THE ROLE OF NON-MHC-RESTRICTED CYTOTOXIC EFFECTOR CELLS IN THE PATHOGENESIS OF ACUTE GRAFT-VERSUS-HOST DISEASE IN F_1-HYBRID MICE

BY

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A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree of

Doctorate of Philosophy

Department of Immunology

University of Manitoba

Winnipeg, Manitoba

ISBN 0-315-81659-7

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

Previous studies have shown that acute GVH reactions produced in parental-F_1 hybrid mice result in the activation of two non-MHC-restricted cytotoxic cells: a Thy-$1^{+/-}$ NK cell that kills NK-sensitive tumor targets, and a Thy-1^{+} NK-like cell that kills both NK-resistant and NK-sensitive tumor targets. Experiments described in this thesis were designed to: (1) identify some of the cell-surface markers on NK-like cells; (2) determine the host/donor origin of splenic NK and NK-like cells; (3) study the relationship between cytokine production and augmented NK cell activity and the activation of NK-like cells; (4) determine the effect of purging NK and NK-like cells from the graft on the development of acute GVH disease. Measurement of *in vitro* cytokine production showed a close temporal relationship between peak levels of IL-2 secretion by spleen cell cultures from mice with GVH reactions and maximal levels of splenic NK cell activity. NK-like cell activity, which was only present in mice with acute GVH reactions, correlated with IFNα/β production, but not with IL-2 or IFNγ. Augmented NK activity was depletable with host-strain specific antibody; however, most of the NK-like cell activity was resistant to depletion and therefore of donor origin. The effect of depleting NK1.1^{+} cells from a lymphoid cell allograft on the outcome of acute GVH disease was examined. Results showed that while the recipients of NK1.1-

depleted grafts prepared from donors stimulated with poly I:C developed some of the characteristic features of acute GVH disease they did not develop cachexia and exhibited longterm survival. Survival in this treatment group was associated with an absence of splenic NK-like cell activity. Experiments also showed that splenic NK-like cells expressed CD3 and TCRγ/δ. A hypothetical model describing the role of NK-like cells in the effector phase of acute GVH disease is proposed.

ACKNOWLEDGEMENTS

I would like to thank the members of my committee, Drs. A.H. Greenberg, E. Sabbadini, D. Rayner, D. Bowden and W. Lapp, for their helpful comments over the course of my study and careful evaluation of this thesis.

Most of all, I would like to thank my supervisor and friend, Dr. John Gartner. I thoroughly enjoyed working with John and now that I am finished, I hope I will be taking with me the same philosophical and enthusiastic approach to research that he has shown me over my tenure in his laboratory. What I thank John for the most is that he taught me that research should be "fun", and in his lab it was.

A special thanks to Veronica Sanders for her expert technical assistance. I would also like to thank my mother-in-law, Florence McCormick and my sister-in-law, Shelagh McCormick, for taking care of the kids so I could work on my thesis.

The financial support of the University of Manitoba Graduate Fellowship Program and the Manitoba Health Research Council is gratefully acknowledged.

This thesis is dedicated to my parents, John and Lorraine MacDonald, and to my wife Laura and my children Maggie and Jane.

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ABBREVIATIONS

AIDS, acquired immune deficiency syndrome

$ASGM_1$, ganglio-N-tetraosylceramide

B6, C57BL/6

BAF_1, (CBA x A/J)F_1

BCG, bacillus Calmette-Guerin

BDF_1, (C57BL/6 x DBA/2)F_1

bg, beige

BM, bone marrow

BMT, bone marrow transplant

C, complement

Con A, concanavalin A

CSF, colony-stimulating factors

CTI, cold-target inhibition

CTL, cytotoxic T lymphocyte

CTLL, cytotoxic T lymphocyte cell line

DTH, delayed-type hypersensitivity reaction

FACS, fluorescence activated cell sorter

FAM, function associated molecule

GVL, graft-versus-leukemia

GVH, graft-versus-host

Hh, hybrid histocompatibility

HSP, heat shock proteins

HSV, herpes-simplex virus

IEL, intraepithelial lymphocyte

IFN, interferon

IL-1, interleukin-1

IL-2, interleukin-2

IL-12, interleukin-12

LAK, lymphokine-activated killer

LCM, lymphocytic choriomeningitis

LGL, large granular lymphocyte

LPL, lipoprotein lipase

LPS, lipopolysaccharide

LU, lytic units

mHA, minor histocompatibility antigens

MHC, major histocompatibility complex

MLC, mixed-lymphocyte culture

mls, minor lymphocyte stimulatory

MLR, mixed-lymphocyte reaction

MoAb, monoclonal antibodies

NDV, New Castle disease virus

NK, natural killer

NKSF, natural killer cell stimulatory factor

NS, natural suppressor

$P \rightarrow F_1$, parental $\rightarrow F_1$

PBL, peripheral blood lymphocyte

PFC, plaque-forming cell

PHA, phytahemagglutinin

poly I:C, poly inosinic:cytidylic acid

SLE, systemic lupus erythematosus

SRBC, sheep red blood cells

TCGF, T cell growth factor

TCR, T cell receptor

TH, T helper

TNF, tumor necrosis factor

TNF-R, tumor necrosis factor receptor

VLDL, very low-density lipoprotein

CHAPTER 1

LITERATURE REVIEW

1.1 Historical Perspective

Although bone marrow transplantation (BMT) was originally developed as a treatment for leukemia, it is now used as a method for treating several other diseases including aplastic anemia, immunodeficiency diseases, some inborn errors of metabolism and cancer. The efficacy of allogeneic BMT, however, continues to be limited by a high incidence of graft rejection or the onset of graft-vs-host (GVH) disease. While it is now well understood that engraftment of allogeneic immunocompetent cells into an immunologically compromised recipient may result in GVH disease, much is still unknown regarding the pathologic mechanisms leading to tissue injury and the eventual demise of the recipient.

The effects of allogeneic tissue engraftment were first described following experiments in chick embryos which showed that the engraftment of either spleen, bone marrow, liver and kidney resulted in splenomegaly and the appearance of white nodules on the surface of the chorio-allantoic membrane and in the spleen, skin, liver and kidneys (1). Because the nodules were shown to consist largely of white blood cells the graft was believed to be a source of stimulatory factors responsible for the proliferation of host leukocytes residing at these different tissue sites.

The alternative possibility that allografts contain cells capable of recognizing and proliferating in response to incompatible tissue antigens in the host was later proposed by

two independent investigators (2,3). Both authors suggested that the pyroninophilic lymphocytes which appeared in rejecting dog renal allografts were of local origin and were likely stimulated by the host's tissue antigens. Simonsen later speculated that if the host was immunologically incompetent then the only cells able to mount an immune response would come from the allograft; since this reaction would be directed against host alloantigens the response would be deleterious to the host. This hypothesis was proven to be correct in subsequent experiments with chick embryos engrafted with spleen cell allografts (4). These recipients were shown to develop severe hemolytic anemia and marked splenomegaly with the chicks succumbing 3-4 days after hatching.

Coincidently, a similar conclusion was drawn by Billingham and Brent from studies concerning tolerance (5). These authors observed that while the intravenous injection of neonatal mice with allogeneic adult spleen cells induced tolerance to skin grafts from the spleen cell donor strain many of the animals became moribund soon after, with the severity of the disease varying with the strain combinations used. The onset of disease was characteristically marked by a lack of growth and unhealthy appearance (ruffled fur and abnormal gait), and was followed shortly thereafter by diarrhea, and eventually death. The disease was referred to as "runt" disease since the lack of growth resulted in animals considerably smaller in size than their control littermates.

In addition to the grossly visible changes in the runts, abnormalities in the lymphoid tissue were also apparent. Lymphoid tissue was shown to be involuted and hypoplastic with the lymph nodes being absent in the most severely affected animals.

