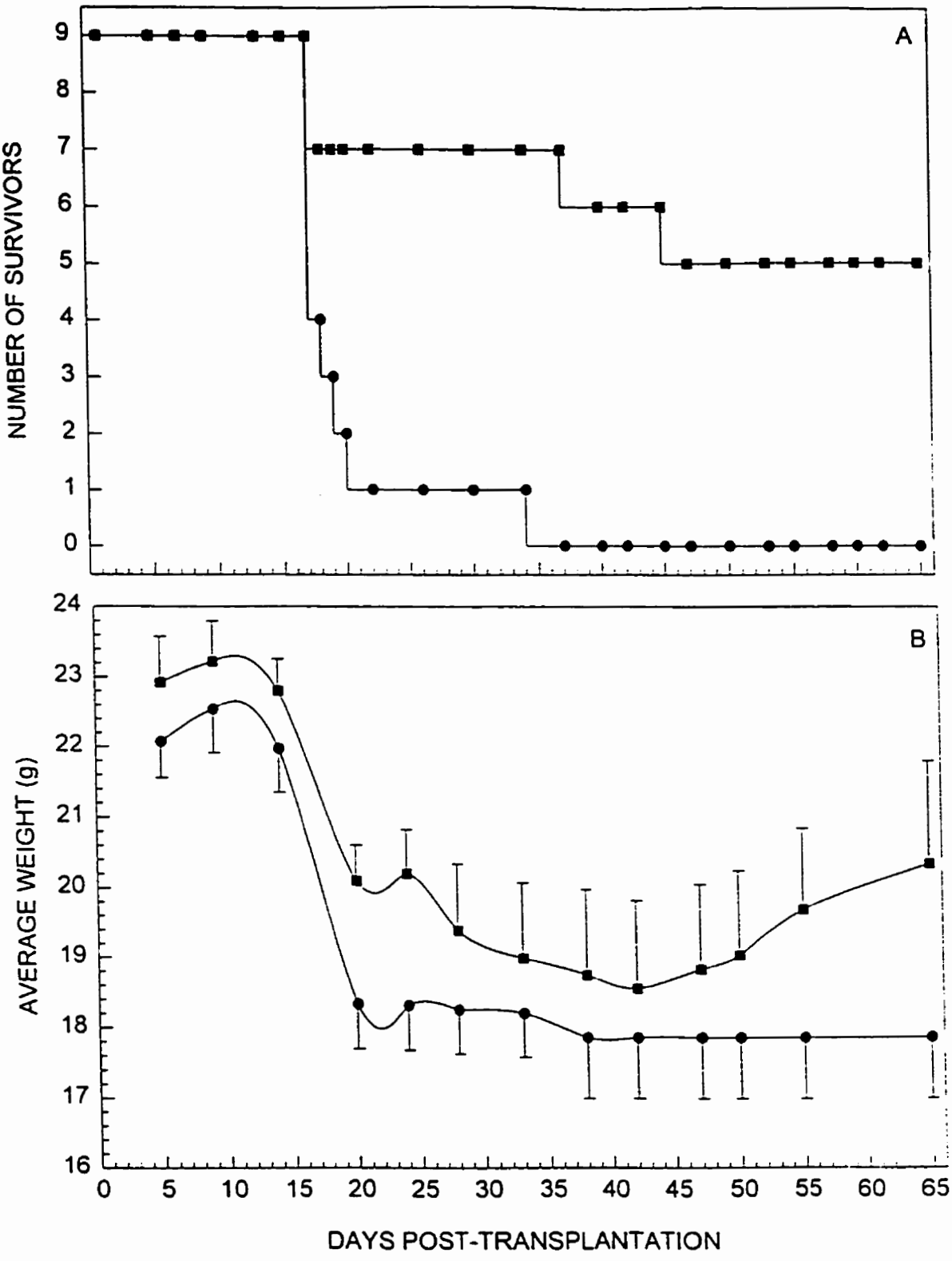

A Unenriched spleen cells
SAL
LGL
NK1.1+
γδTCR+
NK1.1+/γδTCR+
B NK1.1-enriched spleen cells
NK1.1+
NK1.1+/γδTCR+
0
20
40
60
80
100
Percent Labelled

Figure 4.8 Graphs comparing survival (A) and the pattern of weight loss (B) in mice that received grafts from untreated (●) or GL3-treated (■) donors to day 65 post-transplantation. The weights of deceased mice (phantom weights) were included in calculations of group mean body weights. On day 65, the difference in mortality between the two groups was significant ($p < 0.01$) by chi-square analysis.


A
NUMBER OF SURVIVORS
9
8
7
6
5
4
3
2
1
0
B
AVERAGE WEIGHT (g)
24
23
22
21
20
19
18
17
16
0
5
10
15
20
25
30
35
40
45
50
55
60
65
DAYS POST-TRANSPLANTATION

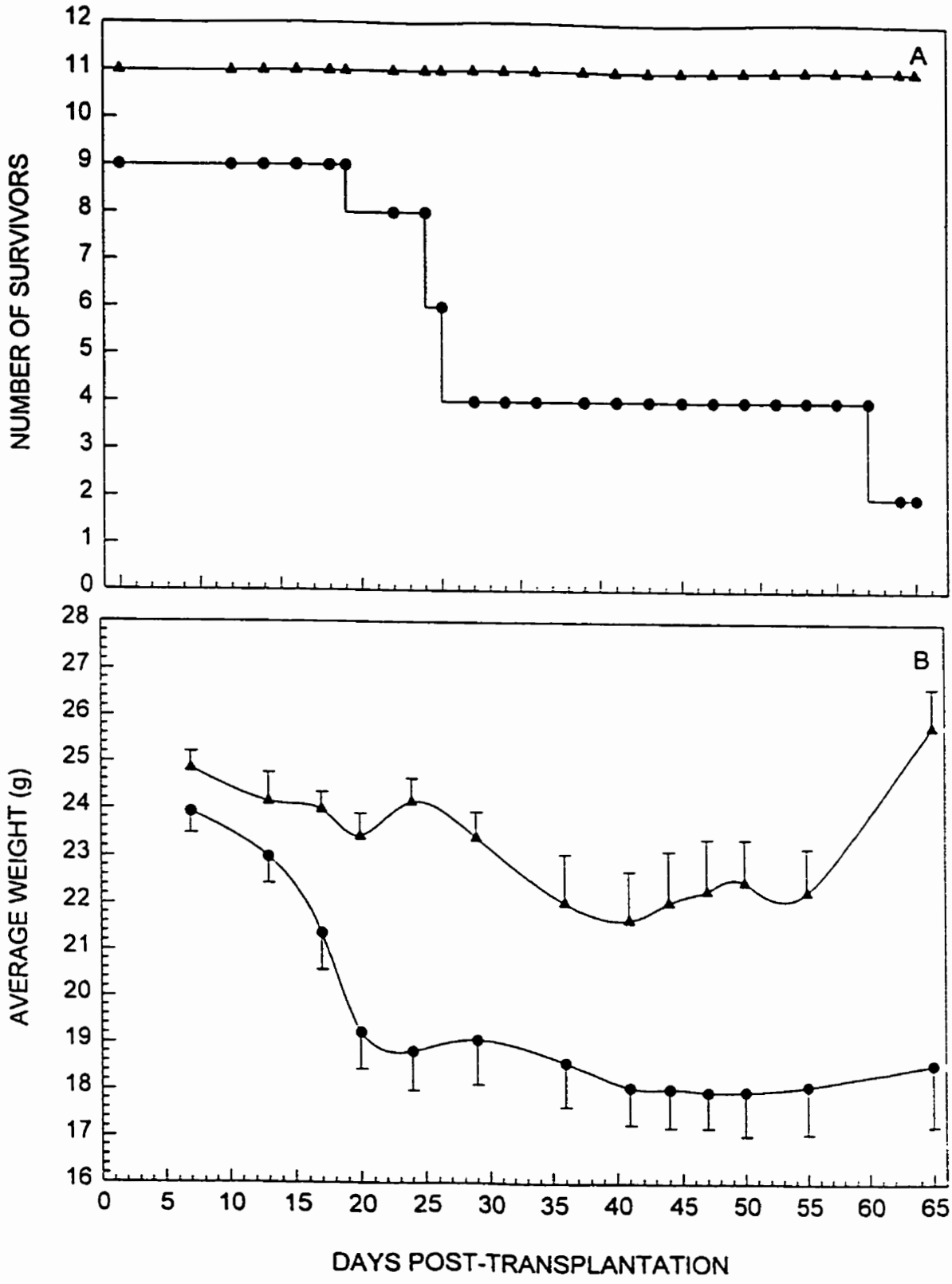
last day of the experiment, 56% (5 out of 9) of the recipients were still alive. Mice that had received grafts from untreated donors began to lose weight rapidly during the second week of the reaction. By day 20, the mean body weight of this group had decreased by 20% and changed very little for the duration of the experiment. The weight of mice transplanted with grafts from anti-γδTCR treated donors also decreased, but began to recover on day 45 and continued to do so until day 65.

The percentage of γδTCR$^+$ cells in grafts harvested from anti-γδTCR treated or untreated donors was determined by subtracting the percentage of cells labelled with hamster IgG and goat anti-hamster IgG-FITC from the percentage labelled with anti-γδTCR mAb and goat anti-hamster IgG-FITC. We found that 2-3% of the cells in grafts from untreated donor mice were γδTCR$^+$, and that only 0.4-1% of the cells expressed γδTCR when donors had been treated with GL3 ascites fluid (data not shown).

Figure 4.9A shows that 7 out of 9 of the mice that received grafts from untreated donors died by day 65 of the reaction, whereas none of the recipients of transplants from poly I:C/anti-NK1.1-treated donors died. Figure 4.9B shows that recipients of grafts from untreated donors began to lose weight rapidly during the second week of the reaction. By day 20 post-transplantation, the group mean body weight had decreased by 20% and remained near this level for the duration of the experiment. Mice that received grafts from poly I:C/anti-NK1.1-treated donors experienced a transient 10% reduction in their group mean body weight, but later recovered to and exceeded their original weight.

Cytotoxicity assays were used to determine the extent to which *in vivo* depletion of donor mice had eliminated NK1.1$^+$ cells from the graft. Poly I:C treatment increased the level of YAC-1-directed lysis from 24 to 48 LU_{10}. This activity was reduced to 0 LU_{10} in grafts harvested from donors treated with PK136 ascites fluid. Similarly, BW1100-directed

Figure 4.9 Graphs comparing survival (A) and the pattern of weight loss (B) in mice that received grafts from untreated (●) or poly I:C/NK1.1-treated donors (▲) to day 65 post-transplantation. The weights of deceased mice (phantom weights) were included in calculations of group mean body weights. On day 65, the difference in mortality between the two groups was significant ($p < 0.01$) by chi-square analysis. The level of YAC-1-directed lysis increased from 24 to 48 LU_{10} following poly I:C stimulation. This was reduced to 0 following the *in vivo* depletion procedure (data not shown).


A
B
NUMBER OF SURVIVORS
AVERAGE WEIGHT (g)
DAYS POST-TRANSPLANTATION
0
1
2
3
4
5
6
7
8
9
10
11
12
16
17
18
19
20
21
22
23
24
25
26
27
28
0
5
10
15
20
25
30
35
40
45
50
55
60
65

lysis increased from a level so low that an LU_{10} could not be calculated to 9.5 LU_{10}, following poly I:C stimulation and was then reduced to 0 LU_{10} by PK136 treatment (data not shown).

4.5 Discussion

These results provide evidence that the "NK-like" activity that develops in mice with acute GVH reactions is mediated by non-MHC restricted cytotoxic cells that bear the γδT cell receptor. Although the number of $\gamma\delta TCR^+$ cells present in the spleens of normal mice is low, our data show that they increase during the development of GVHD. Our immunophenotyping and flow cytometry data also indicate that a significant proportion of LGL in the spleens of GVH mice express both γδTCR and NK1.1. The elimination of γδT cells from donor mice significantly reduced the lethality of this disease, indicating that these cells are involved in its pathogenesis.

Because coexpression of NK1.1 on $\gamma\delta TCR^+$ cells was not observed on LGL in the spleens of normal mice and appeared only during the GVH reaction, we postulate $CD4^-/CD8^-/NK1.1^-/ASGM_1^+/\gamma\delta TCR^+$ cells in the graft migrate to the spleen and lymph nodes during the development of GVHD. Here, under the influence of cytokines released by allogeneically stimulated T cells, they become $NK1.1^+$, non-MHC restricted, cytotoxic cells which have the ability to lyse target cells such as BW1100, which are normally insensitive to lysis by conventional NK cells. It is possible that the cytokines that confer NK-like function on these cells also induce expression of NK1.1 on the cell surface. The cytokines involved in this process remain unknown, but two likely candidates are IL-2 and IFN. Both are released during the allogeneic phase of the reaction, and the secretion of IFNαß is closely associated with the activation of "NK-like" activity (209). It has also been

demonstrated that freshly isolated γδTCR$^+$ cells are not normally cytotoxic, but acquire the ability to lyse both NK-sensitive and NK-resistant targets when stimulated with IL-2 (411).

It is well known that some γδTCR$^+$ cells resemble LGL (411;432) and that some share the CD4$^-$/CD8$^-$ phenotype exhibited by GVH-activated NK-like cells (411;432;433). Cells with this phenotype can mediate non-MHC-restricted lysis of YAC-1 target cells when stimulated by cytokines (360;411). In one study, Koyasu (411) showed that cultivation of either CD4$^-$/CD8$^-$/γδTCR$^+$ or CD4$^-$/CD8$^-$/αßTCR$^+$ cells with IL-2 results in the transformation of these cells into LGL with NK function, as denoted by their ability to lyse YAC-1 cells. In contrast to the CD4$^-$/CD8$^-$/αßTCR$^+$ cells which constitutively express NK1.1, CD16 and FcεRIγ, the CD4$^-$/CD8$^-$/γδTCR$^+$ cells express these markers only after they have been cultured in the presence of IL-2. In Koyasu's study, both αß- and γδTCR$^+$ LGL exhibited conventional NK activity. Our previous experiments showed that the NK-like cells arising during the development of acute GVHD can lyse both YAC-1 and BW1100 target cells, and the results reported here indicate that the cells mediating BW1100-directed lysis express γδTCR, and not αβTCR.

Our finding that *in vivo* treatment of donor mice with GL3 ascites fluid significantly reduced mortality in mice with acute GVHD indicates, to some extent, that γδTCR$^+$ cells are involved in the pathogenesis of this disease. The fact that mortality was not completely eliminated by this treatment may be explained by experiments performed by Kaufmann and colleagues (360). They showed that treatment of C57BL/6 mice with GL3 ascites fluid *in vivo* can reduce the number of brightly staining splenic γδTCR$^+$ detected by flow cytometry, but that some of these cells can re-emerge in the spleens of GL3-treated mice after *in vitro* culture for 4 days. This suggests that GL3 down-regulates γδTCR expression, but does not eliminate the cells *per se*. In our study, some γδT cells may have functionally

recovered in recipients of GL3-depleted grafts and contributed to the demise of these mice. Alternatively, it is possible that the removal of γδT cells from the graft may not be sufficient to completely prevent mortality, and that other cellular populations must also be eliminated in order for complete protection to occur.

Our finding that *in vivo* depletion of NK1.1$^+$ cells from donor mice completely prevented mortality is consistent with our previously published experiments using an *in vitro* method for purging NK1.1$^+$ cells from the graft (209). Prestimulation of donor mice with poly I:C was required in both depletion models in order to decrease mortality, but the role of this treatment in achieving protection from GVHD following NK1.1 depletion of the graft is poorly understood. Our finding that γδT cells in the spleens of GVH mice coexpress NK1.1 would suggest that poly I:C induces expression of NK1.1 on γδT cells, thereby allowing them to be purged from the graft during the NK1.1 depletion procedure. However, our attempts to demonstrate this by flow cytometry have been unsuccessful (data not shown). We postulate that γδT cells acquire NK1.1 expression during the "cytokine storm" that occurs during the early stages of the reaction in response to cytokines other than, or in addition to, those released following *in vivo* stimulation with poly I:C. If this speculation is correct, recipients of NK1.1-depleted grafts from poly I:C-stimulated donors are protected against the lethal effects of acute GVHD, despite the persistence of donor-derived γδT cells. This would suggest that γδT cells are unable to mediate the development of acute, lethal GVHD in the absence of donor-derived NK1.1 cells. We therefore hypothesize that γδT cells contribute to the pathological events that ultimately lead to the demise of mice with acute GVHD, but that other NK1.1$^+$ cells are the key mediators of this disease. The lack of complete protection seen in recipients of grafts from γδT cell-depleted donors provides further support for this idea. Exactly how γδT cells are involved remains elusive, but it has been suggested that they may mediate the epithelial cell injury commonly

seen during acute GVHD because of their tendency to migrate into epithelial compartments of the body (378).

4.6 Appendix.

All experiments were performed by the candidate. Dr. E. S. Rector provided assistance with flow cytometry analyses.

CHAPTER 5

ACUTE MURINE GVHD IN RECIPIENTS OF GRAFTS FROM TCRδ KNOCKOUT DONORS*

* This work by C.A. Ellison and J.G. Gartner is in press in the Scandinavian Journal of Immunology.

5.1 Abstract.

γδT cells have been implicated in the pathogenesis of acute graft-versus-host disease (GVHD). We therefore performed experiments to determine whether mortality from GVHD is reduced in BDF_1-hybrid mice when parental strain, TCRδ KO donors are used. We compared mortality, weight loss, IFN-γ production and cytotoxic activity in recipients of either wild-type or TCRδ KO grafts. In both groups, weight loss and mortality were identical. Elevated NK and NK-like activity was also seen in both groups. Similarly, IFN-γ levels were elevated in both groups, but recipients of TCRδ KO grafts produced twice as much as recipients of wild-type grafts. These results demonstrate that TCRδ KO grafts can induce GVHD as severe as that seen in recipients of wild-type grafts, a finding that is at odds with studies reported in the previous chapter, demonstrating reduced mortality when γδT cells are purged from donor mice. We suggest that the inconsistency may lie in the higher levels of IFN-γ seen with TCRδ KO grafts and that the protection provided by the absence of γδT cells in the graft is overwhelmed by the higher levels of IFN-γ.

5.2 Introduction.

Several recent studies have focused on identifying the role played by γδT cells in the pathogenesis of GVHD. It is known from clinical studies that the number of $\gamma\delta TCR^+$ cells increases in both the peripheral blood (378) and skin (376) of patients with GVHD, suggesting that these cells may be important. In an immunohistochemical study done in mice with acute GVHD, cells with a morphology and surface phenotype similar to γδT cells were identified in tissues known to be targeted (431). In the previous chapter, we reported our own study in an F_1-hybrid model showing that mortality can be reduced if grafts are harvested from donor mice depleted of γδT cells *in vivo* using anti-γδTCR mAb (399).

Other evidence implicating γδT cells comes from experiments performed by Blazar and colleagues, demonstrating that murine grafts containing highly purified γδT cells have the ability to induce lethal GVHD in allogeneic recipient mice (434).

In this study, we investigated the outcome of acute GVH reactions in BDF_1-hybrid recipients of grafts from C57BL/6, parental-strain donors that are deficient in γδT cells due to a mutation in the TCRδ constant region gene segment. Itohara and colleagues developed these KO mice using gene targeting in embryonic stem cells. They are devoid of T cells that react with the anti-TCRδ mAb, 3A10, and of thymocytes reactive with F536, a mAb specific for Vγ5. They do, however, contain numbers of peripheral αβ T cells and B cells that are the same as those seen in the corresponding wild-type controls (435). Our results show that recipients of grafts from γδT cell-deficient donors are not protected from acute lethal GVHD.

5.3 Experimental Design.

Grafts harvested from either TCRδ KO or wild-type, C57BL/6 donors were used to induce GVH reactions in BDF_1-hybrid mice. We monitored and compared weight loss and mortality in these two groups of recipients on several days post-induction. Interferon-γ was assayed in spleen cell cultures established from mice sacrificed on day 8 post-induction. Experiments performed previously in our laboratory have demonstrated that this is the day of reaction on which this cytokine can be detected consistently in spleen cell cultures derived from recipients of wild-type grafts (436). A 4-hr ^{51}Cr-release assay was used to measure cytotoxic activity directed against NK-sensitive, YAC-1, and NK-resistant, BW1100, target cells by splenic effector cells prepared from each group of donors and recipients.

5.4 Results.

5.4.1 Mortality and weight loss.

Figure 5.1 shows that recipients of grafts from TCRδ KO began to die on day 16 post-induction and that survival on day 100 of the reaction was 11%. The mortality rate in mice that received wild-type grafts was similar, with the first mice dying on day 18 post-induction. By day 100, 10% of the recipients in this group remained alive.

Figure 5.2 shows the mean body weights determined for recipients of grafts from either TCRδ KO or wild-type donors on several days post-induction. The means were not significantly different on any of the days shown, except for day 24 post-induction, when the mean for recipients of TCRδ KO grafts was significantly higher than that recorded for recipients of wild-type grafts (Students' t test $p<0.05$). By day 30 of the reaction, recipients of grafts from either TCRδ KO or wild-type donors had lost 15% and 16% or their original body weight, respectively.

5.4.1 IFN-γ production in spleen cell bulk cultures.

Interferon-γ levels were measured by ELISA in supernatants collected from cultures of spleen cells that had been harvested from recipients of either TCRδ KO grafts, or wild-type grafts, on day 8 of the reaction. The cultures were sampled 48 hr after they were established. Figure 5.3 shows that 63.4 IU of IFN-γ were present in cultures of spleen cells from recipients of TCRδ KO grafts, a level nearly three times that seen in recipients of wild-type grafts (21.7 IU). This difference was statistically significant (Student's t test $p<0.05$). Control cultures containing spleen cells from BDF_1-hybrid mice that did not receive grafts contained no detectable IFN-γ (data not shown).

Figure 5.1 Graph showing, on several days post-induction, the percentage of survivors in groups of mice that received grafts from either TCRδ KO (●; n=18) donors or wild-type (■; n=15) donors.

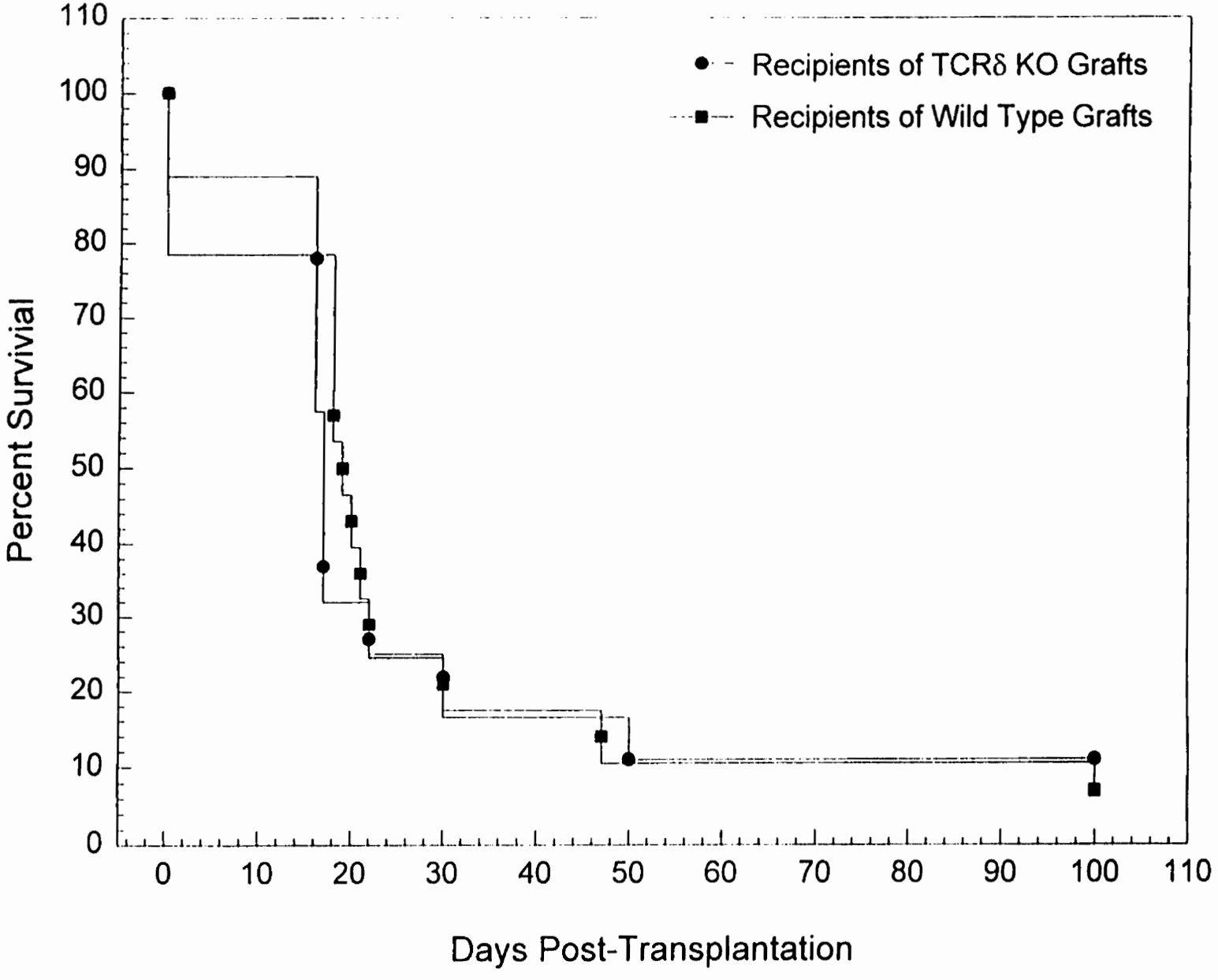

Recipients of TCRδ KO Grafts
Recipients of Wild Type Grafts
Percent Survivial
Days Post-Transplantation
0
10
20
30
40
50
60
70
80
90
100
110

Figure 5.2 Graph showing, on several days post-induction, the mean body weights determined for recipients of grafts from either TCRδ KO (●; n=9) or wild-type (■; n=14) donors.

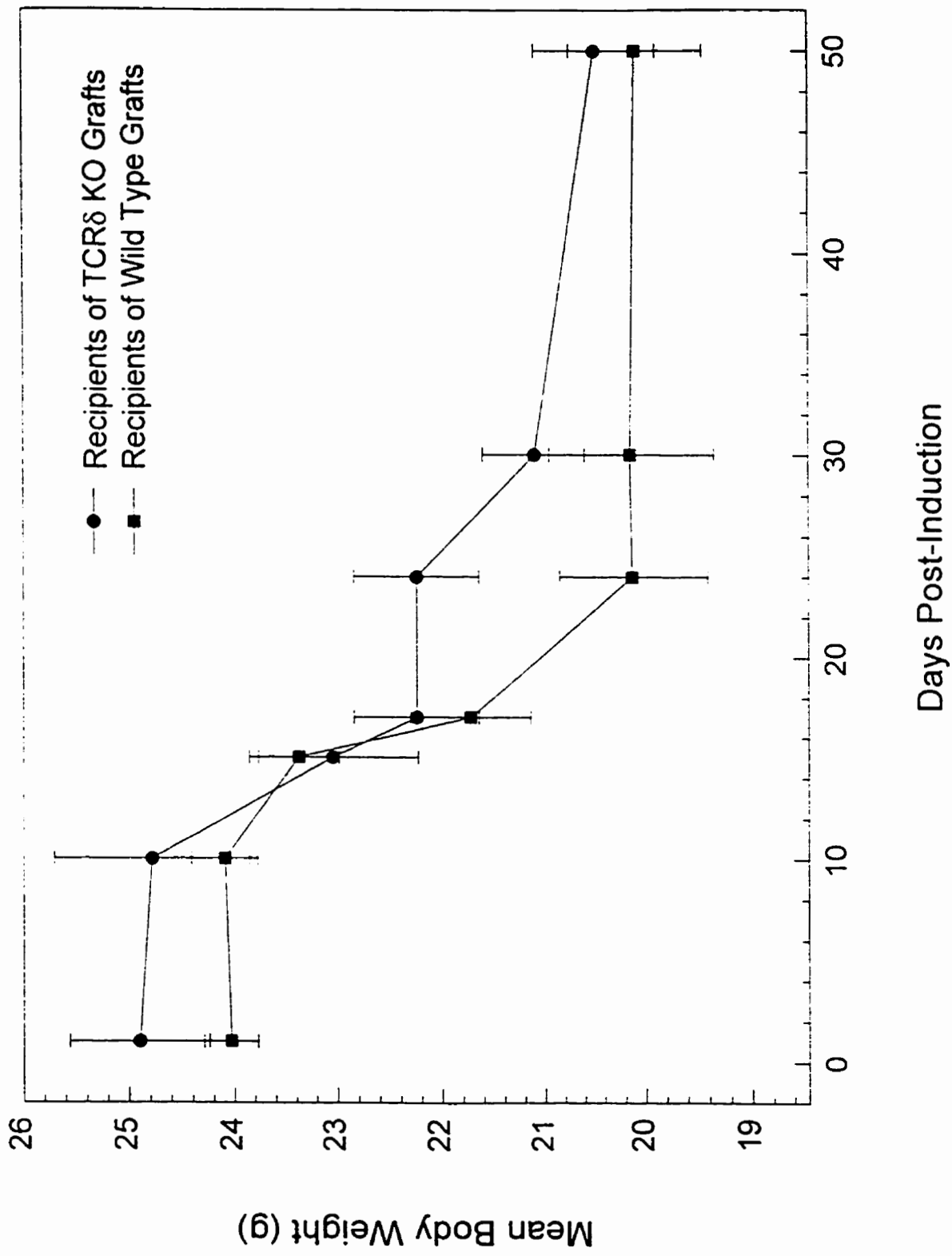
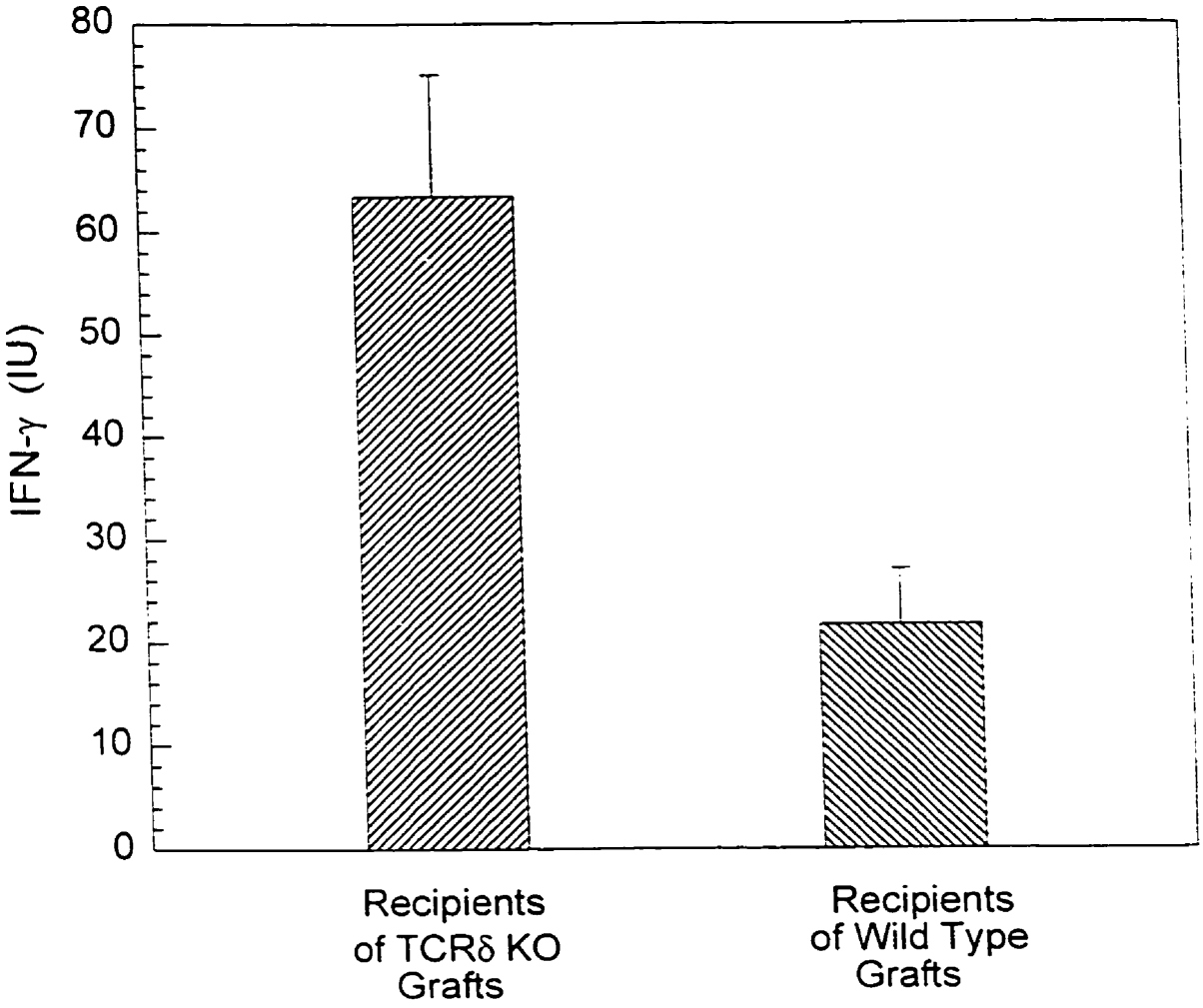

Recipients of TCRδ KO Grafts
Recipients of Wild Type Grafts
Mean Body Weight (g)
Days Post-Induction
26
25
24
23
22
21
20
19
0
10
20
30
40
50

Figure 5.3 Graph showing IFN-γ levels in supernatants from cultures of spleen cells prepared from recipients of either TCRδ KO grafts or wild-type grafts. The difference was statistically significant (Students' t test $p < 0.05$).


80
70
60
50
40
30
20
10
0
IFN-γ (IU)
Recipients of TCRδ KO Grafts
Recipients of Wild Type Grafts

5.4.3 NK cell-mediated lysis by splenic effector cells.

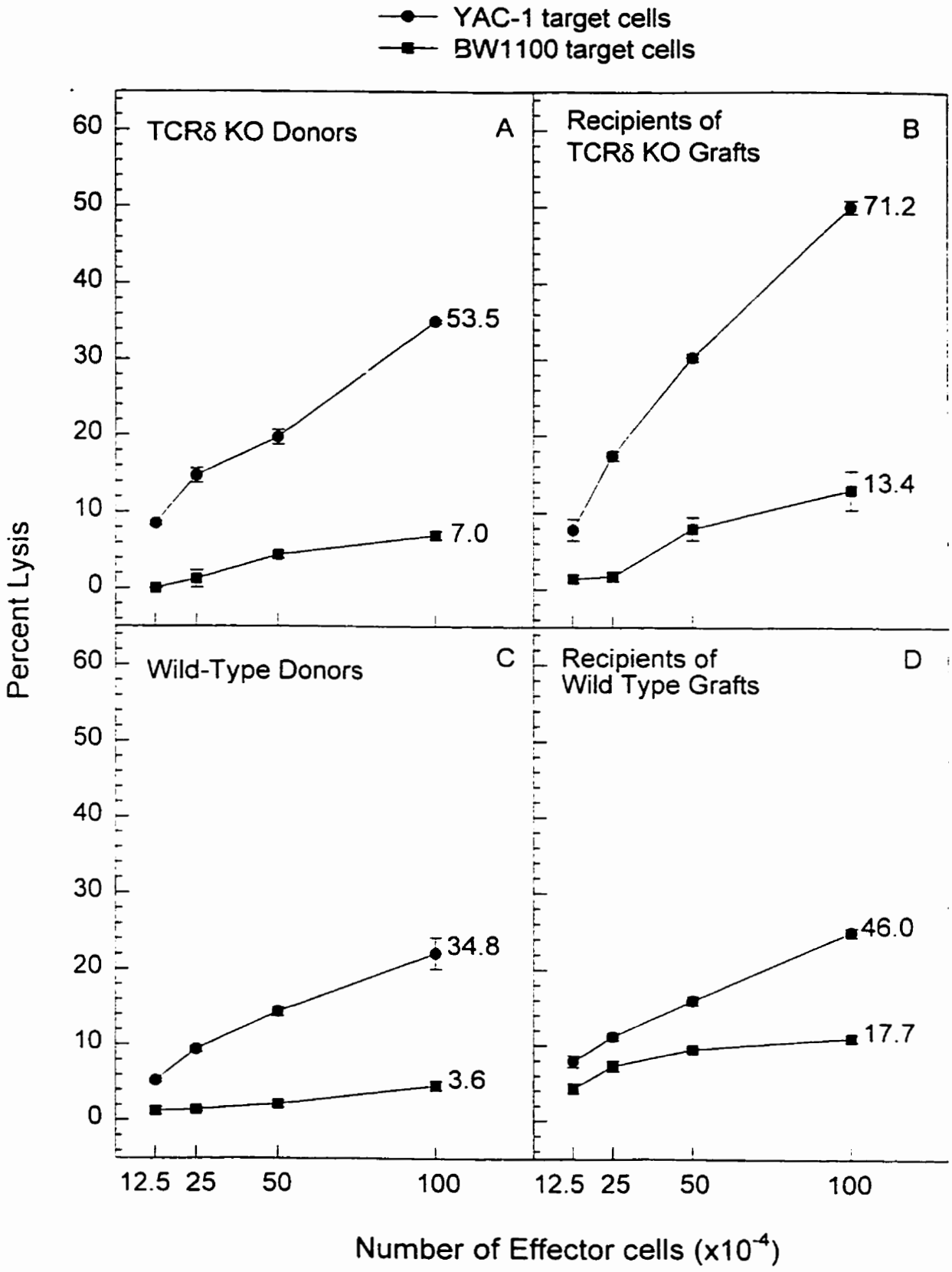
Figure 5.4 shows that the level of YAC-1-directed lysis was somewhat higher in recipients of TCRδ KO grafts than that seen in TCRδ KO donors (71.2 versus 53.5 LU) and that the level of BW1100-directed lysis was nearly two-fold higher than that seen in the same donors (13.4 versus 7.0 LU). The level of YAC-1-directed lysis in recipients of wild-type grafts was more than 5-fold higher than that seen in the wild-type donors (6.0 versus 34.8 LU), and the level of BW1100-directed lysis was elevated nearly 5-fold from 3.6 to 17.8 LU.

5.5 Discussion.

These experiments show that grafts from TCRδ KO donor mice provide no protection from acute, lethal GVHD. Mortality in both groups of recipients reached a level of 90%, and the wasting syndrome characteristic of acute GVHD developed whether or not γδT cells were present in the graft. One important difference we observed was the much higher level of IFN-γ produced by recipients of TCRδ KO grafts, compared to those that received wild-type grafts.

Several studies have implicated IFN-γ in the pathogenesis of acute GVHD. This cytokine is instrumental in the Th1-mediated immune response believed to underlie the development of acute GVHD (159;242;271;402;437). However, the cellular mechanism leading to the production of IFN-γ during GVH reactions has not been completely elucidated. Recipients of TCRδ KO grafts demonstrate IFN-γ levels well above those seen in recipients of wild-type grafts. Why these levels are 3-fold higher is unknown. Evidence from the work of other investigators has suggested that γδT cells control the function of αβT cells, and that some of this regulatory effect is lost when γδT cells are missing during

Figure 5.4 Graphs showing the levels of YAC-1- and BW1100-directed lysis in spleen cells from TCRδ KO donor mice (A), recipients of grafts from TCRδ KO donors (B), wild-type donors (C), and recipients of grafts from wild-type donors (D). Recipients were assayed on day 8 post-induction. The error bars indicate the SE of the mean percent lysis for three replicate samples at each E:T ratio. The number beside each plot represents the LU_{10} calculated from the mean percentage of lysis observed at each E:T ratio.


YAC-1 target cells
BW1100 target cells
TCRδ KO Donors
A
Recipients of
TCRδ KO Grafts
B
Wild-Type Donors
C
Recipients of
Wild Type Grafts
D
53.5
7.0
71.2
13.4
34.8
3.6
46.0
17.7
Percent Lysis
0
10
20
30
40
50
60
12.5
25
50
100
Number of Effector cells (x10-4)

development (348). It is possible that αβT cells from TCRδ KO donors may respond more vigorously during GVH reactions, secreting proportionately more IFN-γ following allogeneic stimulation. Alternatively, NK cells may take over some of the functions performed by γδT cells in their absence. Our work using IFN-γ gene KO donor mice indicates that IFN-γ plays a key role in mediating the rapidity with which mortality develops in acute GVHD. If the cytokine is absent, mice that would otherwise develop rapidly progressive, lethal, acute GVHD develop a more indolent, lymphoproliferative form of GVHD resembling a chronic GVHD-like syndrome (436). Other studies using different experimental approaches have suggested a similar role (271;402). Furthermore, experiments performed by Mowat and colleagues showed that mice with acute GVHD experienced lower levels of weight loss and mortality when treated with anti-IFN-γ, and that the cytokine was necessary for the development of GVHD-associated enteropathy (269).

Our observation that constitutive absence of γδT cells in the graft provides no protection from acute GVHD is inconsistent with our previous observation that depletion of γδT cells from donor mice by antibody treatment significantly reduces mortality (399). It is not inconceivable that the enhanced level of IFN-γ production in recipients of TCRδ KO grafts may abrogate whatever protection the absence of γδT cells from the graft might otherwise have provided. This hypothesis is supported by our observation that the IFN-γ levels present in recipients of grafts from γδT cell-depleted donors are not significantly different those seen in recipients of unmodified grafts (data not shown).

There is evidence suggesting that NK and NK-like cells play a role in the development of acute GVHD. The most convincing studies have involved the use of antibodies such as anti-NK1.1 and anti-ASGM$_1$ to eliminate NK cells from the graft, and of

grafts from Beige mice that are deficient in NK cell function (331-333;428;429). Although conventional NK activity and NK-like activity were elevated in both groups of recipients by day 8 post-induction, a proportionately higher increase in the level of NK-like activity was seen in recipients of wild-type graft. It has been suggested that NK-like cells are mediators of tissue injury in acute GVHD, and our previous study demonstrated that γδTCR cells mediate much of the donor-derived NK-like activity in recipients of wild-type grafts (209;333;399). These results indicate that cells other than γδT cells can mediate this type of cytotoxicity in recipients of TCRδ KO grafts, but that these cells do so to a lesser degree.

In summary, while previous studies have shown that elimination of γδT cells from the graft provides some measure of protection from acute lethal GVHD, we have found that no such protection occurs when grafts deficient in γδT cells are used. Recipients of TCRδ KO grafts demonstrate levels of IFN-γ production that are considerably greater than those observed in recipients of wild-type grafts, which could abrogate the partial protection that the absence of γδT cells from the graft provides. The results of this study therefore do not negate the validity of our previously reported observations, nor do they refute the idea that γδT cells play a role in the pathogenesis of acute GVHD. They do, however, underscore the caution that should be exercised when results from experiments employing gene knockout mice are interpreted.

5.6 Future directions.

A more precise description of the role played by donor-derived γδT cells in acute GVHD could be provided by further experimentation if a method was developed to ensure the complete removal of these cells from a wild-type graft. Possible methods include the MACS isolation technique and indirect complement-mediated lysis. Should the complete elimination of γδT cells from the graft be achieved, it would be possible to compare lesion

development in epithelial tissues targeted by acute GVHD in recipients of untreated and γδT cell-depleted grafts. A comparison of endotoxin levels in the serum collected from these two groups of recipients could also be performed.

5.7 Appendix.

All experiments were performed by the candidate.

CHAPTER 6

THE ROLE OF NK1.1$^+$ CELLS IN THE TH1 RESPONSE, ENDOTOXEMIA AND INTESTINAL PATHOLOGY ASSOCIATED WITH ACUTE, MURINE GVHD*

* This work by C.A. Ellison, K.T. HayGlass, J.M.M. Fischer, E.S. Rector, G.C. Macdonald and J.G. Gartner was published, in part, in Transplantation, 1998, 66:284-294.

6.1 Abstract.

Previous work has shown that GVHD-associated mortality and cachexia can be prevented in F_1-hybrid recipients if donors are stimulated with poly I:C and grafts are depleted *in vitro* of NK1.1$^+$ cells. Because it is known that NK cells can promote the development of a Th1 response, we postulated that removal of these cells from the graft might obviate this response during the development of acute GVHD. GVH reactions were induced in two groups of recipients. The first received NK1.1-depleted grafts that had been harvested from poly I:C-stimulated, C57BL/6 donors. The second received unmodified grafts. We compared IFN-γ levels, IL-10 levels, lipopolysaccharide (LPS)-induced TNFα release, intestinal pathology, endotoxemia and the levels of engraftment in the two groups. Results showed that both IFN-γ production and LPS-induced TNFα release were negligible in recipients of NK1.1-depleted grafts when high levels were seen in recipients of unmodified grafts. However, intestinal lesions containing apoptotic bodies were seen in both groups, and serum levels of endotoxin were not significantly different. Furthermore, engraftment of CD4$^+$ and CD8$^+$ cells in recipients of NK1.1-depleted grafts was equal to or greater than that observed in recipients of unmodified grafts. These results suggest that NK1.1-depletion of the graft confers protection against mortality by interfering with an immunoregulatory mechanism that results in the development of a Th1 response in GVH mice. This treatment does not abrogate GVHD-associated enteropathy or endotoxemia. It does, however, prevent macrophages from becoming primed to release TNFα when stimulated with LPS, thereby protecting recipients from the exaggerated sensitivity to endotoxin seen in mice with acute GVHD. Consequently, these mice do not succumb to the disease.

6.2 Introduction.

The function of NK cells in the pathogenesis of GVHD has been the subject of considerable investigation (209;329-333;427;428). In a previous study using a parental strain→F_1-hybrid model of acute GVHD, it was demonstrated that cachexia and mortality could be prevented if the graft was depleted of cells expressing the NK cell marker, NK1.1. To achieve this protective effect, the depletion protocol required that donors be injected with the IFN-inducer polyinosinic:polycytidylic acid (poly I:C), 18 hours before the graft was harvested (333). Several theories about where NK cells act in the mechanism of acute GVHD exist. The most widely held suggests that they serve as cytotoxic effectors. Removing these cells from the graft should therefore mitigate tissue injury, thereby protecting the host.

In this study we tested an alternative hypothesis to the "cytotoxicity theory" to explain how removal of $NK1.1^+$ cells from the graft may protect recipients from developing lethal acute GVHD. We postulated that elimination of $NK1.1^+$ cells may remove a source of IFN-γ that is instrumental in triggering the Th1 response thought to mediate the pathogenesis of acute GVHD. By preventing the production of Th1 cytokines, NK1.1 depletion should, in turn, prevent Mϕ activation and, consequently, obviate the release of TNFα and other Mϕ derived factors believed to mediate the lethal effects of acute GVHD.

We therefore performed experiments to determine whether elimination of $NK1.1^+$ cells from the graft diminishes or eliminates the production of IFN-γ seen early in the course of acute GVH reactions and whether purging also prevents the priming of macrophages (Mϕ) for LPS-induced TNFα release. We observed that removal of donor-derived $NK1.1^+$ cells from the reaction did indeed eliminate IFN-γ production in the

spleen. It also prevented the excessive LPS-induced TNFα release observed in mice with acute GVHD.

To test the hypothesis that NK cells are involved in the development of intestinal lesions during acute GVHD, we also conducted a histopathological comparison using tissue collected from recipients of NK1.1-depleted and unmodified grafts. The "exploding crypt cell" seen in the intestine is characteristic of the acute GVHD and underscores the apoptotic mechanism involved (113). We therefore tried to identify apoptotic bodies in the intestinal epithelium and measured LPS levels in sera collected from both groups of recipients. We found that endotoxin levels were not significantly different in recipients of either NK1.1-depleted or unmodified grafts, and that intestinal lesions, characterized by apoptotic bodies in the crypts, were also present in both groups of recipients. These results indicate that donor-derived NK1.1$^+$ cells are not required for the development of GVHD-associated intestinal lesions, and that other cell types must be involved.

6.3 Experimental Design.

The first set of experiments was performed to verify our previous observation that depleting NK1.1$^+$ cells from allografts of lymph node and spleen cells, *in vitro*, can prevent acute GVHD. The grafts were harvested from donors injected i.p. 18 hr earlier with poly I:C. We determined the effect of depletion on weight loss, splenomegaly and mortality. To verify our previous observation that this treatment prevents acute, lethal GVHD, we established four experimental groups: (a) recipients of untreated grafts from untreated donors, (b) recipients of NK1.1-depleted grafts from poly I:C-stimulated donors, (c) recipients of untreated grafts from poly I:C-stimulated donors, and (d) recipients of NK1.1-depleted grafts from untreated donors. Mice in groups (c) and (d) were not protected against mortality, confirming our previous observations (333). These control groups were

therefore omitted from subsequent experiments. In our pilot experiments we found that incubation of the graft with complement alone had no protective effect, so this control was also dropped from later experiments.

A second set of experiments was performed to compare the amount of IFN-γ and IL-10 in supernatants from spleen cell cultures harvested from recipients of NK1.1-depleted grafts from poly I:C-stimulated donors (hereafter referred to as recipients of NK1.1-depleted grafts), recipients of unmodified grafts from untreated donors (hereafter referred to as recipients of unmodified grafts), and untreated, BDF_1-hybrid control mice. Interferon-γ and IL-10 levels were measured by ELISA on days 4, 8, 15 and 20 post-transplantation.

When mice with acute GVHD are injected with small doses of LPS they develop very high levels of TNFα in the serum (383). A third set of experiments was therefore performed to determine whether NK1.1-depletion of the graft abrogates this response. LPS-induced TNFα release was measured in recipients of unmodified grafts, recipients of NK1.1-depleted grafts and untreated controls on days 8 and 15 post-transplantation. TNFα was measured by ELISA.

In a fourth set of experiments, we measured serum endotoxin levels in the recipients of either unmodified or NK1.1-depleted grafts, and in untreated BDF_1-hybrid control mice using an LAL assay (438). These studies were particularly difficult because each mouse had to be completely exsanguinated in order to obtain enough serum to assay, making sequential measurements impossible. We therefore had to predict the time-point at which serum LPS levels would be highest. Results from our pilot studies indicated that recipients of unmodified grafts succumbed within 6 to 18 hours following the injection of a low dose of LPS. This suggested that the "window of opportunity" in which LPS could be detected in circulation during an acute GVH reaction is very small. In a typical experiment, approximately 40% of the mortality associated with acute GVHD occurs in a

2-3 week period, beginning around day 15. However, there are no reliable signs to predict when an individual mouse is going to die. Although it is not an entirely dependable indicator that death is imminent, we decided to use severe cachexia as a sign that a mouse was becoming moribund. Recipients of unmodified grafts that had lost more than 20% of their pre-induction weight were therefore sacrificed for the determination of serum endotoxin levels. Recipients of NK1.1-depleted grafts (which do not develop cachexia) were also sampled, randomly, during the same time interval (days 15 and 45). To study the histopathology of the gut in both groups of recipients, we collected intestinal specimens (ileum) immediately after exsanguination

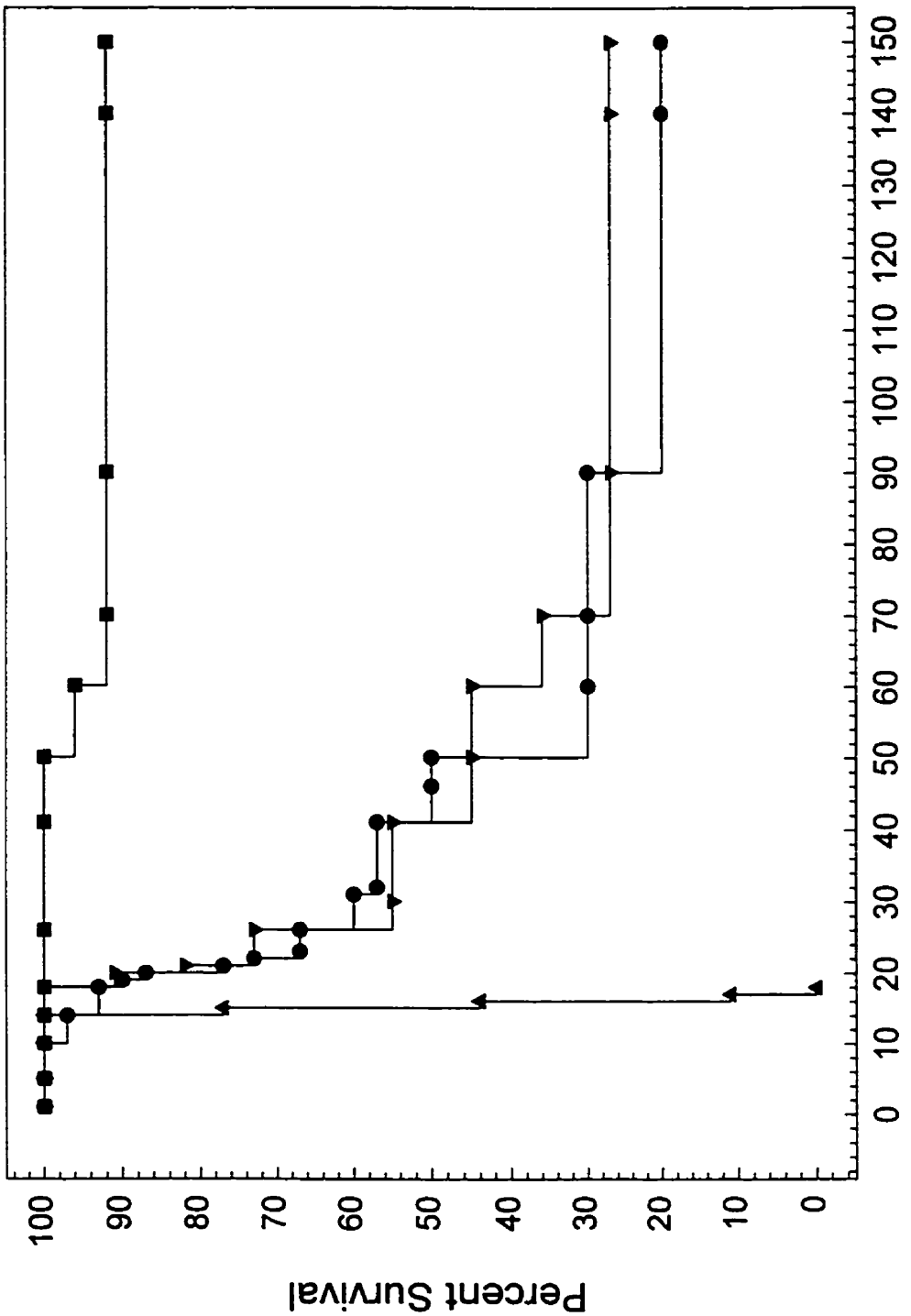
To confirm that the differences we observed in recipients of NK1.1-depleted grafts were not due simply to abortion of the graft, we conducted a fifth series of experiments in which two-colour flow cytometry analyses were used to identify donor- and host-derived cells in the spleens of recipient mice. We further determined the percentages of donor-derived CD4 and CD8 cells present in each group of recipients.