On the basis of the above observations and what was then known about transplantation immunology at that time, Billingham was able to formulate the conditions under which GVH disease would arise (6). First, the graft must contain immunologically competent cells. This is supported by the observation that the severity of GVH disease was augmented with an increase in the number of lymphocytes in the graft (7). Second, the immunologically competent cells of the graft must be able to mount a response against transplantation antigens expressed on host tissues. It should be noted however, that cells from different lymphoid compartments are not equal in their ability to induce GVH disease. For example, thoracic-duct lymphocytes and lymph node cells are the most potent inducers of GVH disease, while spleen cells and bone marrow cells are less effective and thymocytes are ineffective even at high doses (7). Third, the host must be incapable of rejecting the graft (6). Different strategies have been used to prevent graft rejection and, as such, have formed the basis for the different animal models used to study GVH disease.

1.2 Murine Models of GVH Disease

1.2.1 F_1-Hybrid Disease

F_1-hybrid recipients manifest GVH disease-associated features after the injection of adult spleen and/or lymph node cells prepared from either parental strain. Because the recipients codominantly express transplantation antigens encoded by H-2 genes inherited from both parents, grafts from either parent are considered to be self and thus fail to elicit an immune response. However, the immunocompetent cells in the graft are not tolerized to the alloantigens of the other parent and are able to mount an immunological response against the recipient.

1.2.1.1 H-2 differences in class I and class II antigens

The severity and form of GVH disease that presents in unirradiated F1-hybrid recipients after the injection of parental cells depends upon the degree of histoincompatibility between donor and host. Differences across the entire major histocompatibility complex (MHC) result in the development of acute, lethal disease. This form of GVH disease, referred to as "suppressive" GVH disease by Gleichmann (8), is characterized by those features originally described by Billingham in neonatal runts (splenomegaly, pancytopenia, lymphoid hypoplasia) and in addition show immune dysfunction. For example, Lapp and co-workers (9) have described a model of acute GVH disease using (C57BL/6 (B6) x A/J)F_1 (B6AF_1)-hybrid

mice injected with spleen and lymph node cells isolated from either of the parental strains. Recipients in these parental $\rightarrow F_1$ ($P \rightarrow F_1$)-hybrid strain combinations exhibit hunched posture, cachexia and persistent diarrhea with most of the animals succumbing by day 35 - 40 of the reaction. Furthermore, the cellular and humoral immune responses of the recipients are profoundly suppressed as evidenced by their inability to reject skin allografts (10), the absence of a plaque-forming cell (PFC) response to T-dependent foreign antigens such as heterologous red cells (11), and by the inability of the T-cell specific mitogens concanavalin A (Con A) and phytahemagglutinin (PHA) and the B-cell specific mitogen lipopolysaccharide (LPS) to stimulate cellular proliferation (12). Similar observations with other $P \rightarrow F_1$ strain combinations have also been observed (eg. B6 $\rightarrow$ (B6 x DBA/2)F_1 (BDF_1)-hybrids (13).

1.2.1.2 H-2 differences limited to class II antigens

If the H-2 differences between the parental strains is restricted so that the disparity is limited to either class I or class II antigens then the severity and form of GVH disease in the F_1-hybrid recipient is altered or minimized. In the majority of $P \rightarrow F_1$ combinations involving class II differences, for example B6 $\rightarrow$ (B6 x B6.C.H.2^{bm12})F_1-hybrids (14), graft recipients develop a chronic form of GVH disease referred to by Gleichmann (8) as "stimulatory". Animals

suffering from this form of GVH disease do not develop the wasting syndrome characteristic of the suppressive form, but they do go on to develop a variety of autoimmune features characteristic of systemic lupus erythematosus (SLE). Survival times in these recipients (>100 days) is increased over those with the suppressive form; however, the production of autoantibodies in these animals generally leads to premature death as a result of immune-complex glomerulonephritis. In addition to the development of SLE-like features, lesions characteristic of other collagen vascular diseases, such as arteritis, Sjögrens syndrome and scleroderma, also appear depending upon the $P \rightarrow F_1$ combination used. These features have made stimulatory GVH disease an attractive experimental model with which to study autoimmunity.

Stimulatory or chronic GVH disease can also be produced in $P \rightarrow F_1$-hybrid combinations that differ at both class I and II if the donor $CD8^+$ cells have been first depleted from the graft with anti-Lyt-2^+, an antibody specific for the murine cell-surface marker on class I MHC-restricted cells, and complement prior to induction (15). While the injection of B6 spleen and lymph node cells into BDF_1-hybrid recipients normally results in acute GVH disease, the mortality can be prevented by removing $CD8^+$ cells from the graft. However, recipients still go on to develop autoimmune phenomena as indicated by the presence of serum autoantibodies against

nuclear antigen, dsDNA and thymocytes.

In the P $\rightarrow$ F_1 combination DBA/2 $\rightarrow$ BDF_1-hybrids recipients develop stimulatory GVH disease instead of the suppressive form despite class I and class II differences when either B6 or B10 parental strain mice are used (8,13). Although this appears to contradict the rule that disparity across the entire H-2 results in acute GVH disease, limiting dilution analysis on spleen cells from DBA/2 mice has shown almost a complete absence of precursor CTL that can react against B6 cells (16). The absence of allospecific CTL therefore makes this particular P $\rightarrow$ F_1-hybrid combination similar to those experimental models in which Lyt-2^+ were removed cells from the graft. These authors have suggested that the presence or absence of CTL activity determines which form of GVH disease develops; when CTL specific for the host are present in the graft, these cells kill the F_1-hybrid B cells thereby preventing autoantibody production. However, when host-specific CTL are absent, inappropriate helper activity from allo-specific L3T4^+ cells stimulates B cell proliferation (B cell hyperplasia) and differentiation through the release of cytokines and thus leads to autoantibody production and stimulatory GVH disease.

Animals with stimulatory GVH disease do not experience the profound immunosuppression exhibited by those with the suppressive form but do demonstrate partial suppression of cellular and humoral immune responses (14,17). Spleen cells

from mice with stimulatory GVH disease demonstrate suppressed CTL activity against TNP-modified spleen cells but normal allospecific CTL activity (14) indicating that only those CTL responses controlled by $L3T4^+$ cells, and not by $Lyt\text{-}2^+$ cells, are defective (14). Further mixing experiments showed that this immuno-suppression was mediated by an as yet unidentified suppressor cell. As was found with T cell responsiveness, suppression of B cell activity was not complete since only primary antibody responses were shown to be suppressed (17). BDF_1-hybrids injected with T-dependent antigens such as SRBC or TNP-KLH on day 16 or later exhibited suppressed PFC responses. The primary antibody response to the T-independent antigen levan was also suppressed on day 16; however, the suppression was only transient since PFC responses by day 20 were comparable to those demonstrated by normal F_1-hybrid mice. In contrast to the suppression of $L3T4^+$ TH activity, the deficiency in primary B cell responses does not appear to be mediated by a suppressor cell but to be associated with a defect in B cell maturation. In mixing experiments, splenic T cells from either normal F_1-hybrids or F_1-hybrids with GVH reactions cultured with normal F_1-hybrid splenic B cells and SRBC were shown to induce primary PFC responses to SRBC. However, when these T cell populations were incubated with B cells from F_1-hybrids with GVH reactions the SRBC PFC response was suppressed. The authors suggested that, in F_1-hybrid mice with GVH reactions, most B cells specific for exogenous

antigen are driven by excessive TH cell helper factors and, in an absence of antigen, into a terminally differentiated state without a significant increase in cell numbers. In the presence of fewer antigen-responsive B cells the primary antibody response would appear as a decrease in the number of PFC.