6.4 Results.

6.4.1 The effect of NK1.1-depletion on mortality, weight loss and splenomegaly.

These experiments were performed to verify the extent to which NK1.1-depletion of the graft provides protection from the mortality and weight loss that occurs in GVH mice. Our findings were similar to those reported by us previously (333). Figure 6.1 shows that mortality in recipients of unmodified grafts started on day 14 post-transplantation and reached 50% by day 46 of the reaction. All but 20% of the mice in this group died by day 150 post-transplantation when the experiment was ended. The overall survival rate in recipients of NK1.1-depleted grafts from poly I:C-stimulated donors was 92% on day 150. Data shown for these two groups was combined from five experiments.

Figure 6.1 Graph showing the effect of NK1.1 depletion of the graft on mortality from acute GVHD. Data from the following four groups are shown: recipients of untreated grafts from untreated donors (●; n=30); recipients of NK1.1-depleted grafts from poly I:C-stimulated donors (■; n=25); recipients of untreated grafts from poly I:C-stimulated donors (▲; n=11); and recipients of NK1.1-depleted grafts from untreated donors (▼; n=9). GVH reactions were induced by injecting 60 x 10^6 lymph node and spleen cells from C57BL/6 mice into (C57BL/6 x DBA/2)F_1-hybrid recipients. NK1.1 depletion of the graft was performed using anti-NK1.1 mAb (PK-136 ascites fluid) and complement. Donors treated with the IFN-inducer poly I:C were injected with 0.1 mg, 18 hr prior to sacrifice. Data shown for recipients of untreated grafts from untreated donors and recipients of NK1.1-depleted grafts from poly I:C-stimulated donors were combined from 5 separate experiments. Data from each of the two remaining groups is from one representative experiment.


Percent Survival
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
Days Post-Transplantation

The survival rate for recipients of NK1.1-depleted grafts from poly I:C-stimulated donors was 100% in two of the five experiments shown. Figure 6.1 also shows results from one representative experiment performed using two control groups. The first consisted of mice that were injected with unmodified grafts from poly I:C-stimulated donors. Mortality in this group was exacerbated with the first death being observed on day 15, and 100% of the mice dying by day 18. The second consisted of mice injected with NK1.1-depleted grafts from untreated donors. The pattern of mortality in this group was very similar to that observed for recipients of unmodified grafts from untreated donors. Incubation of the graft with complement alone also had no effect on its ability to cause lethal GVHD (data not shown).

Figure 6.2 shows that NK1.1-depletion prevents the weight loss associated with acute GVHD. On day 20, a few days after the onset of mortality, the mean body weight of recipients of unmodified grafts mice (20.61 ± 0.60 g) was 14.1% lower than that of recipients of NK1.1-depleted grafts (23.98 ± 0.20 g). This difference was found to be statistically significant by Students' t test ($p<0.001$). Spleen indices as determined on days 8, 12 and 17 post-transplantation are reported in Table 6.1. There was no significant difference between the SI for recipients of either unmodified or NK1.1-depleted grafts (Students' t test, $p>0.05$).

6.4.2 IFN-γ and IL-10 levels in bulk cultures of spleen cells.

Table 6.2 shows IFN-γ and IL-10 levels in spleen cell bulk cultures on days 4, 8 and 15 post-transplantation. Interferon-γ was detected only on day 8. The highest levels were seen in recipients of unmodified grafts (19.9 ± 3.2 U/ml). The NK1.1-depleted and control groups had day 8 IFN-γ levels of 4.0 ± 4.0 and 1.4 ± 1.4 U/ml respectively. The

Figure 6.2 Graph showing individual and mean total body weights for recipients of unmodified grafts (n=17), recipients NK1.1-depleted grafts from poly I:C-stimulated donors (n=28) and BDF_1-hybrid control mice (n=14). The means are indicated by solid horizontal lines and the SEM by broken horizontal lines. Mice were weighed daily in all experiments, but only data from day 20 are shown for comparison. The day 20 mean body weights of recipients of either unmodified or NK1.1-depleted grafts were compared using Students' t test and the difference was found to be significant ($p<0.001$). The results shown represent data combined from 4 separate experiments.

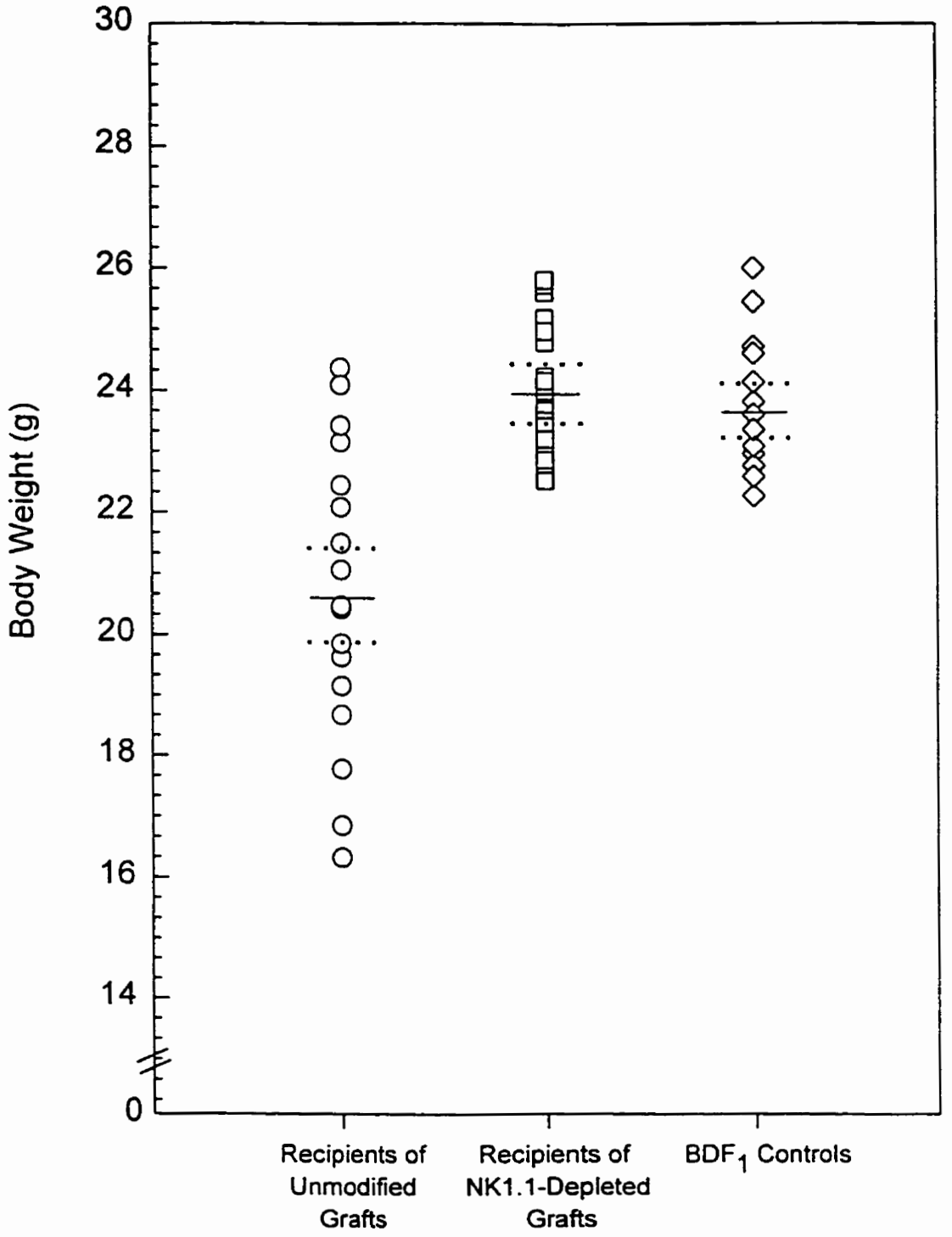

30
28
26
24
22
20
18
16
14
0
Body Weight (g)
Recipients of Unmodified Grafts
Recipients of NK1.1-Depleted Grafts
BDF1 Controls

Table 6.1 Spleen indices in recipients of unmodified[a] and NK1.1-depleted grafts[b]

Treatment Group	Day Post-Induction	Number of Mice/Day	Spleen Index[c]
Recipients of Unmodified Grafts	8	3	2.2 ± 0.1
	12	3	3.1 ± 0.5
	17	3	1.4 ± 0.3
Recipients of NK1.1-Depleted Grafts	8	3	1.7 ± 0.2
	12	3	2.9 ± 0.7
	17	3	1.6 ± 0.1

[a] This group received unmodified grafts from untreated donors.

[b] This group received grafts from donors that had been injected with poly I:C 18 h before the grafts were harvested. The grafts were depleted *in vitro* with anti-NK1.1 mAb and complement.

[c] The differences between the spleen indices for the two recipient groups were analyzed by Students' t test for each day. No significant differences ($p > 0.05$) were observed.

Table 6.2 IFN-γ and IL-10[a] levels in spleen cell cultures from BDF_1 control mice, recipients of unmodified grafts[b], and recipients of NK1.1-depleted[c] grafts on days 4, 8, 15 and 20 post-transplantation.

Cytokine	Treatment Group	Number of Mice (group/day)	Unit of Cytokine ± SEM			
			Day 4	Day 8	Day 15	Day 20
IFN-γ	BDF_1 Control Mice	5	<0.5	1.4 ± 1.4	<0.5	<0.5
	Recipients of Unmodified Grafts	5	<0.5	19.9 ±3.2[d]	<0.5	<0.5
	Recipients of NK1.1-Depleted Grafts	4	<0.5	4.0 ± 4.0[d]	<0.5	<0.5
IL-10	BDF_1 Controls	3	<0.2	<0.2	<0.2	<0.2
	Recipients of Unmodified Grafts	3	14.5 ± 1.7	<0.2	<0.2	<0.2
	Recipients of NK1.1-Depleted Grafts	3	13.0 ± 0.8	<0.2	<0.2	<0.2

[a] IFN-γ and IL-10 levels were measured by ELISA. The units shown are in U/ml. The values <0.5 and <0.2 for IFN-γ and IL-10 respectively indicate levels below which cytokines could not be measured reliably.

[b] This group received unmodified grafts from unstimulated donors.

[c] This group received grafts from donors that had been injected with poly I:C 18 hr before the grafts were harvested. The grafts were depleted with anti-NK1.1 mAb and complement. Recipients of unmodified grafts from poly I:C-stimulated donors showed IFN-γ levels of 44 ± 8.2 in d 8 spleen cell cultures.

[d] The difference between the mean U/ml of IFNγ in the two groups of recipients was statistically significant (Students' t test, $p < 0.02$).

difference between the mean U/ml of IFN-γ in recipients of either unmodified or NK1.1-depleted grafts was found to be statistically significant by Students' t test ($p < 0/02$).

Interleukin 10 was detectable only on day 4 in cultures from recipients of either unmodified (14.5 ± 1.7 U/ml) or NK1.1-depleted grafts (13.0 ± 8 U/ml). The difference in the mean U/ml of IL-10 between these two groups was not statistically significant by Students' t test ($p > 0/05$).

6.4.3 TNFα levels in sera following LPS injection.

Table 6.3 shows the amount of TNFα detected in serum collected from recipient mice that either had, or had not, been injected i.v. with LPS 90 min before sacrifice. On day 8 of the reaction, recipients of unmodified grafts that had not received LPS had only 2.9 ng/ml of TNFα in their serum. When stimulated with LPS, this level was increased approximately 13 fold to 38.5 ng/ml. On day 15 no TNFα was detectable in serum from uninjected recipients of unmodified grafts whereas 505.2 ng/ml was detected following treatment with LPS. Without LPS injection, recipients of NK1.1-depleted grafts had no detectable TNFα in their serum on either day 8 or day 15 of the reaction. The corresponding amount of TNFα measured in the serum of those injected with LPS was 3.6 ng/ml and 2.4 ng/ml. We did not detect any TNFα in serum from control mice either before or after injection with LPS (data not shown).

6.4.4 Endotoxin levels in sera.

In Table 6.4 the data was sorted into three categories, each representing a range of endotoxin units (EU) within which the data were clustered. A chi-squared analysis was then performed to determine whether any significant differences existed. Results indicate

Table 6.3 The effect of NK1.1 depletion[a] on TNFα levels in serum collected from untreated and LPS-injected[b] recipients.

Experimental[c] Group	Day Post-Transplantation	LPS	Number of Mice	TNFα[d] (ng/ml ± SEM)	p-value[e]
Recipients of Unmodified Grafts	8	-	3	2.9 ± 1.8	<0.02
		+	3	38.5 ± 9.3	
	15	-	3	<0.06	<0.001
		+	3	505.2 ± 55.5	
Recipients of NK1.1-Depleted Grafts	8	-	3	<0.06	>0.1
		+	3	3.6 ± 1.8	
	15	-	3	<0.06	<0.001
		+	3	2.4 ± 0.1	

[a] Recipients of NK1.1-depleted grafts received grafts from poly I:C-stimulated donors. Recipients of unmodified grafts received grafts from unstimulated donors.

[b] Mice were injected with 10 μg of LPS and bled after 90 min.

[c] A third group consisting of normal control mice was also included. These mice (n=6) showed no detectable TNFα in the serum (i.e. values <0.06) whether or not they had been injected with LPS.

[d] Serum TNFα levels were measured by ELISA. Values shown as <0.06 indicate a level below which cytokine could not be measured reliably.

[e] The difference between serum TNFα levels with and without LPS injection was analyzed by Students' t test.

Table 6.4 Serum endotoxin levels in recipients of either unmodified[a] or NK1.1-depleted[b] grafts and in untreated, BDF_1 control mice.

Group	No. of mice/ Group	# of mice with LPS=0 U/ml	# of mice with 0<LPS<2 U/ml	# of mice with LPS>2 U/ml
Recipients of Unmodified Grafts	21	10	2	9
Recipients of NK1.1-Depleted Grafts	26	13	6	7
Untreated BDF_1 Control Mice	9	8	1	0

[a] This group received unmodified grafts from untreated donors.

[b] This group received grafts from donors that had been injected with poly I:C 18 h before the grafts were harvested. The grafts were depleted *in vitro* with anti-NK1.1 mAb and complement.

that none were present ($p > 0.05$). They also show that endotoxin was undetectable in all but one of the BDF_1-hybrid control mice tested.

This type of statistical analysis was chosen because of the wide range of values detected in these samples (from 0 EU to values too high to accurately quantify using this assay). We postulate that this occurred because of the reason described in the Experimental Design section, namely that a mouse becoming endotoxemic would experience a rise in serum LPS levels until they became fatal. Detecting the peak level of endotoxin for a particular mouse will therefore depend on the investigator's ability to predict when a mouse is about to succumb to sepsis.

6.4.5 Intestinal pathology.

Figure 6.3 shows that apoptotic bodies characteristic of acute GVHD are visible in hematoxylin and eosin-stained sections of ileum collected from recipients of either unmodified grafts (6.3A and 6.3B), or NK1.1-depleted grafts (6.3C and 6.3D). Similar pathological changes are not present in sections of ileum collected from BDF_1-hybrid control mice that did not receive grafts (6E).

6.4.6 Engraftment of donor-derived cells.

We used flow cytometry to determine the percentage of cells in the spleen that expressed the H-$2D^d$ haplotype, and were of host origin. Cells in the recipient that did not express H-$2D^d$ were therefore considered to have come from the donor. Control experiments showed that 100% of spleen cells from BDF_1-hybrid mice expressed H-$2D^d$, whereas no H-$2D^d$-positive cells were detected in C57BL/6 donors (data not shown). Figure 6.4 shows representative flow histograms from one individual from each of the two groups of recipients on day 8 post-induction. Data comparing the rate at which donor-

Figure 6.3 Pathology of the small intestine in recipients of either unmodified (A and B) or NK1.1-depleted grafts (C and D), and of BDF_1-hybrid (E) control mice that did not receive grafts. Recipients of unmodified grafts were sacrificed on days 18 (A) and 20 (B). Recipients of NK1.1-depleted grafts were sacrificed on days 30 (C) and 35 (D).

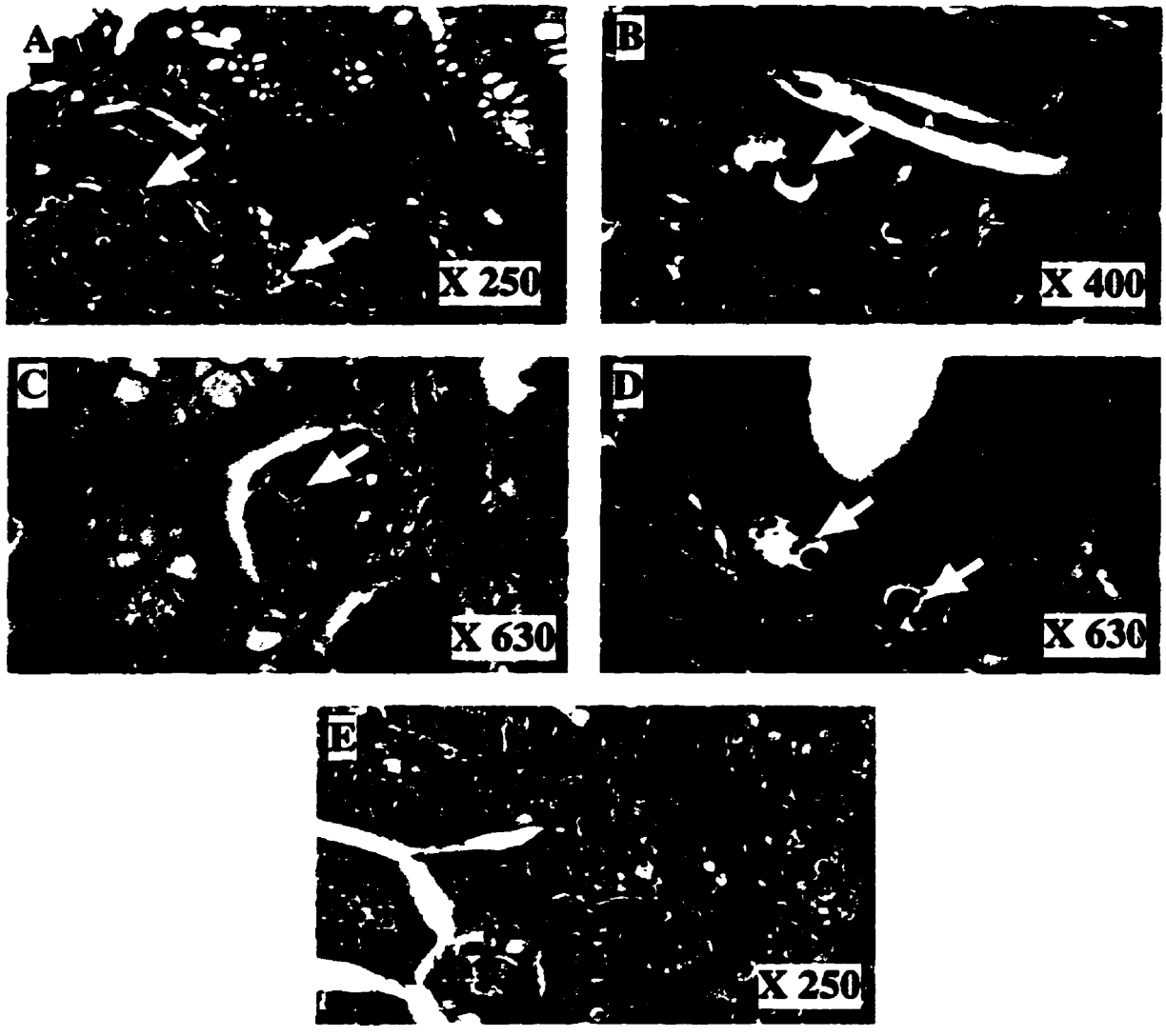
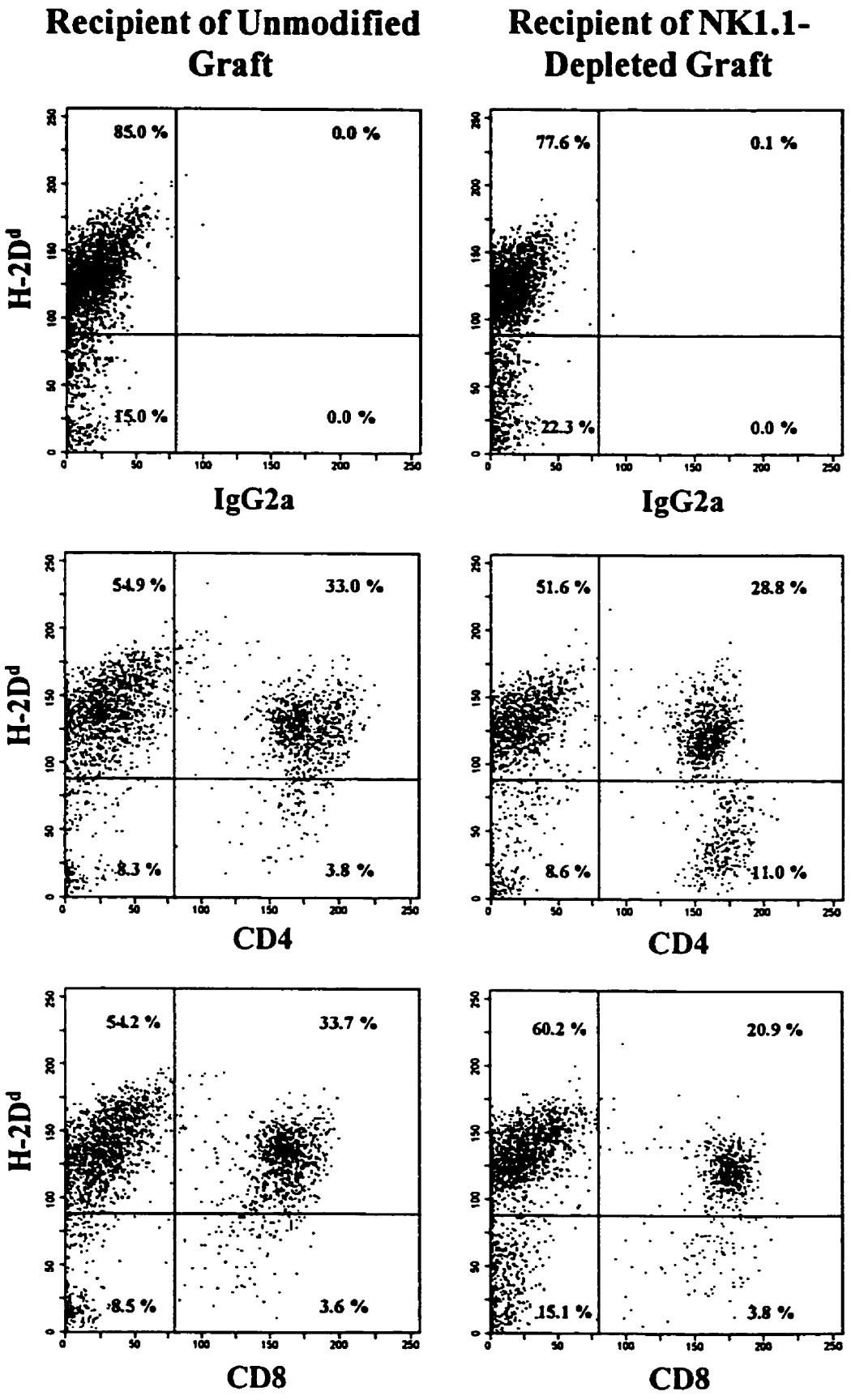

A
X 250
B
X 400
C
X 630
D
X 630
E
X 250

Figure 6.4 Six two-parameter histograms showing the pattern of analysis used to detect engraftment of donor T cells in recipient mice on day 8 post-induction. Representative data from two individual mice that received either an unmodified or NK1.1-depleted graft is shown. Intensities of red (PE) and green (FITC) fluorescence are on the horizontal and vertical axes, respectively. The two histograms in the top row show the percentage of host and donor derived non-adherent cells in the spleen appearing left upper (H-2D^d-positive) and left lower (H-2D^d-negative) quadrants, respectively. The lower 4 histograms show the proportion of CD4$^+$ and CD8$^+$ cells in the host and donor cell populations. The PE conjugated rat IgG2a used in the top two histograms served as an isotype control for the anti-CD4 and CD8. FITC-conjugated mouse IgG2a was used as an isotype control for the anti-H-2D^d. Non-specific labeling was $< 1\%$ (histograms not shown). Each histogram was drawn from 6000 gated events. The value in each quadrant indicates the percentage of gated cells appearing in that quadrant.

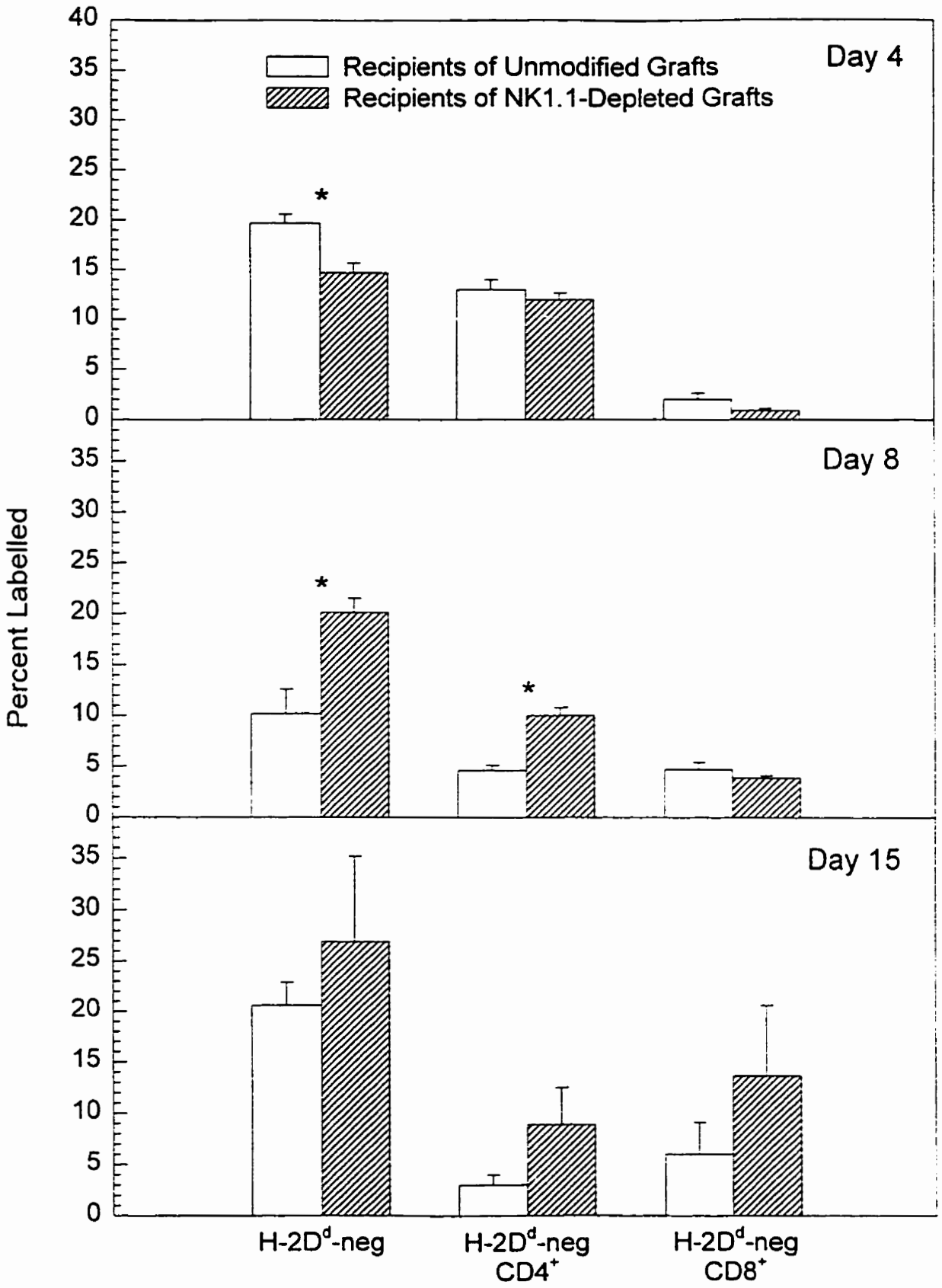

Recipient of Unmodified Graft
Recipient of NK1.1-Depleted Graft
85.0 %
0.0 %
15.0 %
0.0 %
77.6 %
0.1 %
22.3 %
0.0 %
H-2Dd
IgG2a
54.9 %
33.0 %
8.3 %
3.8 %
51.6 %
28.8 %
8.6 %
11.0 %
H-2Dd
CD4
54.2 %
33.7 %
8.5 %
3.6 %
60.2 %
20.9 %
15.1 %
3.8 %
H-2Dd
CD8

derived cells from either unmodified or NK1.1-depleted grafts populated the spleens of recipient mice are shown in Figure 6.5. On day 4 post-induction, recipients of unmodified grafts showed a greater total percentage of donor-derived cells than that seen in recipients of NK1.1-depleted grafts. However, the percentages of $CD4^+$and $CD8^+$ cells were similar. By day 8 of the reaction, both the total percentage of donor-derived cells, and the percentage of donor-derived $CD4^+$ cells in recipients of NK1.1-depleted grafts were twice that seen in recipients of unmodified grafts. The percentages of $CD8^+$ cells were not different in these two groups of mice on day 8. The percentages of total donor-derived cells and the percentages of donor-derived $CD4^+$ and $CD8^+$ cells were not significantly different in these two groups of recipients on day 15 of the reaction.

6.5 Discussion.

In this study we confirmed our previously published work demonstrating that the wasting syndrome and high mortality associated with acute GVHD in F_1-hybrid mice can be prevented if $NK1.1^+$ cells are depleted from the grafts. We also showed that IFNγ production and LPS-induced TNFα release are markedly reduced in spleen cell cultures prepared from recipients of NK1.1-depleted grafts. We modeled the latter experiment after a study by Nestel and colleagues who found that doses of LPS that are sublethal in normal mice are fatal in GVH mice (383). This exquisite susceptibility to endotoxin in GVH mice was associated with the release of large amounts of TNFα into the serum from IFN-γ-primed Mϕ. In our experiments, GVH mice behaved similarly, secreting very large amounts of TNFα in response to LPS. However, recipients of NK1.1-depleted grafts injected with the same dose of LPS were found to have serum TNFα levels very similar to those seen in controls.

Figure 6.5 Histograms showing the mean percentages of H-2D^d negative (donor-derived) cells, as seen on days 4, 8 and 15 post-induction, in spleens from recipients of either unmodified or NK1.1-depleted grafts. Also shown are the percentages of H-2D^d negative cells co-expressing either CD4 or CD8. Analyses were performed using two-colour flow cytometry. Error bars represent the SEM of the mean percentage of positively-stained cells for three mice. Statistically significant differences ($p<0.05$) are indicated (*).


Recipients of Unmodified Grafts
Recipients of NK1.1-Depleted Grafts
Day 4
Day 8
Day 15
Percent Labelled
40
35
30
25
20
15
10
5
0
*
H-2D^d-neg
H-2D^d-neg CD4$^+$
H-2D^d-neg CD8$^+$

These findings suggest that NK1.1$^+$ cells play a critical role in promoting the Th1 response underlying the pathogenesis of acute GVHD. They further suggest that the protective effect conferred on the recipient by removing these cells from the graft results from interference with the cytokine cascade mediating this type of immune response. From our data we cannot determine whether the reduction in IFN-γ was due directly to elimination of an NK1.1$^+$, IFN-γ-producing cell in the graft, but there is evidence that NK cells can produce this cytokine (244;245). As attractive as this idea may be, it is equally likely, if not more likely, that the IFN-γ was produced by alloresponsive CD4$^+$ Th1 cells. Nonetheless, our results indicate that NK1.1$^+$ cells are associated with its production in the pathogenesis of acute GVHD.

It has been suggested that cytokines produced during the initial stages of an immune response commit CD4$^+$ Th0 cells to either a Th1 or Th2 response. For example, IFN-γ and IL-12 promote a Th1 response, whereas IL-4 elicits a Th2-like response (187;236;249;439-441). One hypothesis suggests that IFN-γ released from NK cells after initial contact with antigen drives the development of a Th1-mediated immune response (323). Evidence supporting this idea comes from studies showing that NK cells respond to infection with a number of protozoa, bacteria and viruses by synthesizing and releasing IFN-γ very early in the course of the infection, before an antigen-specific Th1-response has developed (442;443).(243;321-323) In two separate studies, NK cell-derived IFN-γ was also found to be the main factor governing resistance to *Leishmania Major* in mice. It was therefore suggested that NK-cell-derived IFN-γ mediates differentiation of naive CD4 T cells into Th1 cells, thereby facilitating an effective immune response to this pathogen (323;444). The specific "ligands" stimulating IFN-γ secretion by NK cells have not been identified, but in one study bacterial superantigen was suggested (445). Although the

GVHD model is considerably different, it is possible that NK cell-derived IFN-γ may also be a factor in promoting the Th1 response that drives development of acute GVHD.

Studies to detect and measure cytokines released during acute GVH have found high levels of IFN-γ, IL-1, IL-2, TNFα and IL-12, a pattern characteristic of a Th1 immune response (106;127;210;242;446). IFN-γ is believed to be involved in the pathogenesis of acute GVHD because it has the ability to activate Mϕ and enhance their production of proinflammatory cytokines such as IL-1, IL-6 and TNFα, and cytolytic molecules such as NO (447-449). Systemic Mϕ activation is characteristic of acute GVHD and many of the lethal effects of the disease are believed to be mediated by Mϕ-derived TNFα and NO (449). More specifically, TNFα has been implicated in GVHD-associated venoocclusive disease, cachexia, septic shock and disseminated intravascular coagulation (450;451). The role of NO is not as well defined but it is thought to be involved in the development of the intestinal lesions in acute GVHD (449). Macrophages may play an additional role in sustaining acute GVHD by secreting IL-12, a cytokine that potentiates IFN-γ production by NK cells, thereby establishing a positive feedback signal that can maintain and even amplify the intensity of the Th1 response (242). From this, it is obvious that any modification of the graft mitigating Mϕ activation would have a profound effect on the course of the disease.

Experiments performed by Fowler and colleagues showed that LPS-triggered TNFα release was reduced if IFN-γ production in GVH mice was prevented by the injection of polarized donor Th2 cells (271). This is similar to our finding that recipients of NK1.1-depleted do not secrete TNFα into their serum when injected with a sublethal dose of endotoxin. However, our results also indicate that NK1.1-depletion of the graft does not prevent LPS from entering circulation, which is thought to occur when the intestinal

mucosa becomes damaged during acute GVHD (113). From this, one could conclude that a recipient of an NK1.1-depleted graft can survive an episode of sepsis, whereas a mouse with GVH-associated hypersensitivity to endotoxin will succumb even if the episode is low-grade.

The transient appearance of IL-10 in day 4 spleen cell cultures from recipients of either unmodified or NK1.1-depleted grafts is of some interest. In their study of cytokine production in acute and chronic GVHD, Rus and colleagues found that IL-4 and IL-10 were the first cytokines to appear in both forms of the disease (402). IFN-γ appeared later, and only in reactions destined to develop into acute GVHD. They suggested that all GVH reactions default to the Th2 pathway and the development of chronic GVHD, unless IFN-γ is produced to initiate a Th1 response. At this time, we do not know whether recipients of NK1.1-depleted grafts develop Th2-mediated, chronic GVHD, but long-term survivors do not exhibit any obvious signs of this disease.

Our flow cytometry results indicate that NK1.1-depleted grafts are not aborted. The total number of donor-derived, non-adherent, spleen cells in recipients of NK1.1-depleted grafts was lower near the beginning of the reaction (day 4), was greater by day 8, and then decreased to a level that was not significantly different by day 15. The percentage of $CD4^+$ cells surpassed that seen in recipients of unmodified grafts on day 8, but was not different by day 15. The reason why these mice are protected against the lethal effects of acute GVHD must therefore involve interference with the mechanism through which NK cells mediate the pathogenesis of this disease. This is supported by the observation that IFN-γ was absent from spleen cells cultures in recipients of NK1.1-depleted grafts on day 8, when it is normally seen, even though the percentage of $CD4^+$ cells was higher than that seen in recipients of unmodified grafts. Since $CD4^+$ cells are known to be a primary source of IFN-γ during Th1-mediated immune responses, the absence of IFN-γ in these cultures may

very well have been due their inability to become activated and produce Th1 cytokines. If NK cells are involved in promoting a Th1 response during the early stages of a GVH reaction, it is possible that CD4 and/or CD8 cells fail to produce IFN-γ because they have not been appropriately stimulated by NK cells from the graft. Furthermore, the increased number of $CD4^+$ cells seen on day 8 may have been associated with the absence of IFN-γ, since this cytokine is known to have antiproliferative effects on T cells (99;253).

It is clear that poly I:C stimulation of the donor is necessary if NK1.1-depletion is to protect recipients from the lethal effects of GVHD (333). How poly I:C is involved is still not known, but it is conceivable that it either upregulates NK1.1 expression on cells already expressing the marker, or induces expression of NK1.1 on $NK1.1^-$ precursors, or both. In either instance, the net result would be a more thorough purging of $NK1.1^+$ cells from the graft. Because the NK1.1 marker is known to appear on different cellular populations including conventional NK cells, αβT/NK cells and γδT/NK cells, experiments performed to determine the role played by each subset during the development of this disease would be very interesting. This would allow one to prevent GVHD by removing only the relevant cells from the graft. Experiments designed to study the involvement of γδT cells have already been described in a previous chapter.

αβT/NK cells, referred to in the literature as NK1 T cells, are present in low numbers in murine spleens and lymph nodes. The TCR expressed is comprised of an invariant TCRα chain in association with polyclonal Vβ8, Vβ7 or Vβ2 chains. These cells therefore have a limited repertoire of TCR. Sixty percent of NK1 T cells express CD4, whereas the remaining 40% are $CD4^-/CD8^-$. The other markers present at intermediate-to-high levels on the surface of NK1 T cells in NK1.1-expressing strains of mice include Thy-1, CD5, CD44, CD45RB, Ly6C, NK1.1, Ly6C, 3A4, IL-2Rβ and CD69. Several lines of evidence indicate that NK1 T cells are specific for CD1 molecules encoded by a family of

MHC-like genes that are expressed in the thymus, liver, spleen and lung in a β_2-microglobulin-dependent fashion (410). Functionally, they have been reported to produce IL-4 very rapidly *in vitro* following TCR cross-linking (452). *In vivo* studies employing mice that are deficient in NK1.1$^+$ T cells have further demonstrated that IL-4 and IgE are not produced in response to an antigen that normally elicits a Th2 type of response (453;454). It has therefore been suggested that these cells are involved in initiating Th2-mediated immune responses. However, other reports indicate that they can also produce the Th1 cytokines IFN-γ and TNFβ (455-457). Very recently, it was reported that NK1.1$^+$ T cells in the liver constitutively express both IL-12 and IFN-γ receptors, and that IL-12 stimulates production of high levels of IFN-γ and perforin in these cells (458). Other *in vivo* studies support a role for IL-12 in modulating NK1 T cell function (228;459). In addition to their ability to produce immunoregulatory cytokines, NK1 T cells have been shown to induce slow, Fas-mediated lysis of cortical thymocytes, which are known to express CD1 (460). Following short-term culture with IL-2, they may upregulate CD16 expression and mediate redirected lysis through TCR or NK1.1 (411;461). Their level of YAC-1-directed lysis also increases concomitantly following exposure to IL-2 (461).

It is possible that NK1 T cells might contribute to the development of the Th1-mediated immune response that mediates the development of acute GVHD. Furthermore, their cytotoxic function supports the idea that they may mediate injury to CD1-expressing tissues such as the thymus, liver and lung. Although expression of CD1 protein has been reported in the intestinal epithelium, another study found very little CD1 mRNA in this tissue by *in situ* hybridization (461;462).

Our observation that NK1.1 depletion of the graft does not prevent LPS from entering circulation or enteropathy suggests that a non-NK cell is responsible for mediating gut lesions in GVH mice. We speculate that $\gamma\delta$T cells may perform this function.

In summary, we have shown that *in vitro* depletion of NK1.1$^+$ cells from the graft prevents the development of lethal acute GVHD in recipient mice. This protective effect is associated with the absence of IFN-γ production in spleen cell cultures derived from recipients of purged grafts. These mice do not secrete large amounts of TNFα into circulation when injected with a sublethal injection of LPS, unlike GVH mice, which develop very high serum levels when similarly challenged. The presence of endotoxin in sera and intestinal pathology in recipients of NK1.1-depleted grafts further underscores the decreased vulnerability to endotoxin seen in these recipients. We suggest that purging of NK1.1$^+$ cells from the graft prevents the development of acute GVHD by removing cells necessary for the initiation a Th1 immune response. This is sufficient to prevent mortality despite the presence of GVHD-associated enteropathy. Based on our observations, adaptation of this method for the complete prevention of clinical GVHD would probably involve stimulating the graft with poly I:C and then depleting the cells expressing the human homologue of NK1.1 (463), as well as those that mediate the development of intestinal lesions.

6.6 Future directions.

Future experiments could include those designed to measure the levels of other Th2 cytokines and autoantibodies in recipients of NK1.1-depleted grafts. Others could explore the role that NK1 T cells play in the pathogenesis of acute GVHD, and whether γδT cells mediate the GVHD-associated enteropathy seen in recipients of NK1.1-depleted grafts.

6.7 Appendix.

These experiments were designed by the candidate under the supervision of Dr. Gartner. Contributions made by others include the performance of ELISA assays for IFN-γ, IL-10 (W. Stefura) and TNFα (J. Fischer), and technical assistance with the engraftment study (J. Fischer and E. Rector). Data from the LAL assays were combined from experiments performed by the candidate and R. Amadeo, a medical student trained by the candidate.

CHAPTER 7

MURINE GRAFT-VERSUS-HOST DISEASE INDUCED USING IFN-γ GENE KNOCKOUT DONORS*

* This work by C.A. Ellison, J.M.M. Fischer, K.T. HayGlass, and J.G. Gartner was published in the Journal of Immunology, 1998, 161:631-640.

7.1 Abstract.

These experiments were done to determine how the absence of donor-derived IFN-γ would influence the outcome of acute GVHD. GVH reactions were induced in BDF_1-hybrids using grafts from either IFN-γ *gko* or wild-type, C57BL/6, parental-strain donors. GVHD was equally lethal in both groups, but IFN-γ *gko* graft recipients developed a more protracted form of the disease. These mice developed an early wasting syndrome that persisted until death. IFN-γ was present in spleen cell cultures from wild-type graft recipients, but was undetectable in cultures from IFN-γ *gko* graft recipients. Both recipient groups showed macrophage priming for LPS-induced TNFα release. Engraftment of donor-derived $CD4^+$ and $CD8^+$ cells was greater in IFN-γ *gko* graft recipients. Pathologic changes in IFN-γ *gko* graft recipients were different from those typically seen in acute GVHD. The syndrome developing in IFN-γ *gko* recipients consisted of patchy alopecia, corneal dryness and clouding, and lymphocytic infiltration of the liver, salivary gland, lung, and kidney. Some of the lesions closely resembled those seen in the "sicca"/Sjogren's-like syndrome associated with chronic GVHD; however, there was no evidence of immune complex deposition in the kidney. These results indicate that IFN-γ *gko* graft recipients experience a type of GVHD with a longer duration and pathological manifestations different from those seen in wild-type graft recipients. They also suggest that IFN-γ plays a significant role in the pathogenesis of acute GVHD by increasing the rate at which mortality develops.

7.2 Introduction.

In both BMT recipients and experimental animals, acute GVHD is a rapidly progressive, unrelenting, systemic illness characterized by immunosuppression, cachexia,

and tissue injury in skin, liver, intestinal mucosa and occasionally the lung (127;139;450;464-467). The histopathology of acute GVHD is characterized by mononuclear cell infiltrates and epithelial injury in target organs. Its pathogenesis involves the development of a Th1-type, cell-mediated immune response in which IFN-γ is thought to play a prominent role (160). Chronic GVHD has a more indolent course, involves a wider range of organs, and has more diverse pathologic manifestations. The clinical presentation can resemble SLE and scleroderma, and is characterized by autoantibody formation and immune complex disease. This form of GVHD may, in part, be mediated by a Th2-type, humoral immune response (50).

It has been suggested that the balance between Th1 and Th2 cytokines in the initial stages of GVHD may be one factor determining whether the disease follows either an acute or chronic course. Recent findings indicate that all GVH reactions start out with the production of Th2 cytokines and the activation of B cells. Early events that favor the development of acute GVHD are engraftment of $CD8^+$ cells and production of IFN-γ by donor $CD4^+$ cells. Otherwise, there is no transition to acute GVHD and the disease continues to evolve into the chronic form (402). Experiments performed in our laboratory suggest that donor-derived NK cells may be instrumental in the development of acute GVHD, possibly by producing IFN-γ early in the reaction, thereby promoting a Th1 response (468). Very recent data indicate that early production of IL-12 may also be involved in this process (239).

As discussed in the previous chapter, exquisite sensitivity to endotoxin is a key feature of acute GVHD and central to understanding why it is almost always lethal. This effect was attributed to priming of macrophages by Th1 cytokines, particularly IFN-γ (271;383). While these investigations have indicated that IFN-γ serves to promote acute GVHD, other studies have shown that exogenously administered IFN-γ can mitigate some

clinical manifestations and reduce mortality associated with the disease (469). The role of IFN-γ in the pathogenesis of GVHD is therefore still equivocal. The purpose of our study was to explore the role of donor-derived IFN-γ in GVHD by using IFN-γ gene knockout (IFN-γ *gko*) donor mice.

7.3 Experimental design.

GVH reactions were induced in BDF_1-hybrid mice using grafts from either wild-type or IFN-γ *gko*, C57BL/6 donors. Weight loss, mortality and splenomegaly were compared during the course of the reaction. Splenic NK activity was compared at periodic intervals using a 4 hr ^{51}Cr-release assay. TNFα levels were measured by ELISA in sera collected from recipients that either had or had not been injected with a sublethal dose of endotoxin. Interferon-γ and IL-10 levels were also measured by ELISA in spleen cell cultures prepared from each recipient group on several days post-induction. Moribund animals in the agonal stages of GVHD were sacrificed and autopsied. Tissue samples from the skin, liver, lung, and salivary gland were collected for a histopathological study using light microscopy, and from the liver and kidney for electron microscopy. To verify that any differences seen in recipients of IFN-γ *gko* grafts were not due to a delay in or abrogation of engraftment, we compared both the overall level of engraftment, and engraftment $CD4^+$ and $CD8^+$ cells in IFN-γ *gko* and wild-type graft recipients using flow cytometry. Levels of $CD4^+$ and $CD8^+$ cells were also compared in grafts from wild-type and IFN-γ *gko* donor mice using flow cytometry.

7.4 Results.

7.4.1 Comparison of survival, weight loss, and splenomegaly.

Data comparing these indices of GVHD are shown in Figures 7.1 and 7.2. Mortality was 100% in both groups. As illustrated in Figure 7.1A, most recipients of grafts from wild-type donors died 15-20 days post-induction and all had succumbed by day 40. In contrast, the first recipient in the group receiving grafts from IFN-γ *gko* donors died on day 4 post-induction. The remaining mice died between days 50 and 90. There were no survivors beyond day 90. Weight loss data are shown in Figure 7.1B. Recipients of grafts from wild-type donors started to lose weight on day 15. The most rapid reduction occurred between days 15 and 20, corresponding to the period of greatest mortality. IFN-γ *gko* graft recipients experienced a transient episode of rapid and severe weight loss early in the course of the disease, between days 2 to 15. They then recovered, and the group mean returned to pre-induction levels by day 25. This was followed by a second period of wasting that was slower, and sustained over the remaining course of the disease. Figure 7.2 shows that splenomegaly developed in both groups, but occurred later in IFN-γ *gko* graft recipients and persisted until death.

7.4.2 Splenic NK and NK-like activity.

These results are shown in Figure 7.3. Cytotoxic activity directed against YAC-1 target cells was greater in IFN-γ *gko* graft recipients on all three days. The differences were greatest on days 4 and 8. NK-like activity, as determined by the level of BW1100-directed lysis was present in both groups of recipients. It was seen on day 4 in IFN-γ *gko* graft recipients and on day 8 in wild-type C57BL/6J graft recipients. Results from experiments performed to determine the relative contribution of host and donor cells to the NK and NK-like activity are shown in Table 7.1. These phenotyping experiments were

Figure 7.1. Graph showing the effect of using wild-type (□) and IFN-γ *gko* (■) donors on mortality and weight loss associated with acute GVHD. Error bars indicate the standard error of the mean body weights in the two treatment groups on each day.