1.2.1.3 H-2 differences limited to class I antigens

In the majority of P $\rightarrow$ F_1-hybrid combinations involving only class I differences, or where disparity exists across the entire MHC, but $L3T4^+$ cells have been depleted from the graft, no effect has been observed on either immune function or survival (18). The injection of B6 cells or B6.C.H-2^{bm1} into (B6 x B6.C.H-2^{bm1})F_1-hybrid mice however is one of the exceptions to this rule. In this donor/host combination immune dysfunction is thought to result from a defect in L3T4 (TH) function.

The transfer of A/J donor cells into (CBA x A/J)F_1 (BAF_1)-hybrid mice produces chronic GVH disease, the severity of which is dependent upon cell dose; the majority of recipients survive beyond 100 days after injection of 50 x 10^6 donor cells, while less than 50 % remain alive by day 50 of the reaction if 70 - 100 x 10^6 cells are given (19). These recipients also exhibit lasting T and B cell immune dysfunction similar to animals with suppressive GVH disease (20). However, these animals show only minimal weight loss

(9). The strength of GVH reactions in this P → F_1 combination, despite disparity at a single class I loci (*H-2D*), has been attributed to the combined effects of multiple minor histocompatibility antigen differences, including those encoded at the minor lymphocyte stimulatory (*mls*) loci (18).

1.2.1.4 Hybrid Resistance

In the F_1-hybrid model, the cell dose required to produce GVH disease varies with the P → F_1-hybrid strain combination used. For example, as few as 10^7 A/J lymphoid cells are required to induce acute GVH disease in B6AF_1-hybrid mice while a minimum of 3 x 10^7 B6 cells are required (9,21). The requirement for a minimum donor cell number to induce a GVH reaction has been attributed to the necessity to override the capacity of F_1-hybrid animals to reject lymphoid cell grafts, a phenomenon called "hybrid resistance". Hybrid resistance can occur despite histocompatibility at the H-2 locus and codominant expression of H-2 genes; it has been attributed to allorecognition of hybrid histocompatibility (Hh-1) antigens encoded by genes which map close to H-2*D* (22). Unlike class I antigens, Hh-1 antigens are expressed only when an animal is homozygous for *Hh-1* genes (as in the case of inbred strains) thus making parental strain lymphoid grafts susceptible to immune recognition by F_1-hybrids.

Another possible explanation for hybrid resistance involves the differential binding of a self peptide by allelic

MHC gene products (23). Mice from each inbred parental strain express a single allelic form of each MHC class I and class II antigens. These MHC molecules when complexed with self peptide can induce tolerance to self. In the F_1-hybrid, two allelic forms of each MHC molecule are expressed equally. If both of these allelic MHC gene products are able to bind the same peptide, then it is predicted that each will compete for binding of this peptide. Because of this competition it is also predicted that most of the peptide will be complexed with the one MHC molecule having the greater affinity for the peptide. If the concentration of the peptide complexed with the MHC product having the weaker binding affinity is too low, then tolerance is not induced. Therefore, F_1-hybrid mice engrafted with cells from parental strain mice expressing the MHC gene product with the weaker binding affinity will view these cells as non-self since they have not been tolerized to the peptide in the context of that MHC molecule.

It is believed that NK cells are the principal cellular mediators of hybrid resistance. Cells bearing an NK cell phenotype, isolated from SCID (severe combined immunodeficiency) mice and expanded in IL-2 *in vitro* have been shown to restore hybrid resistance when adoptively transferred into mice rendered unable to reject bone marrow grafts by *in vivo* depletion with anti-NK1.1 (24). It appears that immune recognition of specific *Hh-1* alleles is not a property shared by all NK cells but restricted to a particular NK cell subset.

In vivo depletion of (C3H x B6)F_1-hybrid mice with SW5E6, a monoclonal antibody (MoAb) specific for 50 % of NK1.1$^+$ cells, has been shown to abrogate the capacity to reject *Hh-1^d* BM grafts, but not to prevent the rejection of *Hh-1^b* BM grafts (25).

Recently, a cell bearing both NK and T cell markers has also been implicated in mediating hybrid resistance (26). Cells expressing NK1.1, $ASGM_1$, CD3 and T cell receptor (TCR), but lacking CD4 and CD8, have been shown to transfer graft resistance into syngeneic F_1-hybrid recipients rendered non-responsive after treatment with cyclophosphamide.

1.2.2 Parabiosis

The engraftment of one complete individual to another so that the circulation is shared between the two animals is known as parabiosis. If sufficient histoincompatibility exists between the animals and if one of the animals is immunologically nonreactive to the other, as would be the case in a parental-F_1-hybrid pairing, GVH disease will develop (6). The outcome of acute GVH disease in this model is similar to what is observed in the F_1-hybrid model. Affected animals develop a severe wasting syndrome, referred to as parabiosis intoxication, and become severely anemic.

1.2.3 Secondary Disease

As an alternative to models employing genetic manipulation, host tolerance to the graft can also be achieved by ablating the host's immune system. In this model of GVH disease the graft will not be rejected, whether the graft is allogeneic or only semiallogeneic. It has long been recognized that animals who have been lethally irradiated succumb rapidly to radiation sickness unless they are reconstituted with hemopoietic stem cells (27). Not long after demonstrating this protective effect it was observed that grafts other than those from a syngeneic donor did not provide longterm survival. It was noted that CBA mice given allogeneic grafts prepared from A/J strain mice developed a severe wasting syndrome and skin lesions, with mortality reaching 100 % before day 100 (28). This form of GVH disease was called "secondary disease" because GVH disease followed the radiation sickness and was originally thought to be a secondary consequence of irradiation.

The secondary disease model of GVH disease most closely resembles the treatment regimen used in bone marrow transplantation in man. However, it is difficult to separate those changes in the host due to X-irradiation from those caused by the effects of the graft since many of the tissues susceptible to the effects of X-irradiation are also targeted during GVH disease (6,9).

1.2.4 Tolerance Induction in Neonates

Animals can be shown to acquire tolerance towards allografts if they are sufficiently young, and have been injected with an allograft cell suspension prepared from the same donor (29). For example, the transfer of adult Lewis rat strain BM cells into neonatal (1-3 days old) BN strain rats was shown to tolerize the recipients to a skin allograft given four weeks later (7). The immunologically immature state of the neonates rendered them incapable of rejecting the allograft. For this reason the model used to study tolerance can then also be used to induce runt disease in neonatal mouse recipients; however, instead of giving adult BM cells which are lacking in mature T cells, adult spleen, LN or thoracic duct lymphocytes must be used (7). The intravenous injection of homologous adult spleen cells into newborn recipients has been shown in some donor-recipient combinations to cause acute lethal GVH disease (30).

1.3 Features of Acute GVH disease

1.3.1 Splenomegaly

The enlargement of the spleen after engrafting homologous lymphoid cells was shown by Simonsen (4) to be a reliable indicator of GVH disease. It was first believed that the massive accumulation of cells in the spleen and other secondary lymphoid organs resulted from the proliferation of

donor lymphocytes in response to host antigens. However, the transfer of spleen cells from $B6AF_1$-hybrid mice injected with A/J strain spleen cells failed to induce splenomegaly in either secondary $B6AF_1$ hosts or in (A x ASW)F_1-hybrid semiallogeneic recipients. This would have been expected had the proliferating cells in the spleen of the $B6AF_1$-hybrid recipient been of donor origin (31). It was thus concluded that donor cells contribute very little to the enlargement of the spleen and that this is due mainly to proliferation of host cells. This was later confirmed in F_1-hybrid mice with GVH disease by analyzing dividing spleen cells for the presence of marker chromosomes specific to the donor strain or for sex-chromosomes in sex-unmatched P $\rightarrow$ F_1-hybrid combinations (male donors and female recipients) (32,33). Both techniques demonstrated that donor cells comprise the majority of the proliferating cells from day 2 to 8 of the reaction after which the host cells predominate.

Host cell proliferation has been shown to require donor T cells since treatment of the graft with anti-Thy-1 ablates the proliferative response (34). However, release of the mitogenic signal does not require a proliferative response by alloreactive donor T cells since mitomycin-treated donor lymphocytes are able to both stimulate proliferation in one-way mixed-lymphocyte cultures (35), and induce splenomegaly *in vivo* (34).

The extent to which different populations of host cells

contribute to splenomegaly appears to vary with time. For example, early in the reaction (days 1-10) macrophages comprise 20-30 % of the cells in the spleen and are believed to mediate the suppression of T-dependent responses in the spleen at this time (36). Later, host B lymphocytes comprise the majority of cells in the spleen (34,37). It has been shown that only B lymphocytes that express MHC antigens to which the alloreactive response is directed are induced to proliferate, suggesting that F_1-hybrid B cell activation is a two step process (38). It has been suggested that T cell recognition of allo-MHC determinants on F_1-hybrid B cells leads to the activation of both cell populations and to the release of lymphokines by T cells which stimulate the proliferation of activated B cells.

The size of the spleen diminishes in the latter stages of the reaction. This has been attributed to the hypocellularity of the organ. The lymph nodes being similarly affected.

1.3.2 Immune Suppression

It was well established quite early in studies of GVH disease that both cellular and humoral immune responses are profoundly suppressed by the reaction (10,39). For example, complete suppression of the PFC response to SRBC has been observed by day 7 of the acute GVH reaction using the $P \rightarrow F_1$-hybrid combination B6 into $B6AF_1$-hybrids (21). It was noted that the level of suppression varied with cell dosage and that

only 50 x 10^6 cells were required to produce 100 % suppression. Mitogen-induced proliferation of both splenic T and B cells has been shown also to be suppressed by this reaction (40).

Different cell types, acting at different times in the reaction, are believed to contribute to the immune suppression. In the initial stages (day 5 to 18), immune suppression is thought to be mediated by activated macrophages (36) since removal of this cell population, either by plastic adherence or by iron particle phagocytosis from spleen cells isolated from animals with GVH reactions, can restore the *in vitro* PFC response to SRBC to normal control values.

The mechanism by which macrophages cause immune suppression is believed to result from the release of prostaglandin (PG) E (41). Negative selection of splenic macrophages by adherence to rayon columns up to day 15 of the reaction was shown to partially restore T and B cell mitogenic responses. The effectiveness of this treatment declined with time so that by day 20 no recovery from suppression was observed. GVH animals treated with indomethacin, a drug which blocks PG synthesis, produce a significantly greater PFC response than non-treated animals, indicating that suppression of antibody responses was, in part, due to increased PG synthesis.

It has been shown that non-specific suppressor cell activity is also associated with non-adherent spleen cell

populations (42). A Thy-1$^-$ plastic non-adherent spleen cell population isolated from (C57BL/6 x CBA)F_1-hybrid mice with acute GVH disease 15 - 20 days post-induction was shown to significantly contribute to suppression of the *in vitro* PFC response against SRBC generated by donor or host genotype responder cells (40). Similarly, in a model of chronic GVH disease across multiple minor loci, a Thy-1$^-$, plastic non-adherent cell was shown to suppress mitogenic, MLR and CTL responses in a non-MHC-restricted manner (42). This cell was termed "natural suppressor" (NS) because it co-fractionated with NK cells in Percoll. NS cells were surface Ig$^-$, and therefore were not B cells. Despite their morphological similarity to NK cells, NS cells did not express NK1.1 and failed to exhibit cytolytic activity against YAC-1 target cells. Peak levels of NS cell activity occurs two weeks post-induction with the activity decreasing slowly thereafter. It is believed that NS cells mediate suppression through the release of soluble mediators. It has been observed that NS cells separated from an MLR culture by a semipermeable membrane still suppressed CTL activity in the culture. It was shown that indomethacin could partially restore immune responsiveness suggesting PGE may have been one of the mediators involved. The release of PGE by NS cells might explain why rayon columns were unable to deplete all PGE production from GVH spleen cell cultures after the first two weeks of the reaction (41).

NS cell activity has also been shown to be inducible with IFNγ (43). Recently, IFNγ production by spleen cells isolated from mice with acute GVH disease have been shown *in vitro* to be enhanced in response to Con A (44). It was suggested that IFNγ production by TH1 cells, which are preferentially activated during acute GVH reactions, induces NS cell activity.

It has been suggested that $CD8^+$ cells exhibit an allospecifc suppressor function in $P \rightarrow F_1$-hybrid combinations where cytotoxic cells specific for host antigens are generated (18,40). It has been shown that in such parental-strain combinations, $CD8^+$ cells, expressing $ASGM_1$ but negative for NK1.1, comprise approximately 20 % of the splenic lymphocyte population and possess strong host-specific cytotoxic activity (45). It is believed that these cells suppress immune functions by eliminating host-derived responder cells. This idea is supported further by the observation that PFC responses generated by cells of donor genotype are not suppressed to the same extent as those by cells of host genotype when the responder cells are co-cultured with GVH spleen cells during the first 10 days of the reaction (40).

The period in GVH reactions in which specific and non-specific suppressors cells function is of relatively short duration. All suppressor cell activity disappears between 20 - 30 days post-induction (9). However, animals with GVH reactions remain profoundly immunosuppressed for the rest of

their lives. It is believed that this permanent immunosuppression results from defects in T and B cell maturation (9). For example, unlike thymocytes isolated from mice in the early stages of GVH disease, thymocytes from GVH mice 40 days post-induction and beyond fail to restore the ability of adult thymectomized, BM reconstituted BAF_1 mice to reject skin allografts and to provide TH cell activity. It was suggested that the cause of this lasting immunosuppression is injury to the thymic epithelium. It was postulated that as yet unknown, cytolytic effectors enter the thymus and cause lesions in both the cortex and medulla resulting in thymic epthelial injury. With the resulting defect in the maturational environment of the thymus, T-cell development and function is impaired (46). An analogous mechanism has been described for the development of longterm B cell suppression (47,48). In the BM stromal, cells are believed to be a source of growth and differentiation factors necessary for normal B cell development. So, like the medullary epithelial cells in the thymus, the stromal cells appear to be the target of effector cells during GVH reactions. This results in an arrest of B cell maturation.

1.3.3 Cachexia

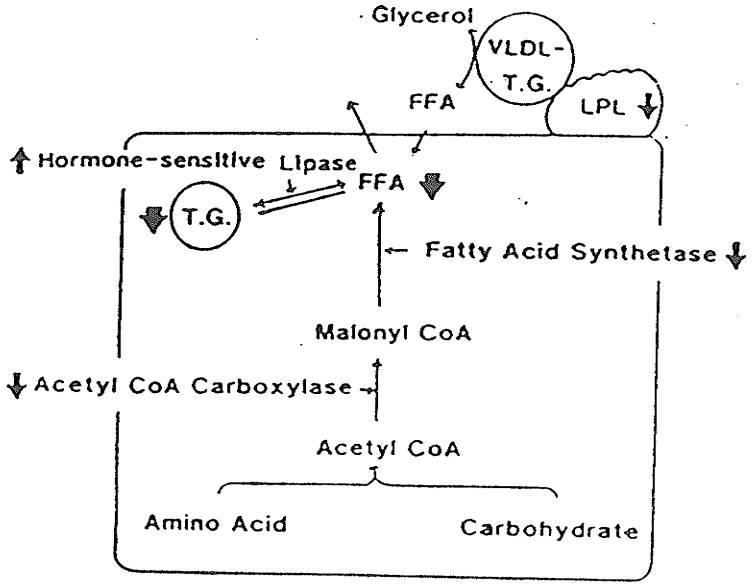
Animals with acute GVH disease develop a severe wasting syndrome (cachexia) characterized by excessive weight loss, weakness and anemia (49). The same condition also has been

described in patients with cancer, acquired immune deficiency syndrome (AIDS), and chronic parasitic infections (50-52).