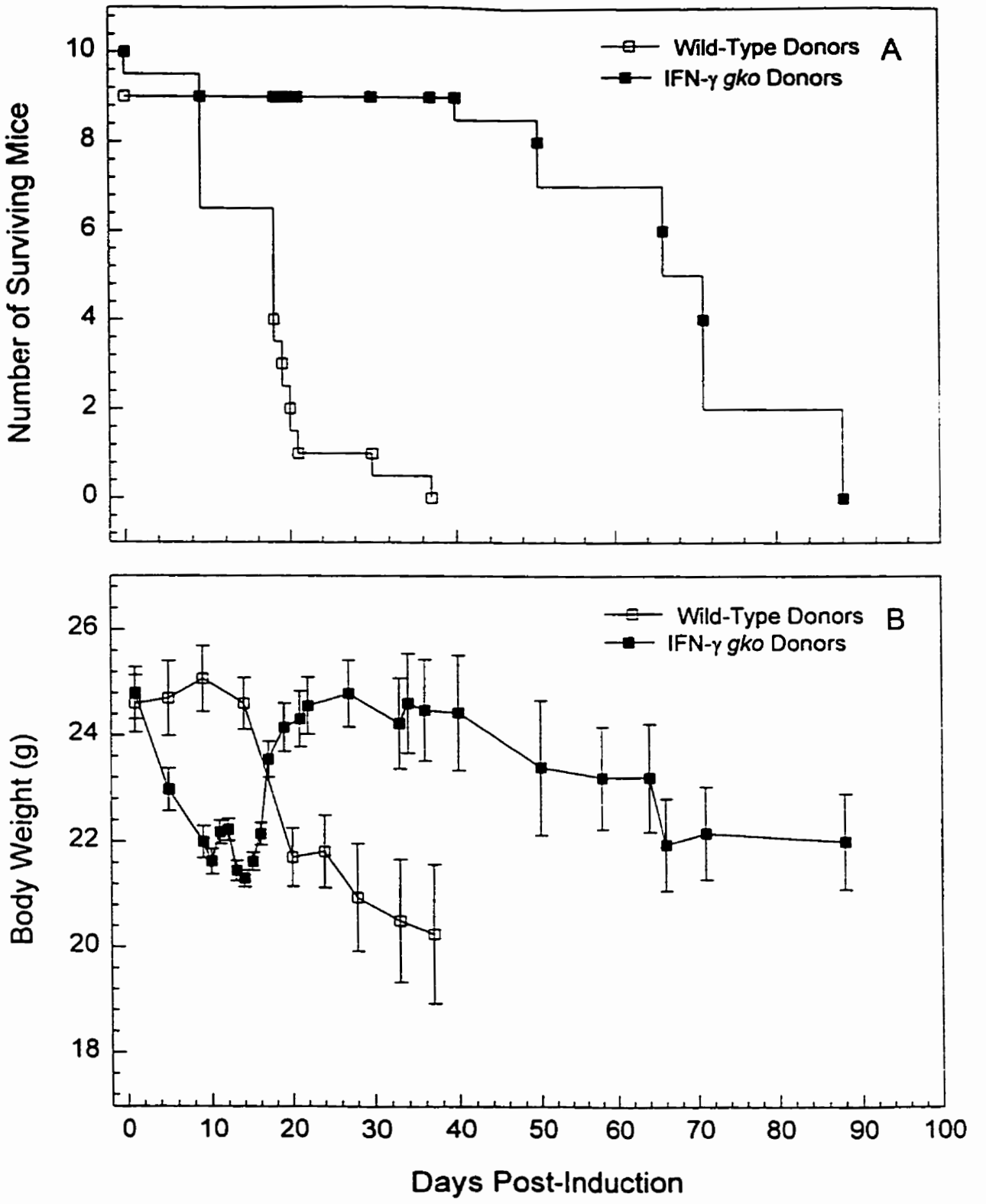

Wild-Type Donors
IFN-γ gko Donors
A
Number of Surviving Mice
0
2
4
6
8
10
Wild-Type Donors
IFN-γ gko Donors
B
Body Weight (g)
18
20
22
24
26
0
10
20
30
40
50
60
70
80
90
100
Days Post-Induction

Figure 7.2 Graph showing changes in spleen indices observed on several days post-induction in recipients of grafts from either wild-type (□) or IFN-γ *gko* (■) donors. Spleen indices for each group were calculated using the formula described in the Materials and Methods and compared using Student's t test. Error bars indicate the standard error of the mean SI obtained for 3 mice in each group, at each time point. Differences in SI between the two recipient groups were statistically significant on days 5 and 10 only ($p < 0.001$ and $p < 0.02$, respectively). No further values are reported for recipients of wild-type grafts beyond day 15, the period of maximum mortality from GVHD in this group, since no further increase in spleen weight was observed in moribund mice sacrificed after this point in the reaction.

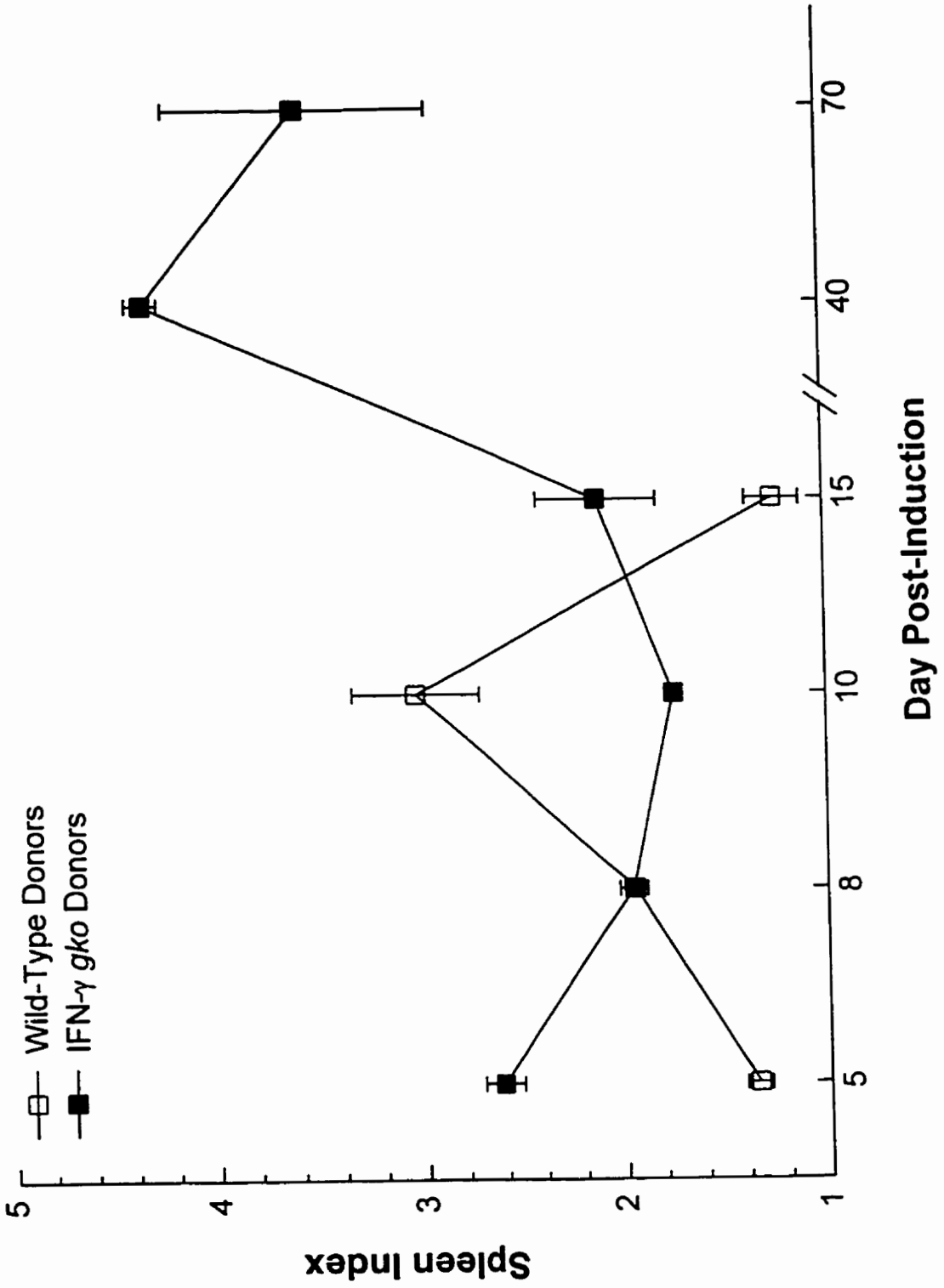

Wild-Type Donors
IFN-γ gko Donors
Spleen Index
Day Post-Induction
1
2
3
4
5
5
8
10
15
40
70

Figure 7.3 Graphs showing YAC-1- and BW1100-directed lysis in spleens from recipients of wild-type (□) or IFN-γ *gko* (■) grafts. Error bars indicate the SE of the mean percentage lysis for the three replicate samples, at each E:T ratio. The bar graphs to the right show the corresponding LU_{10} values for wild-type graft recipients (open bars), and IFN-γ *gko* graft recipients (closed bars). Dose-response curves for which an LU_{10} could not be calculated are indicated by an asterix. Control BDF_1-hybrid mice showed 23 LU_{10} of YAC-1 directed lysis. BW1100-directed lysis in these animals was too low to permit calculation of a lytic unit.

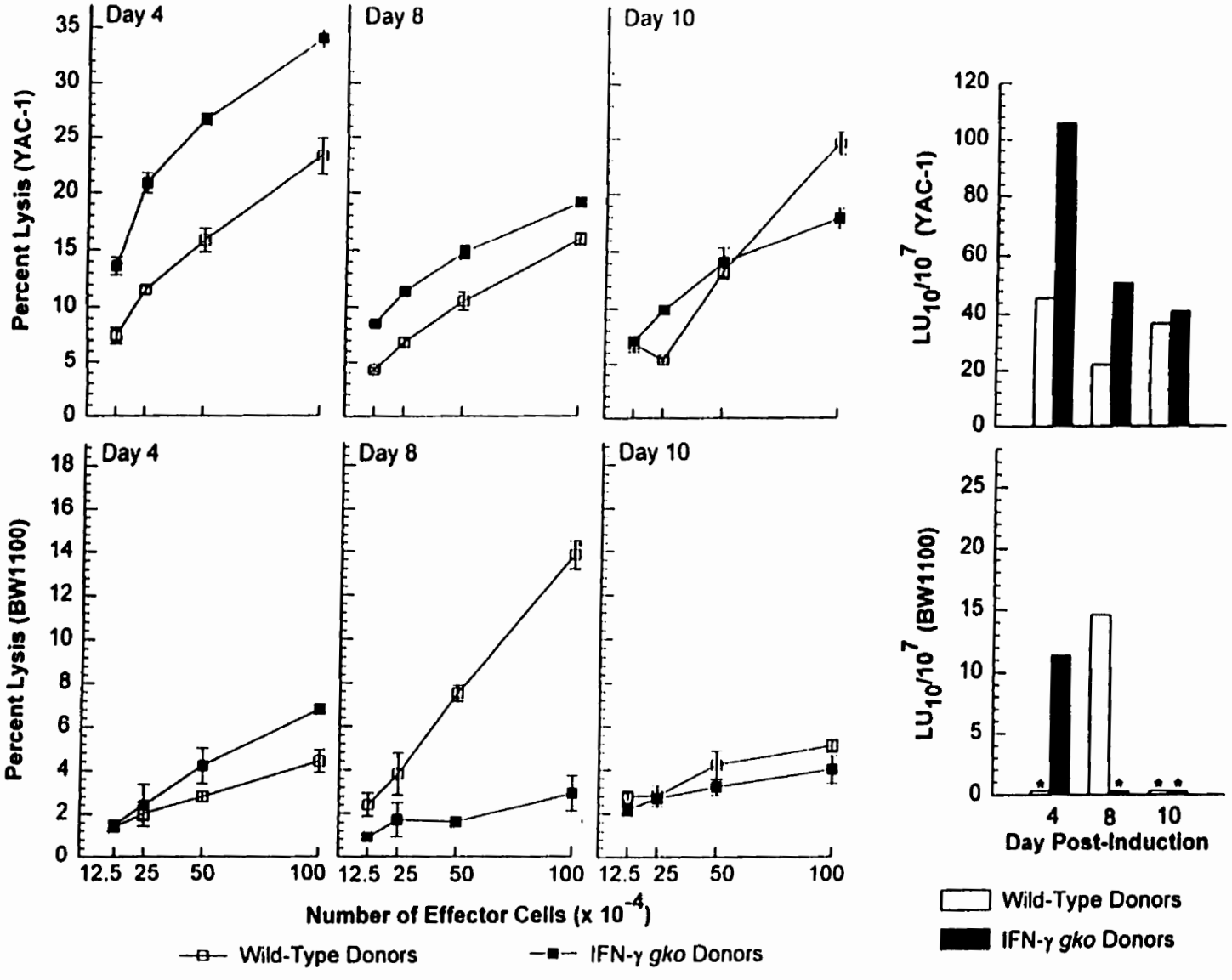

Day 4
Day 8
Day 10
Percent Lysis (YAC-1)
Percent Lysis (BW1100)
Number of Effector Cells (x 10^-4)
Wild-Type Donors
IFN-γ gko Donors
LU10/10^7 (YAC-1)
LU10/10^7 (BW1100)
Day Post-Induction
Wild-Type Donors
IFN-γ gko Donors

Table 7.1 Table showing the relative contribution of donor (H-2D^d-negative) and host (H-2D^d-positive) cells to cytolytic activity directed at YAC-1 and BW1100 target cells in the spleen of mice that received grafts from either wild-type or IFN-γ *gko* donors.

Recipient Group	Treatment of Effector Cells	YAC-1-directed lysis $LU_{10}/10^7$	BW1100-directed lysis[2] $LU_5/10^7$
Wild-Type Graft	No Treatment	63.0[a]	23.6[b]
	C Only	60.8	20.2
	Anti-H-2D^d + C	28.1	13.8
	Anti-ASGM$_1$ + C	0	0
IFN- *gko* Graft	No Treatment	105.2[a]	28.7[a]
	C Only	91.6	21.1
	anti-H-2D^d + C	30.1	5.2
	anti-ASGM$_1$ + C	0	0

[a]Performed on day 4 post-induction

[b]Performed on day 8 post-induction

performed on the days when the levels of NK and NK-like activity were highest. Approximately half the day 4 YAC-1 directed lysis in wild-type graft recipients was donor in origin whereas almost 70% of the day 8 NK-like activity was donor-derived. In IFN-γ *gko* graft recipients one third of the day 4 YAC-directed lysis and approximately 25% of the BW1100-directed cytotoxicity could be attributed to donor cells. The data indicate a comparatively smaller contribution to both NK and NK-like activity by donor-derived cells in the latter group of recipients.

7.4.3 IFN-γ and IL-10 production by spleen cell cultures.

Table 7.2 shows the amount of IFN-γ and IL-10 produced in spleen cell culture supernatants from wild-type and IFN-γ *gko* graft recipients. The measurements were made on days 4, 8, and 15 post-induction. Culture supernatants derived from recipients of wild-type grafts contained 21.6 U/ml of IFN-γ on day 8 post-induction. Interferon-γ was undetectable in cultures from IFN-γ *gko* graft recipients on all of the days assayed, and in cultures from normal BDF_1-hybrid control mice. Interleukin-10 was observed in both groups of recipients on day 4 only. The level seen in recipients of wild-type grafts was significantly higher than that seen in recipients of IFN-γ *gko* grafts (Student's t test $p < 0.02$). Interleukin-10 was undetectable in controls.

7.4.4 Serum levels of TNFα following injection of endotoxin.

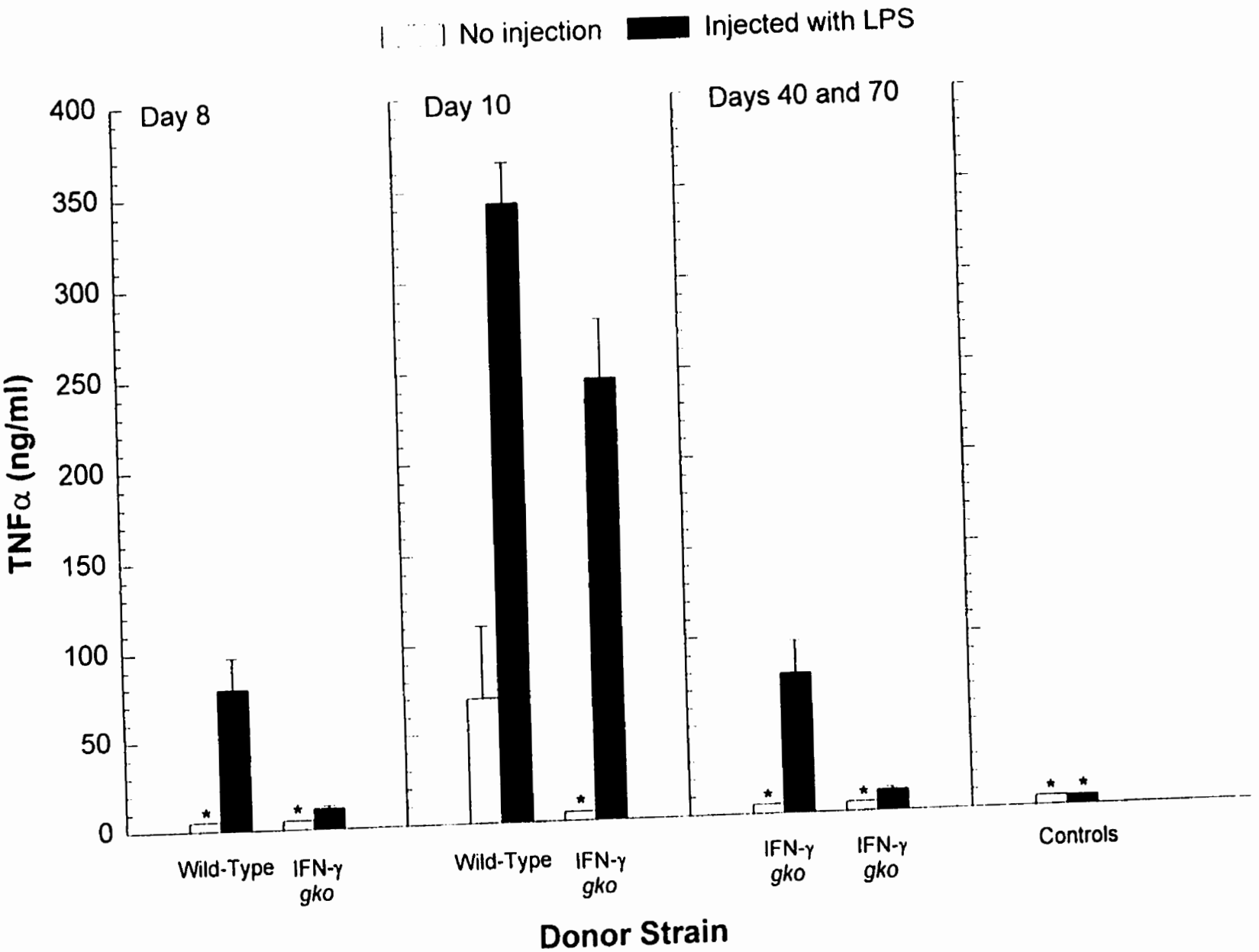
Results are shown in Figure 7.4. Sera from normal control mice did not contain any detectable TNFα, nor did injection of these animals with 10 μg of endotoxin produce any increase in serum levels. Sera from uninjected recipients of grafts from wild-type donors showed no detectable TNFα on day 8 and moderate levels on day 10. Injection of LPS resulted in moderate and marked increases in serum TNFa levels on days 8 and 10,

Table 7.2 IFN-γ and IL-10 levels measured on days 4, 8 and 15 post-induction in spleen cell culture supernatants derived from GVH mice that received grafts from either wild-type, or IFN-γ *gko* donors, and from recipient mice that did not receive grafts (Controls).

			Units of Cytokine (U/ml) ± SEM[a]	
Recipient Group	Day Post-Induction	Number of mice/group	IFN-γ	IL-10
Wild-Type Graft	4	3	<0.5	14.5 ± 1.7
	8	3	21.6 ± 0.9	<0.2
	15	3	<0.5	<0.2
IFN-γ *gko* Graft	4	3	<0.5	7.0 ± 1.0
	8	3	<0.5	<0.2
	15	3	<0.5	<0.2
No Graft (Control)	-	3	<0.5	<0.2

[a]The values <0.5 and <0.2 indicate levels below which IFN-γ and IL-10 respectively could not be measured reliably by ELISA.

Figure 7.4 Graphs showing LPS-induced TNFα levels in serum samples collected from wild-type graft and IFN-γ *gko* graft recipients on days 8 and 10 post induction, IFN-γ *gko* graft recipients on days 40 and 70 post induction, and normal control mice. On each of the days tested, three mice from each recipient group were given a 10 μg of LPS i.v. in 200 μl of PBS. The amount of TNFα appearing in the serum 90 min after LPS injection was compared with that observed in three uninjected mice from the same group. Serum samples for which the level of TNFα was below the lower limit of detection in the assay (<0.06 ng/ml) are indicated by an asterix. Error bars indicate the SE of the mean serum TNFα level in each group. The level of TNFα following LPS injection observed in each recipient group on each of the days tested was compared by Student's t test. The difference in LPS-induced TNFα release between recipients of wild-type and IFN-γ was statistically significant on day 8 only (Student's t test, $p<0.02$). The difference seen on day 10 was not statistically significant. Only recipients of IFN-γ *gko* grafts were analyzed on days 40 and 70, since all the wild-type graft recipients had succumbed to the reaction.


No injection
Injected with LPS
Day 8
Day 10
Days 40 and 70
400
350
300
250
200
150
100
50
0
TNFα (ng/ml)
Wild-Type
IFN-γ gko
Wild-Type
IFN-γ gko
IFN-γ gko
IFN-γ gko
Controls
Donor Strain

respectively. Sera from uninjected IFN-γ *gko* graft recipients contained no detectable TNFα on either day 8 or day 10. Injection of LPS resulted in a marked increase in the serum levels of TNFα seen on day 10, but not on day 8. Overall, the levels of LPS-induced TNFα release observed in IFN-γ *gko* graft recipients were lower than those seen in recipients of grafts from wild-type donors. These elevated levels also persisted until day 40 of the reaction.

7.4.5 Pathology of GVHD in recipients of grafts from IFN-γ *gko* donors.

Beginning at about day 50 post-induction, IFN-γ *gko* graft recipients developed a syndrome characterized by patchy allopecia on the head and neck, with focal excoriation and ulceration of the skin around the snout, ears and back of the neck. Retraction of the eyelids, protrusion of the eyeball from the orbit and clouding and desiccation of the cornea and conjunctiva were observed in several of the animals. Some of these features can be seen in the photograph shown in Figure 7.5. In sections of skin we observed ulceration associated with chronic inflammation and granulation tissue reaction in the ulcer beds. Sampling away from the ulcerated areas revealed lymphocytic infiltration of the dermis. The epidermis showed edema and mononuclear cell infiltration. Dyskeratotic epidermal cells were occasionally observed. Lymphocytic infiltration was also present in the epithelium surrounding the hair follicles. Some of these features can be seen in Figure 7.6. When autopsied, gross examination of the spleens demonstrated marked enlargement and the presence of white nodules. Enlargement and pallor of the liver was also observed and expansion of the portal tracts by a large cellular infiltrate could be seen upon microscopic examination (Figure 7.7A). This infiltrate consisted mostly of lymphocytes, but neutrophils and eosinophils could also be seen (Figure 7.7 B and C). Although confined mainly to the portal areas, the lesions occasionally extended through the limiting plate into the lobules.

Figure 7.5 Gross photograph of the skin and eye lesions observed in an IFN-γ *gko* graft recipient on day 66 post-induction. Note the excoriation and alopecia around the snout as well as the protrusion of the eye from the socket and clouding of the cornea (white arrows). Focal excoriation was also present around the ears (black arrow).

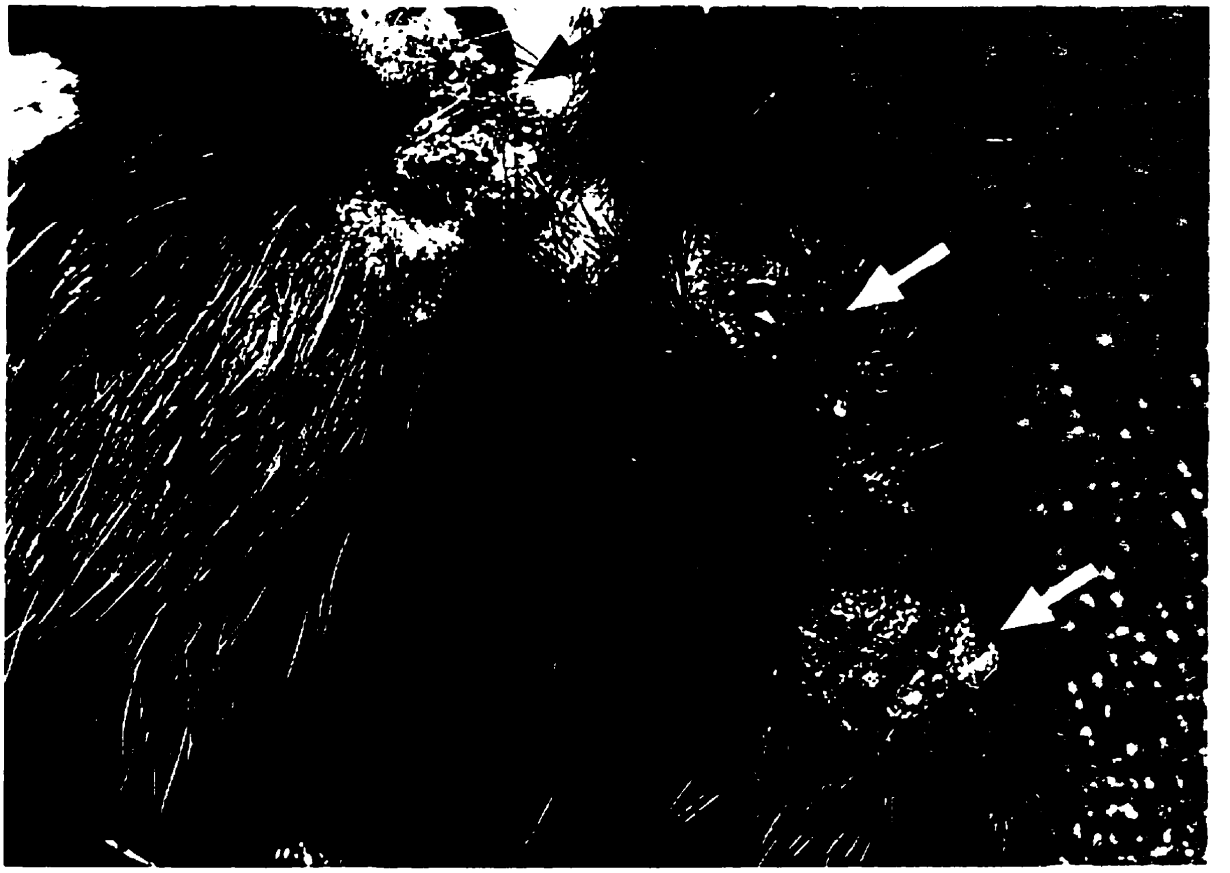


Figure 7.6 Skin pathology in IFN-γ *gko* graft recipients as seen by light microscopy on day 77 post-induction. Note the ulceration in the epidermis and the presence of a cellular infiltrate in the dermis (A). A dyskeratotic epidermal cells is indicated by the arrow in B.

A

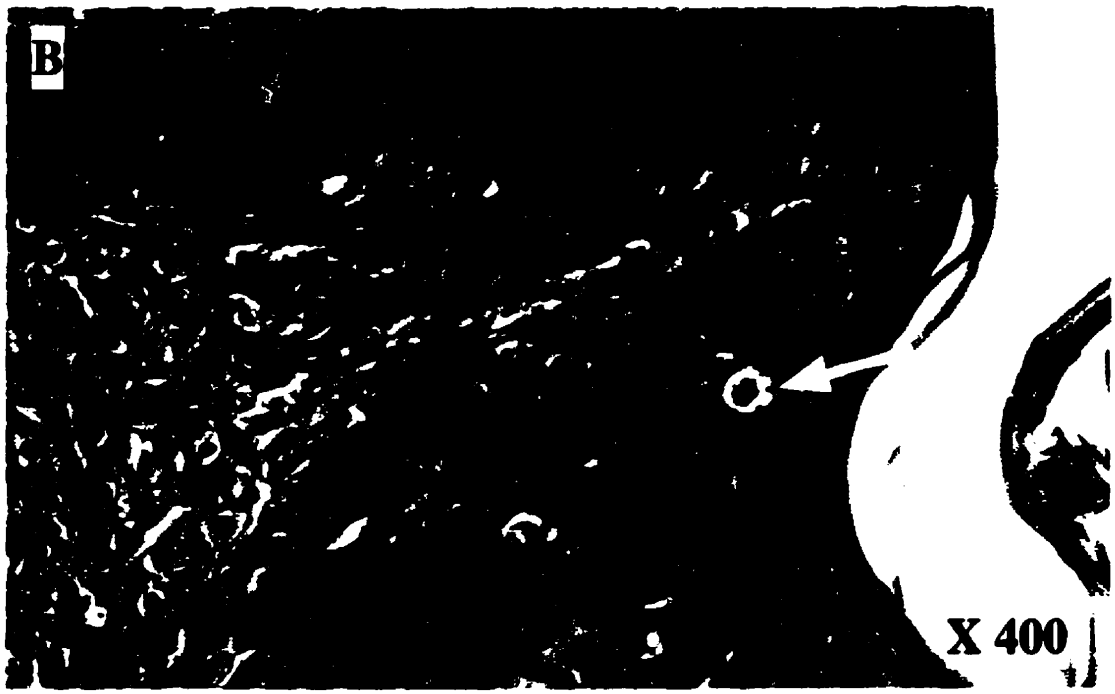
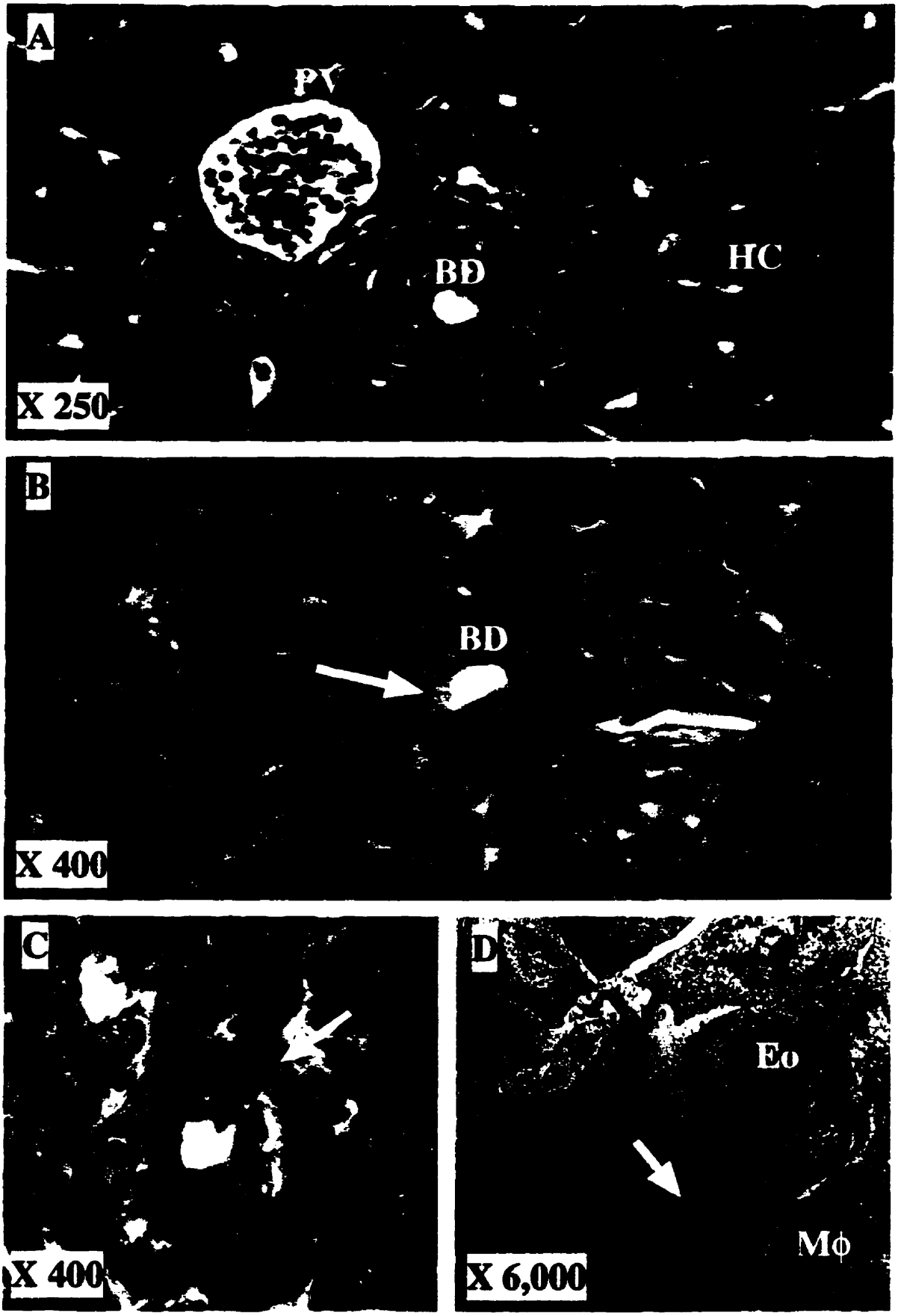


Figure 7.7 Liver pathology as seen by light and electron microscopy in IFN-γ *gko* graft recipients on day 66 post-induction. Note the large mononuclear cell infiltrate surrounding the portal vein and bile duct in (A). Disruption in the bile duct epithelium is indicated by the arrow in (B). The infiltrate seen in (A) consisted mostly of lymphocytes, but eosinophils could also be seen, as indicated by the arrow in (C). The crystalline structure characteristic of eosinophilic granules can be seen within the eosinophils shown in the electronphoto-micrograph (D). These granules are indicated by the arrow in (D).

PV; portal vein, BD; bile duct, HC; hepatocyte, Eo; eosinophil, Mφ; macrophage


A
PV
BD
HC
X 250
B
BD
X 400
C
X 400
D
Eo
Mϕ
X 6,000

Intracanalicular bile stasis was evident and bile ducts could not be identified in many of the portal areas. Disruption of the bile duct epithelium was also seen in some mice. No hepatocellular necrosis was observed. Cellular infiltrates were also identified in salivary glands (Figure 7.8). These were centered on excretory ducts and consisted of lymphocytes, neutrophils, and some eosinophils. Invasion of the duct epithelium by lymphocytes was conspicuous in some mice. Similar infiltrates were also present in the lung where they were localized around the bronchi and pulmonary blood vessels (Figure 7.9).

Several of the animals autopsied also showed unilateral hydronephrosis with obstruction in the lower one third of the ureter. Sections of kidney demonstrated focal lymphocytic infiltrates in the interstitium of the cortex and medulla. The glomeruli appeared normal by light microscopy (Figure 7.10A). Electron microscopic examination of the glomeruli showed no evidence of immune complex deposition in the basement membrane (Figure 7.10B).

Control BDF_1-hybrids, and IFN-γ *gko* donor mice housed under the same conditions as IFN-γ *gko* graft recipients did not develop any overt signs of disease over the course of the experiment. Autopsies of these animals revealed no histopathological abnormalities.

7.4.6 Engraftment of donor cells.

We used flow cytometry to determine the percentage of cells in the spleen that expressed the H-$2D^d$ haplotype, and were of host origin. Cells in the recipient that did not express H-$2D^d$ were therefore considered to have come from the donor. Figure 7.11 shows representative flow histograms from one individual from each of the two groups of recipients on day 4 post-induction. Data comparing the rate at which donor-derived cells from either wild-type or IFN-γ *gko* donors populated the spleens of recipient mice are

Figure 7.8 Salivary gland pathology as seen by light microscopy in IFN-γ *gko* graft recipient on day 77 post-induction. A cellular infiltrate can be seen surrounding an excretory duct. Although the infiltrate consisted mainly of lymphocytes and some neutrophils, eosinophils were present, as indicated by the arrows.

D;duct

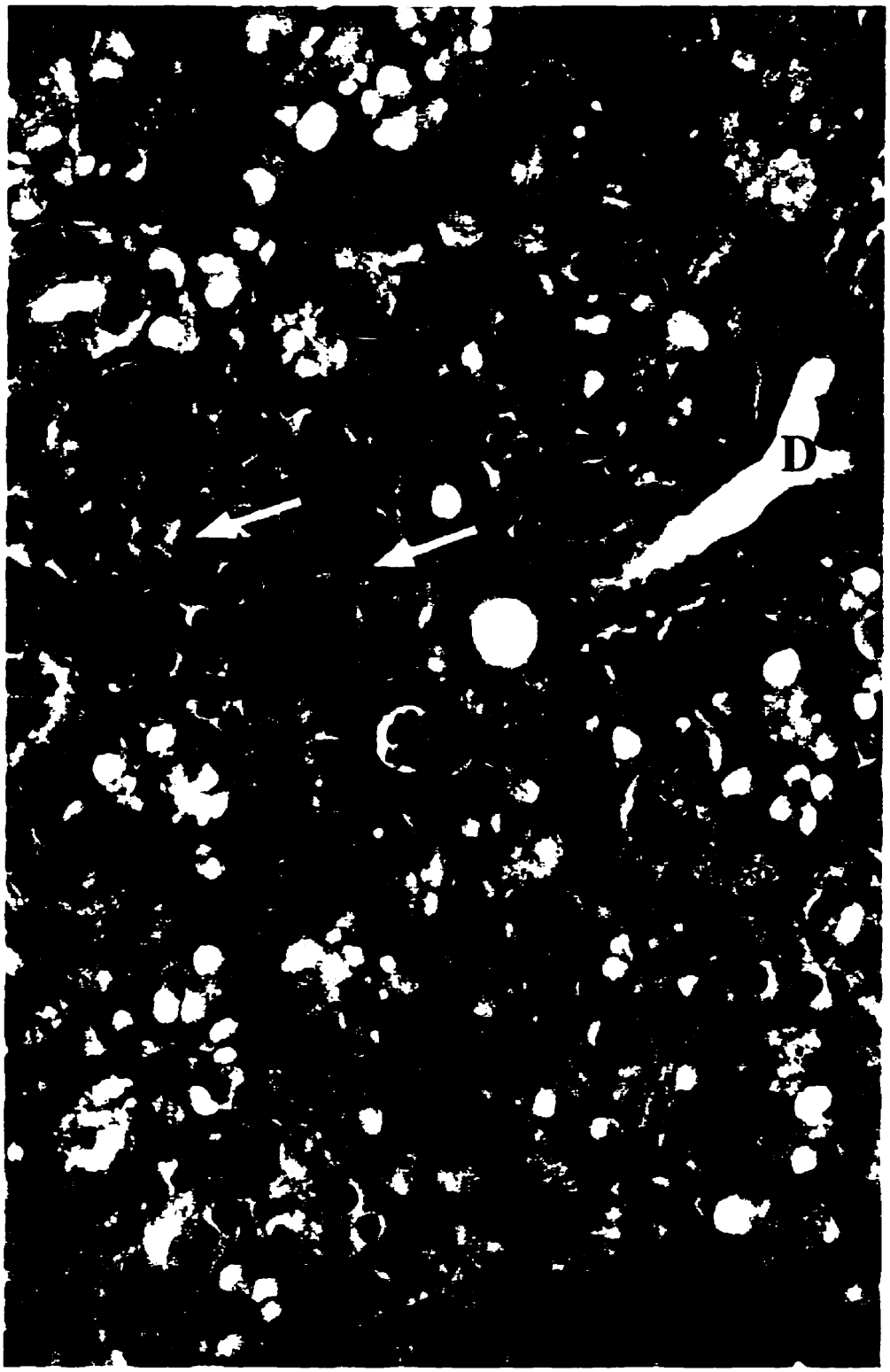

D

Figure 7.9 Lung pathology as seen by light microscopy in IFN-γ *gko* graft recipients on day 66 post-induction. Mononuclear cell infiltrates can be seen in the area surrounding the pulmonary vein and alveoli.

PV; pulmonary vein, A; alveolus

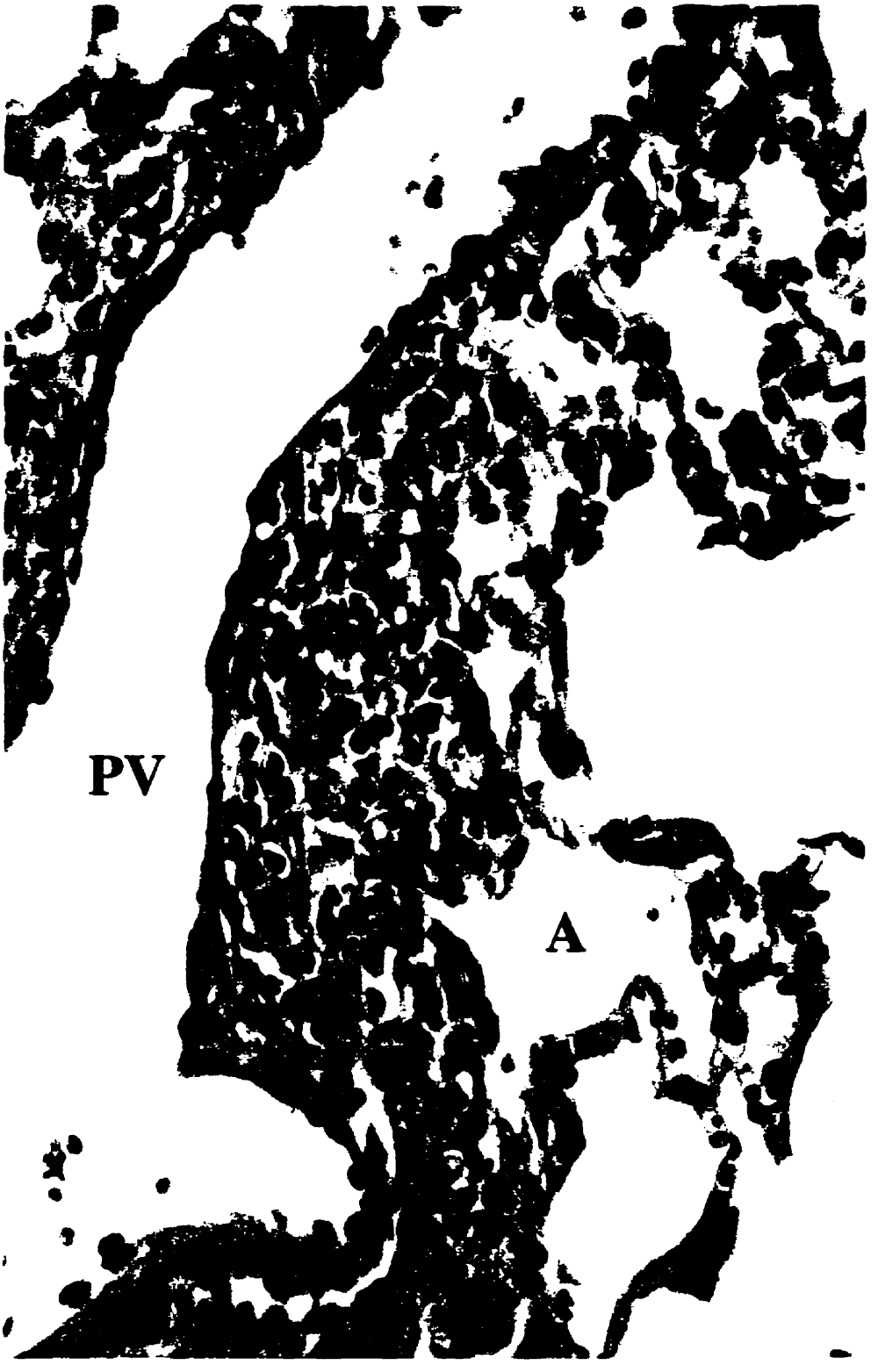

PV
A

Figure 7.10 Kidney pathology as seen by light microscopy in recipients of IFN-γ *gko* grafts on day 73 post-induction (A). A glomerulus is shown (arrow), as well as a cellular infiltrate located within the interstitium. An electron photomicrograph of the glomerular basement membrane from a kidney specimen collected from the same recipient is shown in B. The glomerular basement membrane (arrow) appears normal. No immune complex deposition can be seen.

Cap; capillary

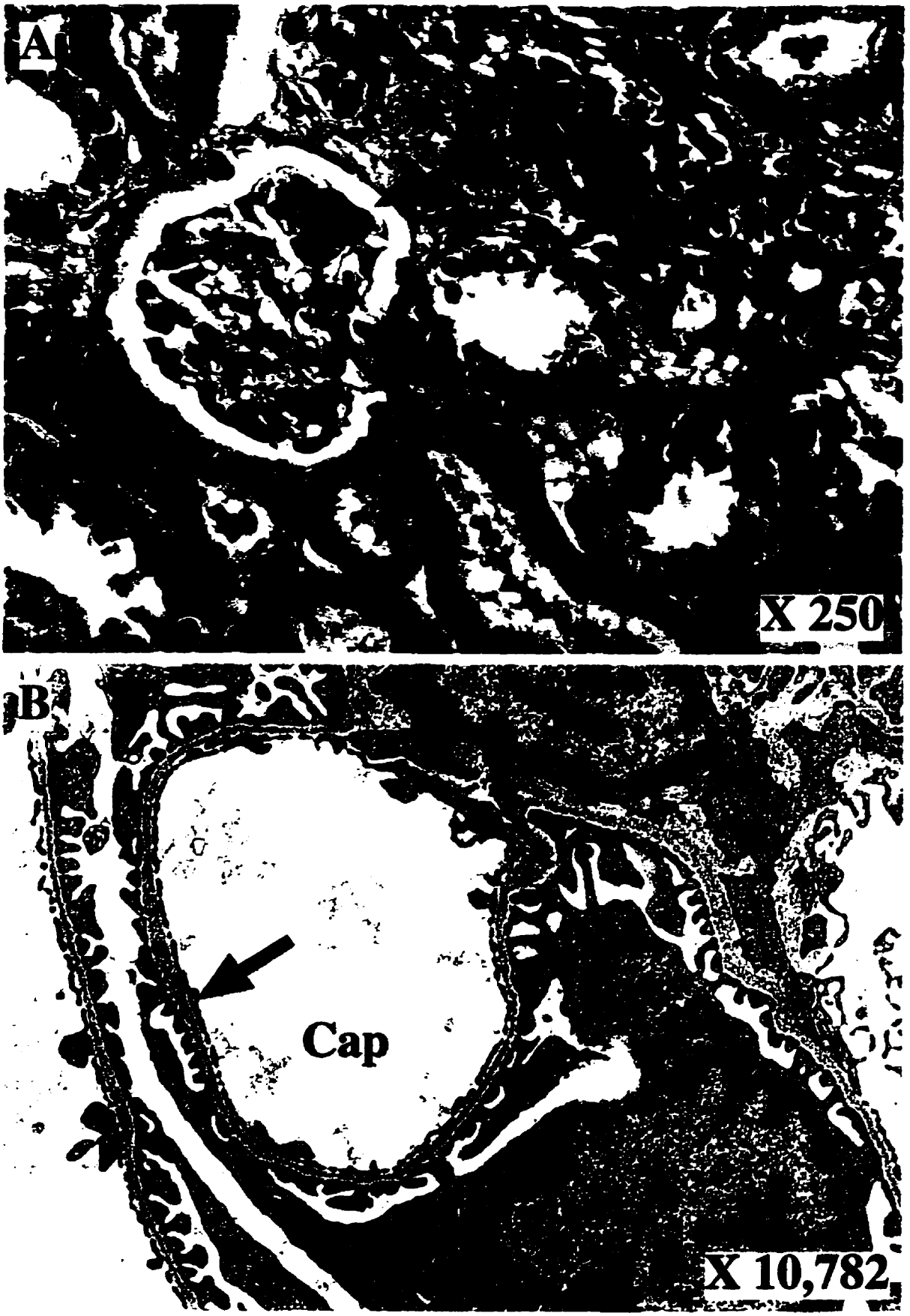
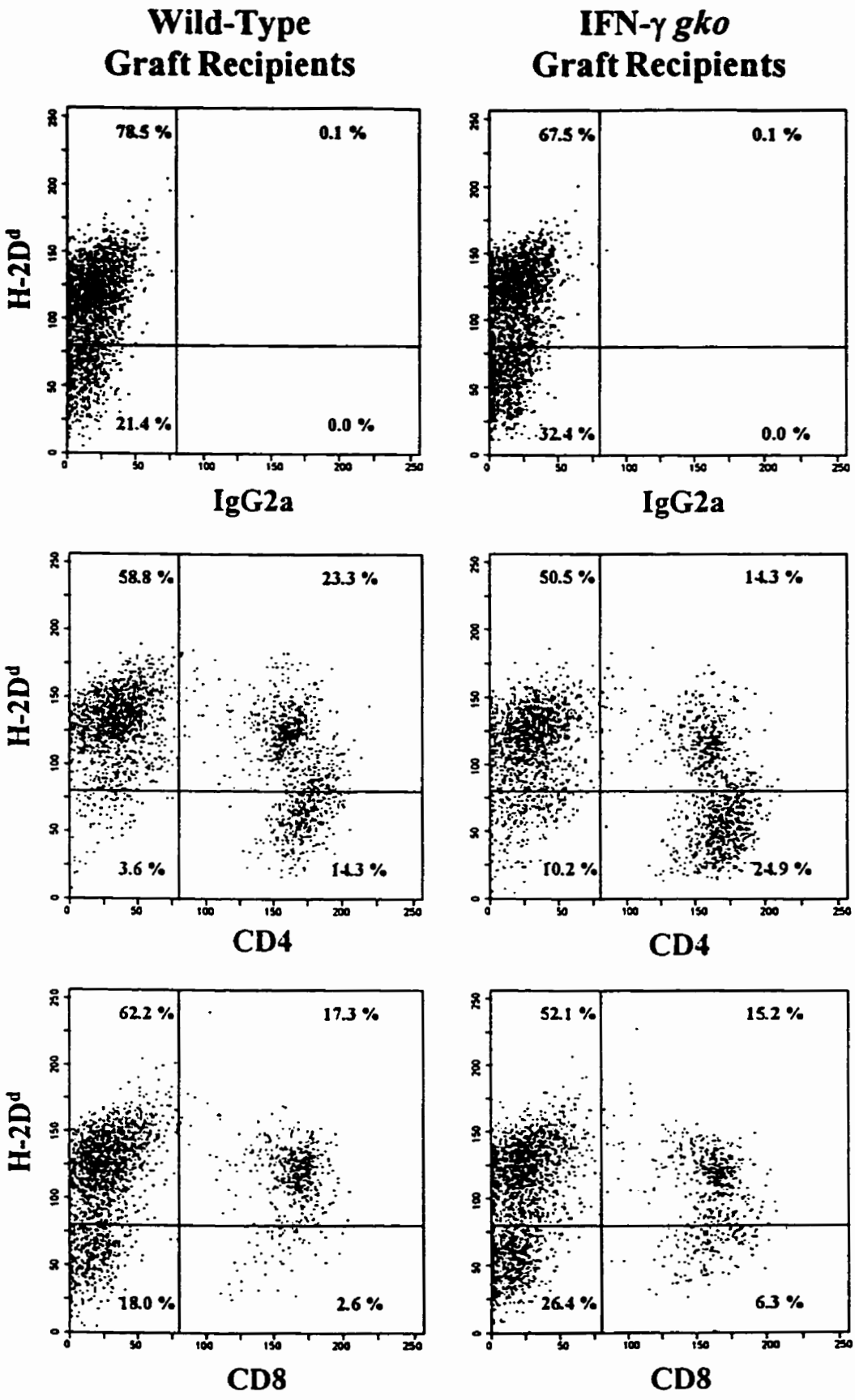

A
X 250
B
Cap
X 10,782

Figure 7.11 Six two-parameter histograms showing the pattern of analysis used to detect engraftment of donor T cells in recipient mice on day 4 post-induction. Representative data from one individual mouse drawn from each of the two recipient groups is also shown. Intensities of red (PE) and green (FITC) fluorescence are on the horizontal and vertical axes, respectively. The two histograms in the top row show the percentage of host and donor derived non-adherent cells in the spleen appearing in the left upper (H-$2D^d$-positive) and left lower (H-$2D^d$-negative) quadrants respectively. The lower 4 histograms show the proportion of $CD4^+$ and $CD8^+$ cells in the host and donor cell populations. The PE conjugated rat IgG_{2a} used in the top two histograms served as an isotype control for the anti-CD4 and anti-CD8. FITC-conjugated mouse IgG_{2a} was used as an isotype control for the anti H-$2D^d$. Non-specific labeling was $< 1\%$ (histograms not shown). Each histogram was drawn from 6000 gated events. The value in each quadrant indicates the percentage of gated cells appearing in that quadrant.


Wild-Type
Graft Recipients
IFN-γ gko
Graft Recipients
78.5 %
0.1 %
21.4 %
0.0 %
67.5 %
0.1 %
32.4 %
0.0 %
H-2Dd
IgG2a
58.8 %
23.3 %
3.6 %
14.3 %
50.5 %
14.3 %
10.2 %
24.9 %
H-2Dd
CD4
62.2 %
17.3 %
18.0 %
2.6 %
52.1 %
15.2 %
26.4 %
6.3 %
H-2Dd
CD8

shown in Table 7.3. Recipients of grafts from IFN-γ *gko* donors showed a greater percentage of donor-derived cells as early as day 4 post-induction. With the exception of day 4, the number of donor-derived cells in the spleen of IFN-γ *gko* graft recipients was more than twice that seen in recipients of wild-type grafts. The percentage of donor-derived $CD4^+$ and $CD8^+$ cells was also greater in recipients of grafts from IFN-γ *gko* donors. In recipients of wild-type grafts, the percentage of donor-derived $CD4^+$ cells declined steadily from day 4 to day 15. A similar decrease was also seen in IFN-γ *gko* graft recipients from days 4 to 8. By day 15, however, the percentage of $CD4^+$ cells in this group began to increase. Both groups showed a steady increase in the number of donor-derived $CD8^+$ cells, but the rate at which the number of these cells increased was greater in IFN-γ *gko* graft recipients. By day 15, the percentage of donor $CD8^+$ cells in IFN-γ *gko* graft recipients was 5 times that seen in recipients of wild-type grafts.

To determine whether these differences in recipient mice were due to different numbers of $CD4^+$ and $CD8^+$ cells in the wild-type and IFN-γ *gko* grafts, we determined the percentages of these cells each of the grafts. Data shown in Table 7.4 indicate that the number of these cells is similar in both groups, an observation consistent with that published previously by Dalton *et al* (253).