Cachexia is also associated with specific biochemical changes. Rabbits exhibiting cachexia after infection with the parasite *Trypanosoma brucei* have high serum levels of very low-density lipoprotein (VLDL) (hypertriglyceridemia). This condition is thought to result from the suppression of cell surface-associated lipoprotein lipase (LPL), the enzyme responsible for the uptake of fatty acids from VLDL, and by the continual catabolism of adipocyte intracellular energy stores due to the suppression of lipogenic enzymes and the up-regulation of a hormone sensitive lipase (53,54). Further studies using endotoxin-sensitive (C3H/HeN) and resistant (C3H/HeJ) mouse strains have shown that the suppression of LPL is mediated by a factor released from macrophages in response to endotoxin, since cachexia could only be induced in endotoxin-resistant strains by transferring serum taken from LPS-stimulated endotoxin-sensitive mice (55).

The LPL-suppressing factor, termed cachectin, has been purified by isoelectrofocusing from endotoxin-stimulated RAW 264.7, a macrophage cell line, and has been shown to have a molecular weight of 17 kDa (56). Scatchard analysis of the binding of iodinated, purified cachectin demonstrated high affinity receptors on adipocytes, as well as muscle cells and hepatocytes, but not lymphocytes or erythrocytes; thus LPL-induced catabolism of energystores is not restricted to

Figure 1.1 The enzymes affected by TNFα leading to the catabolism of fat stores. Taken from Kawakami, M., et al. Specificity in metabolic effects of cachectin/TNF and other related cytokines. Ann. N.Y. Acad. Sci. 587:339, 1990 (54).


Glycerol
VLDL-
T.G.
FFA
LPL
Hormone-sensitive Lipase
FFA
T.G.
Fatty Acid Synthetase
Malonyl CoA
Acetyl CoA Carboxylase
Acetyl CoA
Amino Acid
Carbohydrate

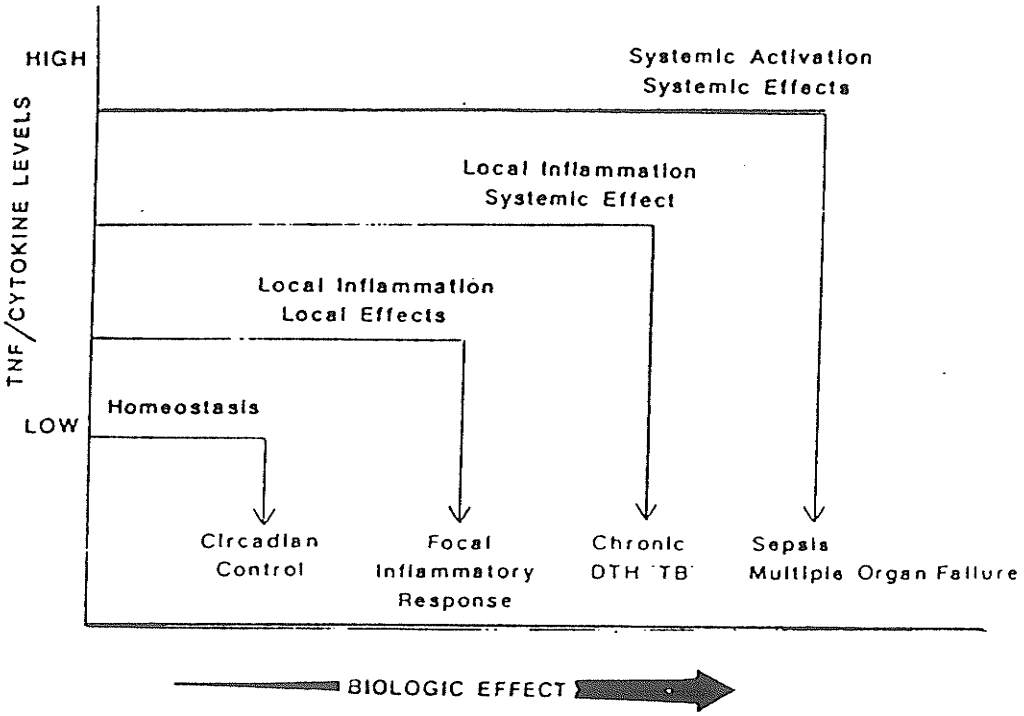
adipocytes (57). Glycogen was shown to be depleted from the muscle cell line L6 after treating the cells with a crude monokine preparation created from endotoxin-stimulated RAW 264.7. The depletion of glycogen was concomitant with the up-regulation of fructose 2,6-biphosphate, an important enzyme in glycolysis. A study incorporating the periodic administration of sublethal amounts of recombinant cachectin has provided strong evidence that this cytokine is responsible for cachexia (58). Rats given 250 μg/kg of cachectin twice daily have been shown to develop features of cachexia (anorexia, weight loss and anemia) which could be prevented by treating the recipients with anti-cachectin antibodies. The catabolic effects induced by cachectin can also be potentiated by IL-1. Studies have shown that IL-1 can augment the catabolic effects of cachectin by decreasing the synthesis of LPL thereby decreasing the utilization of fat for energy (59).

It was not until cDNA for the gene coding for cachectin had been isolated that it was realized that it was identical to that encoding tumor necrosis factor (TNF) (60). TNF, a serum factor induced in bacillus Calmette-Guerin (BCG)-treated mice after stimulation with LPS, has been shown to mediate killing of tumor cells *in vitro* and *in vivo* (61). The existence of a serum factor capable of causing *in vivo* tumor regression had been documented a century earlier by Coley (62) who observed that cancer patients that had developed erysipelas or who had been given a filter-sterilized mixture

of bacterial extracts (Coley's toxin) demonstrated partial, and in some cases complete, resolution of their malignancy. Macrophages and monocytes are believed to be the primary source of TNFα; however, T cells and other mononuclear cells with an LGL morphology have also been implicated in its production. An assortment of stimuli are able to induce TNFα secretion. Included among these are LPS, phorbol esters, and cytokines such as TNF, IL-1, GM-CSF, and IL-2 (63). In addition to the secreted form of TNF, a transmembrane-associated form has been shown to exist on the surface of macrophages and both $CD4^+$ and $CD8^+$ T cells (64,65). Like the soluble 17 kDa TNF molecule, the 26 kDa membrane-associated TNF mediates cytotoxicity of TNF-sensitive target cells (64-66). Membrane-associated TNF has also been shown to be more effective than secreted TNF in the activation of macrophages against Leishmania (67). It is believed that TNF is first expressed in 26 kDa form after activation and that cleavage of this transmembrane molecule during degranulation gives rise to the 17 kDa form (64).

TNF, like many of the cytokines being studied today, has been shown to be pleiotropic, capable of mediating a variety of functions in both immune and non-immune cells, as well as cachexia and tumor cell killing (immune surveillance). Which functions predominate at any given time and whether these functions prove beneficial or deleterious to the host seems to depend on the amount of TNF produced (Figure 1.2, 68).

Figure 1.2 Biological consequences associated with various concentrations of TNFα. Taken from Kunkel, S.L., Remick, D.G., Strieter, R.M., and Larrick, J.W. Mechanisms that regulate the production and effects of tumor necrosis factor-α. Crit. Rev. Immunol. 9:93, 1989 (68).


HIGH
LOW
TNF/CYTOKINE LEVELS
Systemic Activation
Systemic Effects
Local Inflammation
Systemic Effect
Local Inflammation
Local Effects
Homeostasis
Circadian
Control
Focal
Inflammatory
Response
Chronic
DTH TB
Sepsis
Multiple Organ Failure
BIOLOGIC EFFECT

The control of different functions by TNF concentration is believed to result from differential binding to two different high affinity TNF receptors, TNF-R1 and TNF-R2, which exist on most somatic cell (69). IFNγ, IL-2 and certain lectins up-regulate the expression TNF receptors while phorbol esters, IL-1, LPS, glucocorticoids and TNF have the opposite affect (70). These receptors, TNF-R1 (55 kDa) and TNF-R2 (75 kDa), are transmembrane glycoproteins sharing 30 % homology in their extracelluar domains; in particular 4 cysteine-rich repeat units which are believed to comprise the the ligand binding site (71). Little homology is found between the cytoplasmic portions of the two TNF receptors, therefore it has been suggested that certain functions are associated with a particular receptor. In support of this idea, mouse thymocytes and the mouse T cell line CT-6 are induced to proliferate when incubated with rabbit polyclonal antiserum directed against murine TNF-R2, but not with antiserum against TNF-R1 (72). In contrast, treatment with anti-TNF-R1 but not anti-TNF-R2 antiserum induces cytotoxicity, as well as, manganese superoxide dismutase mRNA expression in TNF-sensitive cell lines. Because TNF-induced proliferative responses can be demonstrated at concentrations insufficient to induce TNF-R1-associated functions, TNF in low concentrations is believed to bind to the higher affinity transmembrane TNF receptor, TNF-R2, and to be associated with processes involved in maintaining cellular and/or tissue

homeostasis (68). Because TNF levels increase in response to trauma it is likely that TNF may have some effect on the course of an inflammatory reaction. It has been shown that TNFα can induce expression of the intercellular adherence molecule CDw18 on the surface of both cultured human umbilical vein endothelial cells and neutrophils, thus facilitating margination and migration of inflammatory cells into sites of injury. TNF has also been shown to augment phagocytosis, antibody-dependent cellular cytotoxicity, induce the production of colony-stimulating factors from endothelial cells and fibroblasts and act as a fibroblast growth factor. Fibroblast proliferation, like *in vitro* killing of TNF-sensitive targets, is a property of TNF binding to TNF-R1 receptor (72,73). As already mentioned, binding of TNF to TNF-R1 also induces manganous superoxide dismutase, a mitochondrial enzyme that increases resistance against TNF-mediated lysis (72). Other protective proteins whose expression is known to be up-regulated are ferritin heavy chain and heat shock proteins (HSP) (74). These findings support the hypothesis that cells that bind TNF through TNF-R1 synthesize proteins which protect the cells from TNF-mediated lysis. If, however, transcription of these protective molecules is blocked, as occurs when the cells are treated with actinomycin-D, then the cells become more sensitive to TNF-mediated lysis (72,74).

If the assault on the host is not resolved and TNF

production continues, cachexia ensues. Excessive serum concentrations of TNF, which are observed experimentally following injection of LPS or as a result of a gram-negative septicemia, can lead to septic shock, multiple organ failure and death. Disseminated intravascular coagulation resulting from septic shock has been attributed to TNF-mediated stimulation of a procoagulant factor and the suppression of thrombomodulin expression by endothelial cells (75).

1.3.4 Histopathology

Animals with acute GVH reactions develop numerous pathological changes in different organs. However, the organs that are most vulnerable are the lymph nodes, spleen, BM, thymus, GI tract and liver. Epithelial cells often appear to be selectively targeted by the reaction.

1.3.4.1 Lymphoid organs

During the first week of the reaction lethally-irradiated graft recipients exhibit hypocellularity in primary and secondary lymphoid organs, but no clinical signs of disease (76). The graft enters the lymphoid organs and begins to proliferate. This appears histologically as granulocytic hyperplasia in the bone marrow and red pulp of the spleen, and by lymphoblastoid cell infiltrates in the thymus, lymph nodes and Peyer's patches. By the end of the second week lymph node architecture becomes obliterated with the appearance of

reticular fiber fragmentation and necrosis in the paracortical region. The thymus undergoes stress involution with loss of the cortical medullary demarcation and lymphocyte depletion.

Injury to the thymus can be observed in F_1-hybrid recipients with acute GVH reactions as early as day 6 (77). In these mice, stress involution, resembling "thymic dysplasia", accounts for the reduction in the size of the thymus and the loss of cortical lymphocytes, since neither of these conditions are observed in adrenalectomized mice with GVH disease (78). However, some changes in the thymus, primarily in the medulla, are not stress-related but are mediated by GVH disease-associated mechanisms. These changes include the loss of corticomedullary demarcation, medullary epithelial cell disorganization as a result of infiltrating lymphocytes, loss of Hassall's corpuscles and the incursion of macrophages (78). Injured epithelial cells can be recognized by the presence of a pyknotic nucleus and shrunken cytoplasm (76). All lymphoid organs in the latter stages of acute GVH disease become grossly atrophic and devoid of lymphocytes in both radiation chimeras and F_1-hybrid recipients. A similar sequence of histopathological changes in the thymus has been described for mice with chronic GVH reactions. However, in animals surviving 180 days post-induction, complete regeneration of the cortical and medullary regions was observed (79). Complete regeneration of the thymus was followed by a return of T cell functions.

1.3.4.2 Epithelial tissue

Several histopathogical studies of animals and patients with acute GVH reactions have shown that injury occurs predominantly in the epithelial cells of a wide variety of organs (76,80,81). These changes consist of lymphocytes infiltrating the epithelium. Some of the most frequently affected sites are the liver, skin, lung, intestinal tract, salivary gland, oral mucosa, tongue and esophagus. Infiltrates in the liver appear as clusters of lymphocytes in portal tracts close to bile ducts. Other features of hepatobiliary GVH disease include infiltrates around central veins and coagulative necrosis of hepatocytes (80,81).

Necrosis of crypt epithelium is a distinct feature of intestinal involvement and, if extensive, can lead to the disintegration of the entire crypt (81). Studies of skin and mucosal membrane surfaces by electron microscopy have identified infiltrating lymphocytes in direct contact with epithelial cells undergoing degenerative changes (82). The description of a dyskeratotic cell bordered by one or more lymphocytes has been termed "satellite dyskeratosis" and is strongly supportive of a cytotoxic effector cell in the pathogenesis of GVH disease (80).

1.4 Pathogenesis of Acute GVH Disease

1.4.1 T cells

It is well established that removing T cells from a bone marrow allograft is an effective prophylaxis against GVH disease, suggesting that T cells are important in the pathogenesis of GVH disease (83,84). However, there are a number of disadvantages to this practice. First, leukemic patients exhibit a higher incidence of relapse after receiving a T cell-depleted graft (85). The lower relapse rate with a non-depleted graft is believed to arise from the elimination of residual tumor cells by a process commonly referred to as the "graft-vs-leukemia" (GVL) effect. It is believed that the cells which mediate the GVL effect are allospecific CTL and a population of Thy-1$^+$, CD4$^-$, CD8$^-$, ASGM$_1^+$ effector cells recruited in response to cytokine released during the allogeneic phase of the reaction (86). Second, the removal of T cells leaves the recipients in a severely immunocompromised condition, and susceptible to opportunistic infections (87). Third, an increase incidence of graft failure has also been observed with T-cell-depletion (88). For example, B6 recipients that had been first pre-conditioned by either total body irradiation or total lymphoid irradiation and cyclophosphamide developed GVH disease and died when given a BALB/C BM-spleen cell graft (89). However, if the same graft had been first treated with anti-Thy-1.2 and complement the

recipients survived but without engraftment. It has been suggested that T cells may encourage engraftment by secreting colony-stimulating factors (CSF) such as IL-3 and GM-CSF (89,90).