7.5 Discussion.

The purpose of this study was to determine how the course and outcome of GVHD is altered when grafts derived from IFN-γ *gko* donors are used. The C57BL/6J-*Ifg*^{tm1Ts} mutant employed in these experiments was developed by Dalton *et al* (253). These mice thrive if housed in a clean environment, but show a variety of immune defects including impaired production of antimicrobicidal products, decreased expression of MHC class II antigens by

Table 7.3 Results from flow cytometry experiments determining comparative rates of engraftment of donor-derived non-adherent cells as well as donor-derived CD4$^+$ and CD8$^+$ cells in spleens from mice that received grafts from either wild-type or IFN-γ *gko* donors.

Day Post-Induction	Mean Percent Donor Non-Adherent Cells ± SEM[a]		Mean Percent CD4$^+$ Donor Cells ± SEM[b]		Mean Percent CD8$^+$ Donor Cells ± SEM[c]	
	Wild-Type Graft	IFN-γ *gko* Graft	Wild-Type Graft	IFN-γ *gko* Graft	Wild-Type Graft	IFN-γ *gko* Graft
4	19.7 ± 0.9	33.3 ± 0.6	13.0 ± 1.0	22.6 ± 1.2	2.0 ± 0.6	5.0 ± 0.9
8	10.2 ± 2.4	27.4 ± 2.3	4.6 ± 0.5	11.6 ± 2.3	4.7 ± 0.7	14.2 ± 1.2
15	20.6 ± 2.3	47.0 ± 7.0	3.0 ± 1.0	15.5 ± 0.5	6.0 ± 3.1	31.7 ± 3.1
40[3]	-	77.4 ± 2.5	-	20.2 ± 0.5	-	30.8 ± 4.9

[a]The mean was obtained from one-colour flow cytometry analyses done on three individual mice/group/day. Donor cells were identified as those cells in the histograms that did not express H-2D^d.

[b]The mean was obtained from two-colour flow cytometry analyses done on three individual mice/group/day. Donor CD4$^+$ or CD8$^+$ cells were identified as those cells which expressed CD4 or CD8 but did not express H-2D^d.

[c]No day 40 data is shown for recipients of wild-type grafts because they do not survive beyond day 35 post-induction.

Table 7.4 Percentage of $CD4^+$ and $CD8^+$ cells in grafts from wild-type and IFN-γ *gko* donor mice.

Donor Group	% $CD4^+$ Cells	% $CD8^+$ Cells
Wild-Type	22.2	13.6
IFN- *gko*	23.5	12.1

macrophages, and an increased susceptibility to intracellular pathogens such as *Mycobacterium Bovis*. They also demonstrate uncontrolled proliferation of splenocytes in response to both mitogen and alloantigen, as well as increased T cell cytolytic activity against allogeneic target cells in mixed lymphocyte reactions (253).

Recipients of IFN-γ *gko* graft recipients showed a pattern of mortality and weight loss different from that typically seen in wild-type graft recipients. Although GVHD in both groups was equally lethal, IFN-γ *gko* graft recipients survived twice as long as recipients of wild-type grafts. Because TNFα is allegedly involved in GVHD-associated cachexia, we compared levels of this cytokine in sera collected from both groups of recipients. Our results showed that TNFα was undetectable in sera collected from either group of recipients on day 8 of the reaction, a time when wasting had already begun in IFN-γ *gko* graft recipients. Interestingly, moderate levels of this cytokine were seen on day 10 in wild-type graft recipients that had not yet begun to lose weight, but were undetectable in IFN-γ *gko* graft recipients that had lost more than 13% of their original body weight. These results suggest that factors other than TNFα mediate this effect. Furthermore, it is likely that the factor(s) responsible is/are regulated by IFN-γ, since this early period of wasting was seen only in IFN-γ *gko* graft recipients and not in wild-type graft recipients. This observation is consistent with those of others, showing that TNFα may not be required for the development of the cachexia in all models of wasting, and that other cytokines, glucocorticoids, catecholamines, insulin and insulin-like growth factors may be involved in this process (470).

The presence of LPS-induced TNF-α release in IFN-γ *gko* graft recipients was unexpected, since we had predicted that if donor-derived IFN-γ was responsible for priming macrophages, augmented LPS-induced TNF-α release would not be observed in this group.

We also found that despite the absence of donor-derived IFN-γ, augmented LPS-induced TNFα release was present as early as day 10 post-induction and could still be seen on day 40 of the reaction. To make certain that IFN-γ was not produced by the host, we compared the level of this cytokine in spleen cell cultures from both experimental groups on several days post-induction. As expected, cultures from IFN-γ *gko* graft recipients contained no measurable IFN-γ, even in the very early stages of GVHD, and cultures from wild-type graft recipients contained large amounts on day 8, which was consistent with our previous observations. Nonetheless, we cannot discount the possibility that some IFN-γ was indeed produced by the recipient at a level too low to be detectable by ELISA, and that this amount was enough to prime macrophages. Still, it is more likely that a cytokine other than IFN-γ is involved. Whether this cytokine supplants IFN-γ in IFN-γ *gko* graft recipients or whether it is indeed the real "priming factor" in wild-type graft recipients remains unknown.

Conventional NK cell activity, as measured by YAC-1 directed lysis, appeared in both recipient groups but was higher in IFN-γ *gko* graft recipients. Because both the donor and host can contribute to the YAC-1-directed lysis in these mice, we compared the relative contributions of each to this activity. We found that the donor-derived NK activity remaining after $H\text{-}2^d$ depletion was very similar in both recipient groups. However, the balance of the NK activity, derived from the host was higher in IFN-γ *gko* graft recipients. One possible explanation is that other NK-activating cytokines such as IFN-α/β or IL-15 are produced in greater quantities when IFN-γ is absent from the reaction, and that the host-derived NK cells are better able to respond to this stimulus than the NK cells derived from IFN-γ *gko* donors. The latter hypothesis is supported by Dalton and colleagues (253) who showed that resting NK activity (YAC-1-directed lysis) is significantly lower in IFN-γ *gko* mice compared to wild-type mice. Whether NK cells from IFN-γ *gko* donors respond less

vigorously to alloantigen and/or cytokine stimulation remains unknown. Cytotoxicity directed against BW1100 targets was also present in both groups of recipients. It was slightly higher in those that received wild-type grafts, but appeared earlier in IFN-γ *gko* graft recipients. Considerably less donor-derived NK-like activity was seen in IFN-γ *gko* graft recipients, and the host contributed a greater proportion of the total amount of NK-like activity seen. These results support the idea that host NK-like cells are also activated to a greater extent than donor NK-like cells in IFN-γ *gko* graft recipients. Because our previous observations suggest that NK-like cells are T cells, it is probable that cytotoxic function in this population of cells is also diminished in IFN-γ *gko* mice. Our own experiments using poly I:C to induce NK-like activity in IFN-γ *gko* donor mice indicate that this activity is approximately one third less than that seen in wild-type mice (Ellison and Gartner, unpublished observation). Our present data also reconfirms our previously published observation that most NK-like activity in recipients of wild-type grafts is donor-derived (430).

The pathology of GVHD seen in IFN-γ-*gko* graft recipients was different from that seen in wild-type graft recipients. Two notable differences were the development of skin lesions which we have never before observed in this F_1-hybrid model of acute GVHD, and the development of pathological changes in the eyes of IFN-γ *gko* graft recipients. Also atypical was the size of the cellular infiltrates present in the liver, lung and salivary gland, and the presence of neutrophils, and particularly eosinophils within these infiltrates in the liver and salivary gland. Whether the presence of neutrophils reflects a reaction to the tissue damage, a superimposed bacterial infection, or an idiosyncrasy of GVH lesions in the absence of IFN-γ, is not known. The presence of eosinophils could indicate the development of a Th2-mediated immune response since eosinophilia is associated with production of IL-5, a Th2 cytokine (471). However, our data also show that levels of IL-

10, another Th2 cytokine, are significantly lower in IFN-γ *gko* graft recipients. Although this result appears to be at odds with the idea that these mice develop Th2-mediated chronic GVHD, it could, as suggested by Rus and colleagues, reflect an increased rate of consumption of IL-10 by rapidly proliferating B cells (402).

Some of the histopathological changes we observed in IFN-γ *gko* graft recipients are similar to those seen in BMT recipients with chronic GVHD. This is best exemplified by the large lymphocytic infiltrates we observed in the salivary gland ducts. In chronic GVHD, destruction of the excretory ducts in the salivary and lacrimal glands by infiltrating lymphocytes causes the development of a "sicca syndrome", resembling that seen in Sjogren's disease. Although we did not examine the lacrimal glands, it is quite possible that involvement of these organs may have been instrumental in causing the eye lesions we observed in IFN-γ *gko* graft recipients.

The development of an autoimmune/SLE-like syndrome, characterized by autoantibody formation and immune complex (IC) disease is another feature of chronic GVHD (111). Our EM findings in the kidney demonstrate that IFN-γ *gko* graft recipients do not develop the latter as part of their syndrome. However, the absence of IC disease does not preclude the possibility that autoantibodies are present in these mice, and preliminary data from our laboratory indicates that autoantibodies specific for nucleolar and mitochondrial antigens can be detected in sera collected from IFN-γ *gko* graft recipients (data not shown).

Rus and colleagues demonstrated that both acute and chronic GVH reactions begin with B cell hyperplasia and the development of Th2 cytokines (402). In reactions destined to follow an acute course, this effect was mitigated by donor-derived CD8 cells, which serve to eliminate activated B cells and promote the production of IFN-γ by $CD4^+$ cells. We observed a very large percentage of donor-derived, CD8 cells in recipients of grafts

from IFN-γ *gko* donors, yet these mice developed features of GVHD that were more consistent with chronic GVHD. Dalton *et al* (253) also found that splenocytes from IFN-γ *gko* mice develop very high levels of allospecific CTL activity in MLR. If significant amounts of autoantibody are not present in these recipients, is possible that the large number of donor-derived $CD8^+$ cells abrogated this response. Alternatively, the concommitant appearance of autoantibodies and a large, donor-derived $CD8^+$ cellular population could indicate that the ability of the latter cells to regulate B cell responses may be abrogated.

Our flow cytometry data demonstrated some interesting differences in the pattern of T cell engraftment of the two recipient groups. The number of donor-derived, non-adherent, spleen cells in wild-type graft recipients never exceeded 20%. The percentage of $CD4^+$ cells was 13% on day 4 but gradually declined to 3% on day 15. $CD8^+$ cells showed a modest increase from 2% to 6% over the same interval. The findings were very different in recipients of IFN-γ *gko* grafts. Even in the very early stages of the disease, the level of engraftment was much greater (33% on day 4) and increased steadily to 77% by day 40. The percentage of $CD4^+$ cells on day 4 was also considerably greater (23%). This value declined slightly, but by day 40 had returned to 20%. The percentage of $CD8^+$ cells increased steadily to 31% by day 40. These engraftment data are to some extent mirrored by the patterns of splenomegaly seen in these two groups. Wild-type graft recipients experienced transient splenomegaly early in the course GVHD, which later subsided. This contrasted the splenomegaly seen in IFN-γ *gko* graft recipients, which began later and persisted until the end of the disease. We know from the work of Dalton *et al* (253) that splenocytes from IFN-γ *gko* mice develop an exaggerated proliferative response to allogeneic stimulator cells in MLR. This may explain why we observed both a greater accumulation of donor-derived T cells and persistent splenomegaly in IFN-γ *gko* graft

recipients. It may also provide a cogent explanation for the very pronounced lymphocytic infiltrates in target organs of recipients of IFN-γ *gko* grafts.

In summary, the lethal GVHD developing in recipients of grafts from IFN-γ *gko* donors has some features in common with the acute GVHD. These include, a high mortality rate, cachexia, and macrophage priming for LPS-induced TNFα release, which is thought mediate the development of GVHD-associated septic shock. Other features consistent with chronic GVHD include a more prolonged course of the disease, a greater range of organ involvement, and the development of both cutaneous lesions and a sicca or Sjogrens-like syndrome. The absence of immune complex disease in the kidney indicates that a lupus-like syndrome does not develop, but preliminary results suggest that a Th2-mediated autoimmune syndrome does indeed evolve. Overall, our data supports the idea that donor-derived IFN-γ does play an important role in the pathogenesis of acute GVHD, but refutes the idea that elimination of this cytokine will improve the prognosis for individuals at risk of developing this disease. Instead they suggest that one of the major effects of IFN-γ is to accelerate GVH-induced mortality and that the absence of this cytokine results in a more prolonged course of disease with severe pathological manifestations.

7.6 Future directions.

This work provides a fairly detailed description of the type of GVHD that occurs in the absence of donor-derived IFN-γ, but does not indicate precisely how or where this cytokine acts in the pathogenesis of the disease. Furthermore, the enthusiasm with which we report these results is to some degree tempered by the caution that should be observed when using gene knockout models. The absence of the gene in question during ontogeny can affect the development or function of other components of the immune system. Thus,

an effect observed when a gene knockout is used may not necessarily be due to the absence of the gene or gene product *per se*.

Our results suggest that IFN-γ may serve to increase donor-derived NK and NK-like activity, or that it may be involved in controlling the ability of some cells, particularly donor-derived CD8 cells, to proliferate during acute GVH reactions. A very recent article by Shustov and colleagues indicated that IFN-γ-dependent upregulation of Fas and FasL appears to be required for the elimination of autoreactive B cells by $CD8^+$ cells in the C57BL/6→BDF_1 strain combination (472). A study of Fas and FasL expression and CTL-mediated cytotoxicity in IFN-γ *gko* graft recipients could provide further support for this hypothesis. In addition, a comparison of IL-4 and IL-5 levels in IFN-γ *gko* and wild-type graft recipients would help to elucidate whether the pathological changes observed in the former correlate with the development of a Th2-mediated immune response.

7.7 Appendix.

These experiments were designed by the candidate under the supervision of Dr. Gartner. Contributions of others include the performance of ELISA assays for IFN-γ, IL-10 (W. Stefura) and TNFα (J. Fischer), the preparation of tissues for electron microscopy (Charmaine Hedgecock), and technical assistance with the engraftment study (J. Fischer and E. Rector).

CHAPTER 8

NK1.1 DEPLETION OF IFN-γ GENE KNOCKOUT GRAFTS PROTECTS F_1-HYBRID RECIPIENTS FROM GVHD-ASSOCIATED MORTALITY AND PATHOLOGY

8.1 Abstract.

In this study, we wished to determine how removal of NK1.1$^+$ cells from the graft would alter GVHD in recipients of IFN-γ *gko* grafts. Our previous experiments showed that *in vitro* NK1.1-depletion of the graft was only effective in preventing acute GVHD in recipients of wild-type grafts if the donors were stimulated with poly I:C before the grafts were harvested and depleted. We therefore induced GVH reactions using NK1.1-depleted grafts from IFN-γ *gko* donor mice that either had, or had not been treated with poly I:C prior to sacrifice. Results showed that recipients of NK1.1-depleted grafts from poly I:C-stimulated, IFN-γ *gko* donors did not develop the high levels of mortality, cutaneous lesions or the sicca/Sjogrens syndrome associated with GVHD in IFN-γ *gko* graft recipients. Partial protection against mortality was also seen when the donors were not treated with poly I:C and the graft was depleted of NK1.1$^+$ cells.

8.2 Introduction.

Our previous work has shown that GVHD induced in BDF_1-hybrid recipients using grafts from C57BL/6, IFN-γ *gko* donor mice is more protracted than that seen when wild-type grafts are used. It is also characterized by cachexia, enhanced LPS-induced TNFα release, a Sicca/Sjogrens syndrome, cutaneous lesions, large cellular infiltrates in target organs, and, possibly, by autoantibody formation. Results from these experiments also suggested that IFN-γ was not produced by cells originating in the host, implying that IFN-γ is completely absent from this type of GVH reaction.

Other experiments performed in our laboratory have shown that the mortality and cachexia associated with acute GVHD in this F_1-hybrid model can be prevented if the graft is harvested from poly I:C-stimulated donors and depleted of NK1.1$^+$ cells *in vitro*. This protective effect was associated with a marked reduction in both IFN-γ production and LPS-

induced TNFα release. Based on these results, we hypothesized that the role of NK1.1$^+$ cells in acute GVHD is to drive the development of Th1-mediated response by producing IFN-γ in the formative stages of the immune response.

Our results indicate that although IFN-γ production was either mitigated or abrogated in both the "IFN-γ *gko*" and "NK1.1-depletion" model of GVHD, the outcomes of the disease were very different. Because NK1.1$^+$ cells were present in unmodified grafts harvested from IFN-γ *gko* donors, and absent from NK1.1-depleted grafts harvested from wild-type donors, we hypothesized that some of the pathological changes seen in IFN-γ *gko* graft recipients were mediated by donor-derived cells expressing NK1.1. Our goal in these experiments was therefore to determine whether depletion of these cells from IFN-γ *gko* grafts would prevent the development of these changes, thereby protecting recipients from mortality.

We found that GVH reactions induced in BDF_1-hybrid mice using NK1.1-depleted grafts from poly I:C-stimulated, C57BL/6, IFN-γ *gko* donors were characterized by an initial, transient period of wasting that occurred at the same time as that seen when undepleted, IFN-γ *gko* grafts were used. However, these recipients regained and surpassed their original mean body weight and survived the reaction. Particularly interesting is our observation that none of these mice developed the skin lesions or Sicca/Sjogrens syndrome observed when undepleted grafts were used.

8.3 Experimental design.

Grafts were harvested from C57BL/6, IFN-γ *gko* donor mice that either had, nor had not been injected with poly I:C, 18 hr before the grafts were harvested. NK1.1$^+$ cells were depleted by direct complement-mediated lysis as described in the Materials and

Methods. Recipients of these grafts were monitored periodically for weight loss, mortality, and the development of skin and eye lesions.

8.4 Results.

8.4.1 Weight loss and mortality.

Figure 8.1A shows that NK1.1 depletion of the graft completely prevented mortality in recipients of IFN-γ *gko* grafts, if the donors were stimulated with poly I:C before the depletion was performed. Approximately 60% of the recipients succumbed to the reaction when the donors were not pre-stimulated.

Both recipient groups experienced a transient period of wasting early in the reaction, but recovered somewhat by day 20 (Figure 8.1B). Recipients of NK1.1-depleted grafts from untreated donors then experienced a sustained period of weight loss that lasted until the end of the experiment. In contrast, those that received depleted grafts from poly I:C-stimulated donors regained their original body weight by day 40, and continued to gain weight until the experiment was terminated.

8.4.2 Pathological changes.

The skin lesions and sicca/Sjogrens syndrome seen previously in recipients of IFN-γ *gko* grafts did not develop when the donors were stimulated with poly I:C and the grafts were depleted of NK1.1$^+$ cells. They were, however, present in recipients of NK1.1-depleted grafts from untreated donors. Figure 8.2 shows that large, mixed cellular infiltrates, similar to those seen in recipients of undepleted grafts from IFN-γ *gko* donors were present in specimens of liver (8.2A) and lung (8.2C) collected from recipients of NK1.1-depleted grafts from untreated IFN-γ *gko* donors. Diffuse mixed cellular infiltrates

Figure 8.1 Graphs showing, on several days post-induction, the percentages of surviving mice (A) and mean body weights of mice (B) that received NK1.1-depleted grafts from either untreated (●) or poly I:C-stimulated (■) , IFN-γ *gko* donors.

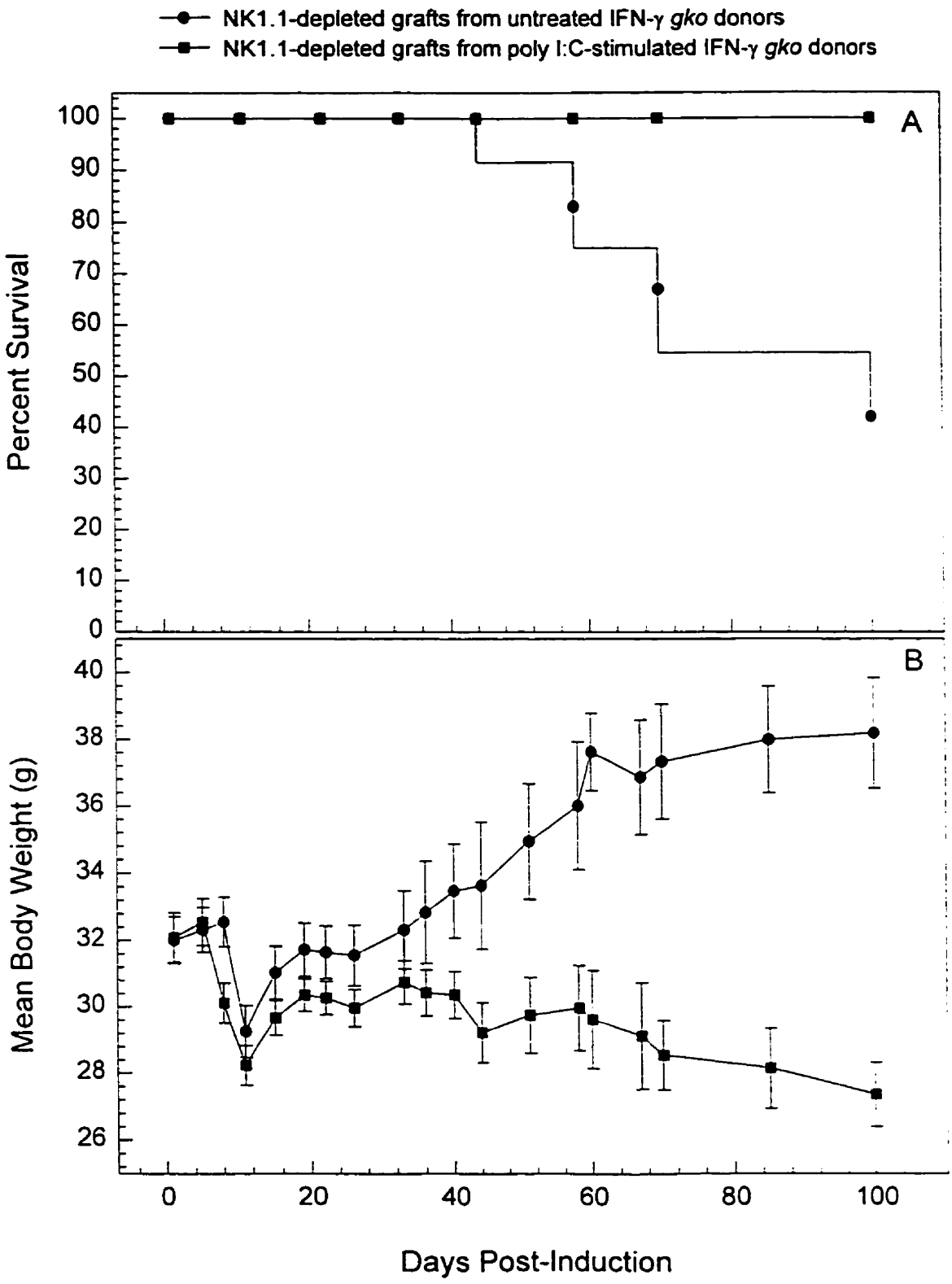
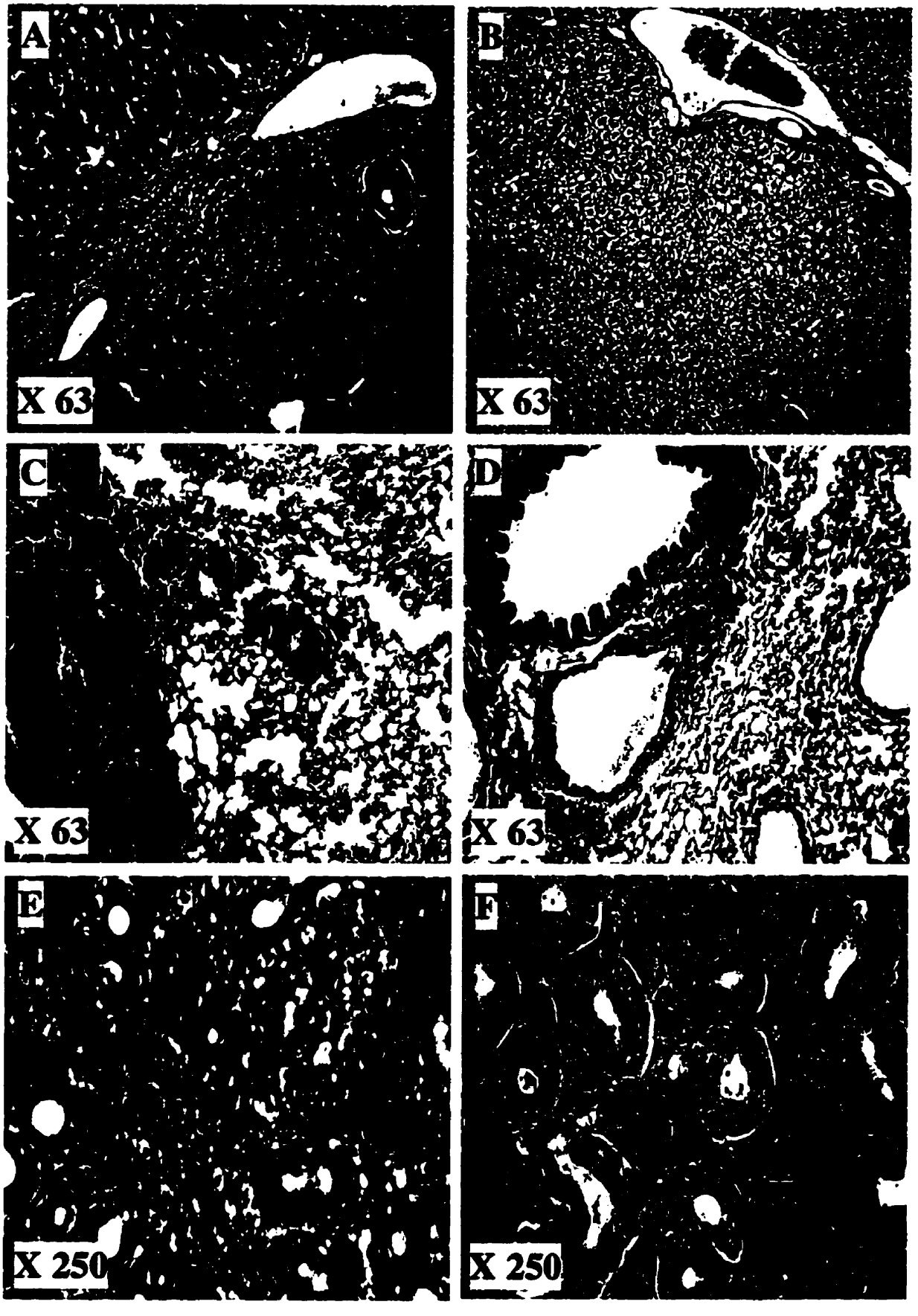

NK1.1-depleted grafts from untreated IFN-γ gko donors
NK1.1-depleted grafts from poly I:C-stimulated IFN-γ gko donors
A
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
B
Mean Body Weight (g)
40
38
36
34
32
30
28
26
0
20
40
60
80
100
Days Post-Induction

Figure 8.2 Comparison of liver (A and B), lung (C and D) and salivary gland (E and F) pathology in recipients of NK1.1-depleted, IFN-γ *gko* grafts from either untreated (A,C and E) or poly I:C-stimulated (B,D and F) donors. Note the extensive mononuclear cell infilatrate in recipients of NK1.1-depleted grafts from untreated donors (A,C,E).


A
X 63
B
X 63
C
X 63
D
X 63
E
X 250
F
X 250

were also seen in the salivary glands of these mice (8.2F). These infiltrates were not, however, observed in either liver (8.2B) or salivary gland (8.2E) collected from recipients of NK1.1-depleted grafts from poly I:C-stimulated, IFN-γ *gko* donors. Some smaller infiltrates could be seen in lung specimens collected from recipients in this group (8.2D).

8.5 Discussion.

Results from these experiments clearly indicate that donor-derived NK1.1$^+$ cells are involved in the pathogenesis of GVHD induced using IFN-γ *gko* donors. Protection from mortality was complete when these cells were depleted from grafts harvested from poly I:C-stimulated donors, and partial when unstimulated donors were used.

Because the early, transient period of wasting persisted in the absence of donor-derived NK1.1$^+$ cells, we must conclude that this event occurs independently of these cells. However, the later, more sustained period of wasting seen in recipients of undepleted, IFN-γ *gko* grafts was abrogated by stimulating donors with poly I:C and then purging the graft of NK1.1$^+$ cells. This treatment also prevented the development of cutaneous lesions and a sicca/Sjogrens syndrome associated with this phase of the disease, indicating that NK cells are required for these pathological changes to occur.

Our previous hypothesis suggested the role of donor-derived NK1.1$^+$ cells in acute, murine GVHD was to promote the development of an IFN-γ-driven, Th1-mediated immune response. However, these results suggest that NK1.1$^+$ cells in the graft can still contribute to the pathogenesis of GVHD when IFN-γ is either absent, or present at very low levels. These results should, however, be verified by demonstrating that engraftment does indeed occur in recipients of NK1.1-depleted grafts from poly I:C-stimulated IFN-γ donors.

The role of donor-derived NK1.1$^+$ cells in GVHD occurring in IFN-γ *gko* graft recipients appears to be related to the development of lesions in the skin, eyes and salivary

glands. Because these features of GVHD are seen only in IFN-γ *gko* graft recipients, and not in wild-type graft recipients, it appears that the NK1.1$^+$ population behaves differently during GVH reactions occurring in an immunological environment that is deficient in IFN-γ. It is also possible that NK1.1$^+$ cells may participate in the development of chronic GVHD, since these pathological lesions resemble those seen in this form of the disease. Our results therefore suggest that GVHD therapies aimed at removing IFN-γ from the reaction may not be successful as long as NK1.1$^+$ cells still remain in the graft.

How NK1.1$^+$ cells mediate the development of cutaneous lesions in IFN-γ *gko* graft recipients is currently unknown. It is possible that they are directly cytotoxic to this tissue, or, alternatively, that they release cytokines that mediate tissue injury. However, because donor-derived NK and NK-like cytotoxic activity are reduced in IFN-γ *gko* graft recipients compared to wild-type graft recipients, it is likely that effects mediated by NK1.1$^+$ cells in the former group are a result of cytokine release, rather than direct cytotoxicity. For example, the role of donor-derived NK1.1$^+$ cells in the sicca/Sjogrens syndrome seen in IFN-γ *gko* graft recipients may be to release factors that help to recruit other cells into the salivary glands and, perhaps, the lacrimal glands. In any event, NK1.1$^+$ cells must become activated in an IFN-γ-independent manner in order to perform these functions.

Since our data shows that NK1.1$^+$ cells contribute to the pathogenesis of GVHD in IFN-γ *gko* graft recipients, it would be interesting to determine how these cells become activated under these circumstances. One cytokine that can influence NK function and may be produced at high levels in IFN-γ *gko* graft recipients is IL-12 (473;474). This is supported by the observation that IFN-γ *gko* mice infected with *Toxoplasma gondii* develop very high levels of IL-12 and elevated NK activity following infection. This response does not, however, appear to assist in controlling infection, since treatment with anti-IL-12 did

not decrease survival. Furthermore, the levels of YAC-1-directed lysis seen in IFN-γ *gko* mice infected with *T. gondii* were higher than those seen in uninfected mice, but lower than those seen in wild-type mice infected with the same pathogen. This observation is consistent with the lower levels of NK activity we observed in IFN-γ *gko* graft recipients compared to wild-type graft recipients. Other support for IL-12 involvement in immune responses occurring in the absence of IFN-γ comes from the observation that IFN-γ *gko* mice infected with *Schistosoma mansoni* experience exacerbated Th2-mediated granuloma formation and eosinophilia when given exogenous IL-12 (475).

Because the pathological changes seen in these recipients appears to be Th2-mediated, it is possible that IL-4-producing NK1 T cells promote the early commitment of $CD4^+$ T cells to a Th2 phenotype. It would therefore be interesting to determine whether NK1/T cells become activated to produce IL-4.

Another cytokine that can activate NK cells, and is also produced by these cells is TNFα (476). In IFN-γ *gko* mice with an active histoplasmosis infection, TNFα was found to be present at higher levels than those seen in wild-type mice with the same infection. It was also shown to play a critical role in secondary immune responses to histoplasmosis that occur in the absence of IFN-γ (477;478). Tumor necrosis factor-α has also been implicated in the pathogenesis of autoimmune diseases such as SLE and type I diabetes, suggesting that it may be a mediator of the autoimmune-like features that we observed in IFN-γ *gko* graft recipients (479;480). Although we were unable to detect this cytokine in sera collected from these recipients, it is possible that it is produced locally, and removed from circulation too quickly to achieve detectable levels in sera. If NK cells are producing TNFα in the skin of IFN-γ *gko* graft recipients, it could certainly be cytotoxic to cells in the vicinity and contribute to lesion development. Alternatively, it may function by increasing cellular adhesion to the vascular endothelium, thereby promoting the development of the large

cellular infiltrates we observed (481). Furthermore, there is evidence that TNFα plays a role in eosinophil accumulation when IL-4 is present (482;483). The eosinophils seen in tissues from IFN-γ *gko* graft recipients may therefore have been recruited by TNFα. An alternative to these hypotheses is the idea that TNFα may play an entirely different role in GVHD by inducing alloresponsive cells to proliferate in the early stages of the reaction. This is supported by *in vitro* experiments showing that this cytokine can cause up to a 6-fold increase in proliferative responses during MLR (484).

8.6 Future directions.

In view of the findings reported in this chapter, it would be interesting to determine whether neutralization of IL-12 would prevent the activation of NK1.1$^+$ cells, and/or the development of the pathological lesions seen in IFN-γ *gko* graft recipients. If a cause and effect relationship between IL-12 and the development of GVHD in these recipients can be demonstrated, it would suggest that strategies aimed at preventing the development of GVHD-associated, Th1-mediated immune responses should be targeted at the very early stages of the reaction, at the time, or before IL-12 is produced. Experiments could also be done to determine how TNFα levels compare in GVHD target tissues collected from IFN-γ *gko* and wild-type graft recipients, and to identify the cellular source of this cytokine. Of significant interest would be a study designed to determine whether NK1 T cells become stimulated to produce IL-4 during GVH reactions induced in IFN-γ *gko* graft recipients.

In summary, the results reported in the last three chapters suggest that interfering with the development of acute GVHD by abrogating the graft's ability to produce IFN-γ does not prevent the disease. Studies are therefore required to further investigate the role of other cytokines involved in the early stages of the GVH reaction, particularly those that either activate, or are produced by NK cells. The elimination of Th1-promoting cytokines

such as IFN-γ, IL-12 and TNFα, in different permutations and combinations, during the formative stages of the GVH reaction is the most promising approach to GVHD prevention that has been provided by this research. One way in which this could be accomplished is by treating the graft with antisense deoxynucleotides (ASO) specific for these cytokines, before the graft is infused. Should this technique be successful in downregulating the graft's ability to produce specific cytokines *in vivo* for a short period of time post-transplantation, it could result in the abrogation of acute GVHD without significantly immunocompromising the host. It would also be very appealing from a clinical perspective, since modification of the graft is more desirable than *in vivo* treatment of the recipient. This approach may also prove to be superior to methods that involve purging cellular populations from the graft, since it takes a considerable time for a cell lineage to be regenerated by the stem cells present in the donor's hematopoietic tissue. The use of ASO in this capacity would also be more specific than graft purging, since it could be targeted only at certain cytokines, and not at an entire cellular population with diverse functional properties.

8.7 Appendix.

The preliminary results presented in this chapter were from experiments performed by the candidate. They will be submitted for publication when more data is available and all outstanding controls have been run.

CHAPTER 9

SUMMARY

The results presented in this thesis were derived from three lines of investigation. The first was to determine the mechanism through which NK1.1 depletion of the graft protects recipients from the effects of acute, lethal GVHD. It was found that removal of $NK1.1^+$ cells from the graft obviates the development of the Th1-mediated immune response that underlies the development of this disease, but that it does not prevent endotoxin from entering circulation or GVHD-associated enteropathy. The second was to determine whether γδT cells play a role in the pathogenesis of acute GVHD. It was found that depletion of these cells from donor mice provided partial protection against mortality. In contrast, recipients of TCRδ KO grafts were not protected, but experienced significantly higher levels of IFN-γ production compared to wild-type graft recipients. The third line of investigation was to determine how the outcome of a GVH reaction would be affected if the Th1 cytokine, IFN-γ is absent from the reaction. To accomplish this, we induced GVH reactions using grafts harvested from IFN-γ KO donor mice. The GVHD observed under these circumstances was lethal, but more prolonged than that seen in recipients of wild-type mice, and had different pathological manifestations, some of which resembled chronic GVHD. The following discussion is an attempt to integrate these findings, to evaluate their clinical and biological significance, and to reconcile any inconsistencies.

The mechanisms involved in the very early stages of an acute GVH are not entirely understood, but likely include the development of a Th1-mediated immune response. Previous clinical studies have shown that interfering with the action of Th1 cytokines participating in the later stages of the reaction, namely TNFα and IL-1, can successfully diminish some of the effects of acute GVHD. However, these benefits are only transient, since withdrawal of the therapy results in GVHD relapse (391). From this, it is likely that strategies aimed at mitigating the GVH reaction during its formative stages would be more

successful, as they would alter the immune response on a more global level. Results from our experiments using grafts depleted of NK1.1$^+$ cells indicate that their elimination decreases IFN-γ production, an early event in the development of cell-mediated immunity. Furthermore, LPS-induced TNFα release is abrogated in recipients of NK1.1-depleted grafts. This phenomenon, which is a more downstream event in the reaction, results from macrophage priming and causes endotoxemic shock. Also significant is the observation that these recipients fail to develop any gross signs of Th2-mediated, chronic GVHD in the absence of a Th1 response.

Until now, very little was known about the mechanism through which NK1.1 depletion of the graft protects recipients against the mortality associated with acute GVHD. The most widely held hypothesis has been that NK and NK-like cells, by virtue of their cytotoxic abilities, were important mediators of injury to tissues targeted by the GVH reaction. Our results clearly indicate that NK1.1$^+$ cells play an immunoregulatory role in the disease. Furthermore, they show that because GVHD-associated enteropathy still develops in recipients of NK1.1-depleted grafts and systemic LPS levels become elevated, cells other than those in the graft expressing NK1.1 are responsible for this type of tissue injury. However, in order to disprove the hypothesis that NK1.1$^+$ are involved in the development of intestinal injury, it would be crucial to determine whether or not donor-derived NK1.1$^+$ cells reappear later in the reaction and contribute to the development of these lesions.

Although our findings implicate NK1.1$^+$ cells in the development of elevated IFN-γ levels in GVH mice, they do not indicate whether they produce this cytokine directly, or stimulate its production by other cells. However, because they are part of the innate immune system, it is hypothesized that they probably respond to alloantigen very early in the reaction by producing IFN-γ, which in turn stimulates CD4$^+$ T cells to differentiate into

Th1 cells and produce more IFN-γ. It is thought that one major function of IFN-γ is to prime macrophages for LPS-induced TNFα release. When IFN-γ-primed macrophages are exposed to LPS, they release large amounts of TNFα. It is known from the work of Kichian and colleagues that IFN-γ mRNA is present in tissues targeted by the GVH reaction by day 14 post-induction (242). Because T cell functions are known to be suppressed when this occurs, it was postulated that local NK cells may be the source of this cytokine. Our results suggest that this may be the case. Experiments are currently underway in our laboratory to determine whether NK1.1$^+$ cells in the graft can produce IFN-γ when stimulated by host alloantigen.

One observation that appears to contradict the hypothesis that IFN-γ primes macrophages for LPS-induced TNFα release comes from our experiments showing that priming can still occur in recipients of IFN-γ *gko* grafts. Whether this means that IFN-γ is not responsible for macrophage priming in recipients of unmodified, wild-type grafts, or that other cytokines compensate for the lack of IFN-γ in recipients of IFN-γ *gko* grafts is not known. Experiments using *in vivo* neutralization of IFN-γ in recipients of wild-type grafts by antibody injection could answer this question.

The observation that donors must be stimulated with poly I:C prior to the NK1.1 depletion for protection to be achieved is also important, and suggests that activation of this cellular population is required if the depletion is to be effective. This observation is consistent with results from another study showing that prevention of acute GVHD by depleting ASGM$_1^+$ cells from the graft also requires pre-stimulation of donor mice. However, in this experiment donors were stimulated with host antigen prior to the depletion (332). This finding is therefore important if this method were to be modified for use in humans receiving allogeneic bone marrow transplants. It also represents an important biological observation. Firstly, reports from the literature indicate that poly I:C may

increase NK cell trafficking from the bone marrow into the lymphoid tissue (485). Secondly, poly I:C appears to induce the production of type I IFN and IL-12 (486). The latter has been shown to induce expression of NK1.1 on NK1 T cells (459;487). Based on these observations, we speculate that poly I:C plays a role in the depletion procedure by stimulating IL-12 production in the donor. This induces NK1.1 expression NK1 T cells thereby allowing them to be effectively eliminated from the graft by NK1.1 depletion. However, to demonstrate that these cells are the relevant cells that are eliminated by the NK1.1 depletion procedure, it would be necessary to selectively purge the graft of NK1 T cells. Unfortunately, there are technical problems associated with this approach because it requires the isolation of cells coexpressing NK1.1 and αβTCR. One way this could be achieved is by isolating the NK1.1$^+$ population from the graft and then depleting the cells in this fraction that co-express αβTCR. The remaining, single positive NK1.1$^+$ cells could then be returned to the graft. Alternatively, this could be done be positively selecting αβT cells from the graft and depleting the cells in this fraction that coexpress NK1.1. A potential problem associated with this method is the observation that antibodies specific for NK1.1 and αβTCR are notorious for causing cellular activation. Another possibly way that cells in the graft coexpressing NK1.1 and αβTCR could be isolated is by using two-colour flow cytometry. However, this technique is constrained by the very large cell numbers required to induce a GVH reaction.

Although it has been shown that NK cells are involved the development of Th1 responses to several infectious diseases, ours is the first demonstration that NK1.1$^+$ cells are involved in the mechanism through which a Th1 response becomes established in mice with acute GVHD. Our experiments also show that LPS levels can become elevated in recipients of NK1.1-depleted grafts. This observation may be particularly important if NK1.1 graft depletion were to be used clinically since the absence of NK1.1$^+$ cells in the

graft may render the host more susceptible to pathogens that require a Th1-mediated immune response in order to be controlled. Theoretically, this deficiency would be transient, as stem cells from the graft should eventually develop into mature NK1.1$^+$ cells. Similarly, our observation that intestinal lesions and endotoxemia still develop in recipients of NK1.1-depleted grafts indicates that this method of prevention should be combined with another that eliminates the effector cell responsible for this type of injury. Alternatively, recipients could be given antibiotic therapy to combat gram negative bacteria in the host.

Our observation that NK1.1$^+$ cells are required to produce a Th1 response during an acute GVH reaction suggests that they may also function to promote cell-mediated immune responses in other immunological responses to alloantigen, for example, following solid organ transplantation. The question of whether NK and/or NK-like cells are required for host-versus-graft reactions is currently under investigation, and there are several reports suggesting that they may be involved. For example: allogeneic heart transplantation activates alloreactive NK cells in the host; patients undergoing renal transplant rejection have a higher proportion of circulating NK-like cells than that seen in individuals not rejecting their grafts; and, porcine cardiac grafts are rejected in primate recipients by a mechanism involving interactions between primate NK cells and the porcine endothelium (472;488;489). Although these reports suggest a correlation between the activation of NK and/or NK-like cells in the host and the rejection of solid organs, they do not indicate precisely how these cells might be involved in the pathogenetic mechanism.

Results from experiments in which GVH reactions were induced using grafts from IFN-γ *gko* donors were also aimed at investigating the contribution made by a cytokine that is involved in the development of the Th1 response. Our study provided a detailed description of the very severe and protracted disease that can develop when donor-derived IFN-γ is absent, and demonstrated that this cytokine accelerates mortality in GVH mice. It

also showed that this type of GVHD has many features that resemble chronic GVHD including skin and eye lesions, cellular infiltrates containing eosinophils, and, possibly, autoantibody formation. These findings represent important considerations in any clinical approaches to GVHD prevention that involve the elimination of IFN-γ from the GVH reaction. From a biological perspective, they may be less significant than observations from our NK1.1 depletion model, because they do not indicate exactly how or where this cytokine participates in the pathogenetic mechanism.

When results from our NK1.1-depletion model and our IFN-γ *gko* model are compared, they appear to be contradictory. Paradoxically recipients of NK1.1-depleted grafts fail to develop a Th1 response or an acute, lethal GVH reaction, yet do not develop any overt signs of chronic GVHD, whereas recipients of grafts from IFN-γ *gko* donors which are incapable of producing this important Th1 cytokine, develop a disease that, in many respects, resembles chronic GVHD. A possible explanation is that NK1.1 T cells are involved in regulating the immune response associated with the development of both acute and chronic GVHD. As previously mentioned, these cells can produce either IFN-γ or IL-4, depending on how they are stimulated. Recipients of NK1.1 depleted grafts from wild-type donors may be protected from GVHD because the grafts do not contain NK1 T cells. This may be critical to GVHD prevention not only because it removes an early source of IFN-γ that would promote acute GVHD, but also because it removes a potential source of IL-4 that could promote the development of chronic GVHD in the absence of a Th1 response. In recipients of IFN-γ *gko* grafts, NK1 T cells are present in the graft and may respond by producing IL-4 instead of IFN-γ, thereby promoting the development of a Th2 response and chronic GVHD. Should this be correct, it would indicate a role for $NK1.1^{+}/\alpha\beta TCR^{+}$ cells in the development of chronic GVHD. This hypothesis is supported by the work of Rus and colleagues who showed that GVH reactions default to a Th2-

mediated immune response and develop into chronic GVHD unless IFN-γ is present in the early stages of the reaction to promote the development of a Th1-mediated response and acute GVHD (402).

Our investigation of the role played by γδT cells in the pathogenesis of acute GVHD is more closely related to the events occurring in the later stages of the GVH reaction. Unlike the NK1.1 depletion and IFN-γ *gko* models used to study the early immune response occurring in GVH mice, this study focussed on the role of donor-derived cytotoxic cells with a broad lytic spectrum. We successfully demonstrated that γδT cells play a role in the pathogenesis of acute GVHD, but that they are probably not the relevant effector cell that is removed from the graft by our NK1.1 depletion protocol. We had initially hoped that this approach would fully protect recipients from acute GVHD, but it appears that γδT cells play a less central role in the pathogenetic mechanism than we had previously thought. However, as mentioned several times throughout this thesis, our results suggest that γδT cells may be the cells that mediate the injury that occurs in the intestinal epithelium. GVHD-associated enteropathy has been studied for some time and it is still unclear how or why the gut becomes more permeable to gram negative bacteria. Because the endotoxemia associated with intestinal injury is a major cause of mortality in humans with acute GVHD, a better understanding of the mechanisms involved in this process are required. An experiments that might be performed would be to determine whether γδT cells are present in the intestine at the site of injury, and whether their removal from the graft abrogates enteropathy and endotoxemia.

The observation that recipients of γδT cell-depleted grafts are partially protected against GVHD-associated mortality, whereas recipients of grafts from TCRδ KO donors are not also seems paradoxical. However, in the latter group of recipients we observed IFN-γ levels that were approximately 3 times as high as those seen in recipients of wild-type

grafts. Because the IFN-γ levels observed in recipients of γδT cell-depleted grafts were not significantly different from those seen in wild-type graft recipients, we hypothesized that excessive IFN-γ production may have overwhelmed any protective effect that may have been provided by removing γδT cells from the graft. It is possible that this phenomenon is associated with the TCRδ mutation, and the effects that this may have had on immune function and development in TCRδ KO mice.

In summary, these findings support the pathogenetic mechanism of acute GVHD presented in Chapter 1 of this thesis. More specifically, they suggest the following sequence of events: $NK1.1^+$ cells, possibly the NK1 T cell subset, become activated to produce IFN-γ during the formative stages of the GVH reaction. We speculate that this involves recognition of alloantigen on host tissues, and the production of IFN-γ inducing factors such as IL-12 by activated macrophages. IFN-γ-producing $NK1.1^+$ cells then promote the commitment of $CD4^+$ Th0 cells to a Th1 phenotype. Macrophages become primed by IFN-γ, or possibly by other cytokines such as type I IFN, that are produced as a consequence of the ongoing GVH reaction. As this reaction proceeds, cells with cytotoxic capabilities, for example γδT cells, become activated and mediate injury to tissues targeted by the reaction. When this occurs, lesions develop in the intestinal epithelium facilitating the entry of gram negative bacteria into the host. High systemic levels of endotoxin then develop as the host's ability to neutralize the effects of LPS become exhausted. The endotoxin then triggers primed macrophages to release excessive amounts of TNFα and other mediators resulting in fatal endotoxemic shock.

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IMAGE EVALUATION
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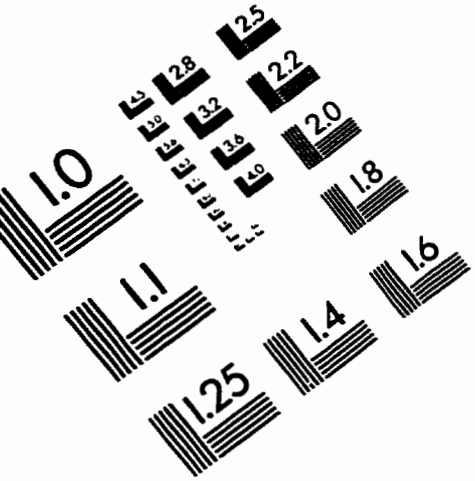

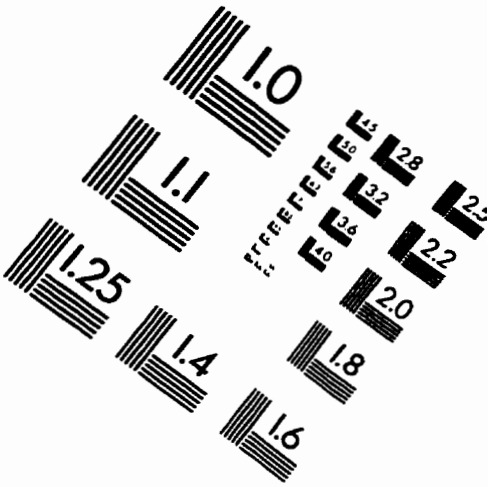

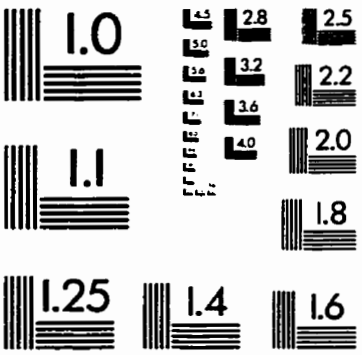

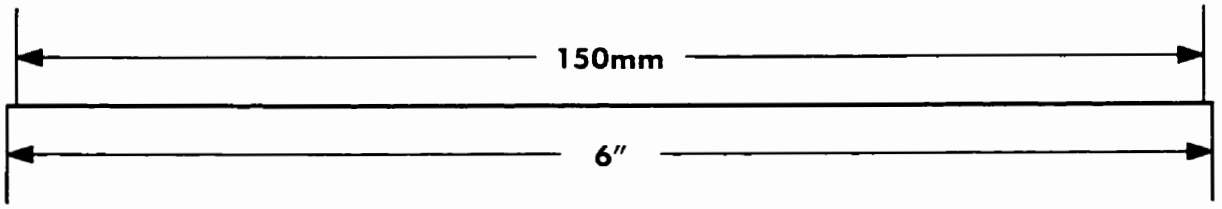

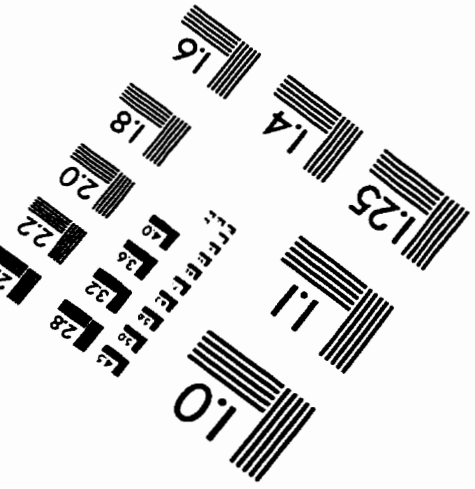

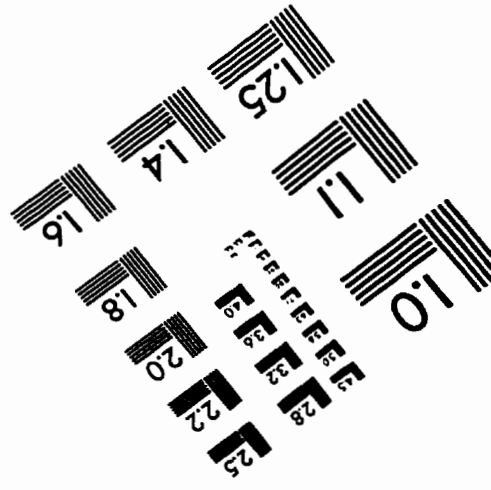

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989