For the most part, T cells with a TH cell phenotype ($CD4^+$) mediate helper cell activity, while $CD8^+$ express CTL activity. Because of the apparent functional dichotomy between these T cell subsets it was originally speculated that elimination of one or the other of these would have a different effect on the outcome of acute lethal GVH reactions. In donor-host combinations involving differences across the entire MHC, both T cell subsets have been implicated in the pathogenesis of acute GVH reactions (91,92), but the severity of the reaction produced by either T cell subset appeared to depend on the strain combination (92). When recombinant and mutant mouse strains where used to examine H-2 differences restricted to either class I or class II antigens, only purified $CD4^+$ cells were able to induce GVH reactions against class II alloantigens while $CD8^+$ cells were only effective against class I differences (93,94). In studies where donor alloreactivity was limited to only minor histocompatibility antigenic (mHA) differences, both T cell subsets were able to initiate acute GVH reactions, but again, their effectiveness varied with the strain combinations.

Immunohistologic studies have revealed that few of the mononuclear cells infiltrating non-lymphoid organs in GVH

disease are $CD8^+$ T cells. Most of these cells appear to have morphological features of LGL and are Thy-1^+, $ASGM_1^+$, Ly-1^-, and Lyt-2^- (95,96). On this basis Ferrara (95) has argued that alloreactive T cells provide help through the production of lymphokines which in turn activate this, as yet, uncharacterized effector cell population. Until recently it was believed that T cell help was provided solely by $CD4^+$ T cells; however, alloreactive $CD8^+$ T cell clones are now know to produce IL-2, IL-3, IL-4, IL-5, IFNγ, GM-CSF and TNFα (97). It is therefore quite conceivable that either T cell subset is able to induce acute lethal GVH disease: but, the specific subtype involved in a given reaction is genetically restricted by the strain combination.

While the function of T cells during the inductive phase of GVH reactions is reasonably well understood there has been no unequivocal demonstration that T cells are the effector cells that mediate the tissue damage associated with acute GVH reactions. Many of the early attempts to characterize the effector cell population focused on CTL. The rationale for these studies stemmed from several observations. It has been shown that the appearance of CTL in mixed-lymphocyte cultures prepared with donor and host cells was predictive of mortality from acute GVH disease (98,99). CTL have also been identified as effector cells in allograft rejection (100). Finally, CTL activity against host MHC has been consistently demonstrated by lymphocytes harvested from mice with acute GVH reactions

directed against either the entire MHC (101) or class I alloantigens (102). One exception though has been described using the P → F_1-hybrid strain combination CBA → (CBA x BALB/C)F_1-hybrids. In these studies, no CTL activity against P815 (H-2^d) was detected in cell populations isolated from the spleen, mesenteric lymph nodes and intestinal intraepithelial lymphocytes (IEL) of F_1-hybrids with acute GVH reactions (103). Attempts to show that CTL activity was also generated in mice with acute GVH reactions directed solely at class II differences have been inconsistent. For example, sublethally-irradiated B10.AQR (Ia^k) mice injected with B10.T(6R) (Ia^q) lymph node cells and T-depleted BM cells did develop host-specific CTL activity, but no such activity could be elicited in sublethally-irradiated (B10.T(6R) x B10.AQR)F_1-hybrid mice reconstituted with B10.AQR splenic lymphocytes (104). It is of interest to note that in a strain combination involving class I differences only, the addition of exogenous IL-2 accelerated mortality without causing a concomitant increase in CTL activity (104). Clearly this observation argues against the idea that CTL are the only effector cell mechanism in GVH disease. Moreover, F_1-hybrid mice with acute GVH reactions which developed more severe intestinal lesions after injection with polyinosinic-polycytidylic acid (poly I:C) did so without showing a change in CTL activity (105). In the light of these experimental observations it is interesting that anti-host CTL activity was measured recently in the

majority of recipients of HLA-identical BM in the absence of GVH disease (106). Collectively, these observations raise questions about the actual role of CTL effector cells during acute GVH reactions.

In a recent study of an infant with combined immunodeficiency disease, the child was observed to have clinical features of acute GVH disease including hepatosplenomegaly, generalized lymphadenopathy and a severe dermatitis (107). Two-color flow cytometry of PBL and immunohistochemical staining of frozen sections of skin and lymph nodes revealed that the majority of the lymphocytes expressed a cell-surface phenotype remarkably similar to what was described by Ferrara (95) in mice with GVH disease; ie. these cells were $CD8^-$ and $CD4^-$. They also showed that these cells expressed CD3 and TCRα/β. Stimulation of these cells with PHA induced the production of IFNγ and TNFα and TNFβ. A cell line developed from the PBL of this patient during the active phase of the disease exhibited non-MHC-restricted cytotoxic activity against both NK-sensitive and NK-resistant tumor targets (108). This pattern of cytolytic activity contrasted with a cell line developed from the same patient during treatment with cyclosporin A which did not exhibit lytic activity against any of the tumor targets (108,109). These findings suggest that an unconventional T cell, lacking CD4 and CD8 expression but expressing CD3/TCRα/β complex, may be involved in the pathogenesis of acute GVH disease.

1.4.2 Natural killer (NK) cells

1.4.2.1 Murine studies

Both animal and human studies have shown that NK cell activity is augmented during the course of the GVH reaction. This has led to the idea that NK cells are involved in the pathogenesis of GVH disease. The induction of non-specific cytotoxic cells during GVH reactions was first reported by Singh et al. (110,111) who showed that spleen cells isolated from B6AF$_1$-hybrid mice inoculated with lymphoid cells from either parental strain could lyse a variety of MHC-unrelated tumour targets. This activity could be depleted with antiserum against F$_1$-hybrid cells or could be prevented by irradiation of the host before induction. These findings suggested that the effector cell involved was host-derived.

Other investigators using the same parental-F$_1$-hybrid combination have studied the time course of NK activity in different lymphoid tissues over the course of the reaction (112). Splenic NK activity was shown to peak on day 3 and rapidly decline and remain at below control levels. The time at which splenic NK activity was maximal was shown to vary with the size of the graft (113). NK activity peaked on day 8 after injection of 3.0×10^7 parental cells, and on day 16 when lower cell doses were used. Histopathological lesions of a moderate-to-severe grade were observed in the pancreas and liver on day 16 of the reaction only in mice with an early augmentation in NK activity (day 3). The authors thus

concluded that the appearance of GVH disease-associated lesions were related more with the fact that NK activity appeared early in the reaction than with the actual level of NK cell activity that was generated. They also went on to suggest that the higher doses of donor lymphoid cells generated levels of T cell activity sufficient to recruit both donor and host NK cells which lead to the formation of GVH disease-associated lesions. NK activity in the lymph node was shown to peak later than splenic NK activity and remained elevated for a longer period of time before returning to control values (112).

One group of investigators has been able to show that NK activity is also elevated in the thymus of F_1-hybrid mice with chronic GVH reactions (112). Thymic NK activity was characterized by an initial burst of cytolytic activity that gradually returned to control levels 30-50 days after induction. This was followed by a second burst of NK activity later in the reaction which was more intense then the first, and remained elevated for as long as 140 days post-induction.

Similar findings regarding the augmentation of NK activity has been reported by other investigators using different parental-F_1 hybrid combinations. For example, augmented NK activity has been measured in spleen cells, mesenteric lymph node cells and intestinal IEL in (CBA x BALB/c)F_1-hybrid mice injected with parental CBA lymphoid cells (103). However, this study failed to demonstrate NK

activity in the thymus. Also, the NK cell activity was insensitive to depletion with anti-Thy-1.2 plus complement and correlated with lymphoid cell proliferation. In a following study the same authors using the same P → F_1-hybrid combination showed that parental strain mice injected into the footpad with spleen cells from F_1-hybrid mice with GVH reactions induced an anti-host delayed-type hypersensitivity (DTH) reaction (114). Because the cells which caused the DTH reaction were activated prior to the appearance of augmented NK activity in the spleen the authors suggest that the cells responsible for the DTH reaction released cytokines which augment NK cell activity. These same authors have also shown that treatment of the F_1-hybrid recipients with anti-$ASGM_1$ and complement is able to remove endogenous NK cell activity, inhibit GVH disease-induced NK activity and cause a reduction in the number of IEL found in the intestine (115). They concluded from their studies that both host and donor NK cells are recruited by lymphokines following an anti-host DTH response and speculated that NK cells are involved in the effector phase of the enteropathology associated with GVH disease.

Whether or not NK cell activity is augmented during GVH reactions appears to depend both on the complement of T cells in the graft and the genetic differences between donor and recipient. NK activity was shown to be suppressed in $B6D2F_1$-hybrids injected with 100 x 10^6 B6 parental cells, while, F_1-

hybrids of the same strain combination given a smaller donor inoculum, 50 x 10^6 cells, demonstrated an enhanced NK activity by day 14 (116). If D2 parental cells were injected, the B6D2F_1-hybrids developed a chronic GVH reaction and demonstrated neither a depressed nor enhanced NK cytotoxicity irrespect of the number of donor cells injected.

Further evidence supporting the idea that NK cells are involved in the pathogenesis of GVH disease has come from experiments employing F_1-hybrid mice carrying the beige (*bg*) mutation (117,118). Mice homozygous (*bg/bg*) at this gene locus have demonstrated impaired NK cell function (119,120). F_1-hybrid recipients (*+/bg* or *bg/bg*) of grafts from *bg/bg* donor mice exhibited increased survival even though they developed splenomegaly, suppressed PFC responses to SRBC, augmented NK cell activity and exhibited only partial suppression of the T cell mitogen response following transplantation than mice that had received grafts from normal donors (118). Similarly, *+/bg* and *bg/bg* F_1-hybrid recipients of *+/bg* grafts developed early splenomegaly, complete suppression of PFC and T cell mitogen responses and augmented NK cell activity. However, moderate-to-severe histopathological changes were only observed when *+/bg* donors were used (117). Because survival depended on the donor mice being *bg/bg*, and since host-NK cell activity was augmented in all recipients, it was suggested that NK cells of donor origin and not host-derived NK cells, when activated during a GVH

reaction, are involved in the pathogenesis of GVH disease. However, it should be noted that, mice homozygous for the beige mutation have also shown a reduced capacity to generate a cytotoxic T cell responses after *in vivo* or *in vitro* (118,121) stimulation. Moreover, beige mice injected with P815 tumor cells displayed significantly lower numbers of lytic units when compared to normal mice and failed to produce an amnestic cell-mediated response following a second challenge with antigen. In defense of their assertion that defective NK cell function accounted for the reduced GVH reactivity in their model, Ghayur et al. (117) contended that the mice used in their study were able to generate a T cell response, since beige mice, when compared to their wild-type counterparts, were able to: 1) generate a PFC response to SRBC, 2) respond to mitogenic stimulation, and 3) reject skin allografts.

Experiments employing cytotoxic antibodies to deplete NK cells have also been used to determine whether removal of NK cell activity would have any affect on the outcome of GVH disease. It has been shown that *in vitro* depletion of the donor inoculum with anti-$ASGM_1$, an antiserum that reacts with NK cells, and complement failed to prevent murine GVH disease (122-126). However, *in vivo* treatment of F_1-hybrid recipients with this reagent effectively prevented the mortality associated with acute GVH disease (115,122,123,125,126). In these experiments, treatment with anti-$ASGM_1$ and complement

resulted in an 80 % depletion of the $ASGM_1^+$ cells from the spleen (122), a reduced proliferative response by spleen cells in mice with GVH reactions (122) and the inability to generate an anti-host CTL response *in vitro* (125). Because immunohistochemical and immunofluorescence microscopy have shown that Thy-1^+ dendritic cells in the epidermis, lung, thymus and spleen are $ASGM_1^+$, these authors speculated that NK cells, as well as other $ASGM_1^+$ cells, may play an important role as antigen presenting cells (122) and in the induction of anti-host CTL responses (125) thereby exacerbating the reaction in tissue sites sensitive to the pathologic effects of GVH disease. However, the reduced prolferative responsiveness of spleen cells and the absence of an anti-host CTL response *in vitro* after treatment with $ASGM_1$ is more likely due to the expression of $ASGM_1$ on the alloreactive T cell precursors and their mature effector cells (see below).

Ghayur and co-workers (117) have suggested that the failure of anti-$ASGM_1$ treatment of the graft to prevent the development of GVH disease may be attributable to the inability of anti-$ASGM_1$ to eliminate precursor NK cells, whereas treatment of the host would remove both the resident and activated NK populations. In a subsequent study they showed that GVH disease could be prevented by $ASGM_1$ antibody depletion of the donor inoculum if the donor mice (B6) had been injected with recipient ($B6AF_1$) lymphoid cells 2 days prior to the induction (127). Although splenomegaly developed

after the induction, PFC responses to SRBC were only partially suppressed and the recipients failed to develop the histopathological lesions associated with GVH disease. In a following study, the induction of $ASGM_1^+$ cells in response to $B6AF_1$ lymphoid cells was shown to be allo-specific since their removal prevented GVH disease in only $B6AF_1$ recipients but not in $B6SJLF_1$ or $B6C3HF_1$ recipients (128). Because allostimulation increased the killing of YAC-1 tumor targets by donor spleen cells and this activity was absent in $ASGM_1$-treated donors, it was suggested that an effector cell with NK activity was responsible for GVH-associated tissue injury. However, this $ASGM_1^+$ NK cell was thought to be distinct from NK cells induced by poly I:C since recipients of grafts from donors treated with poly I:C and then with anti-$ASGM_1$ still developed GVH disease. The authors suggested that this cell was not an NK cell but an NK-like cell, possibly a γ/δ T cell. However, no effort was made to type the cell for any T cell-specific markers. Furthermore, $ASGM_1$ expression is not seen only on NK cells but has also on cytotoxic T cells and activated macrophages (129-133). Therefore the possibility that cytotoxic T cells were also removed by this treatment exists; especially considering these cells are allo-specific. These findings also suggested that anti-$ASGM_1$ and complement depletion failed to remove CTL precursor cells. This idea is contradicted by observations from others who have shown effector CTL and NK cells could not be generated in an MLC if

the responder cells were first treated with anti-$ASGM_1$ and complement (134).

In an effort to minimize the compounding effects of depletion of cell populations other than NK cells, Blazar and co-workers (135) studied what affect *ex vivo* depletion of BM-spleen cell grafts with anti-NK1.1, an allospecific cell-surface marker expressed on NK cells of the $H-2^b$ haplotype, and complement had on the survival of graft recipients. Using antibody and complement depletion techniques, they showed that the removal of endogenous NK cells from C57BL/6 ($H-2^b$) grafts prior to induction did not prevent the mortality associated with acute GVH reactions in B10.BR/SgSnJ ($H-2^k$) irradiated recipients. In fact, mortality was increased in groups of mice that had received NK1.1-depleted grafts. They concluded that donor NK cells were not actively involved in either the induction phase or as effector cells during acute GVH reactions.

While these findings may have represented strong evidence against endogenous NK cells in the pathogenesis of acute GVH disease they did not address the possibility that NK effector cells may be recruited from a precursor cell population during the allogeneic phase of the GVH reaction. These conclusions are also inconsistent with the findings derived from histologic and immunocytochemical studies which have identified membrane-bound cytoplasmic dense granules and parallel arrays of tubules in the infiltrating cells

(136,137). These ultrastructural features are seen in NK cells. Immunohistochemical studies have also shown that the majority of mononuclear cells (>80 %) found in close proximity to dyskeratinocytes stain positively for NK cell-associated surface markers, $ASGM_1^+$, Thy-1^+, and MAC-1^+ and not with antibodies specific for T cell markers (CD4 and CD8).

1.4.2.2 Human studies

The contribution of NK cells to the development of GVH disease following BMT has also been the subject of several human studies. In one such study, BMT patients with high levels of peripheral blood NK cell activity against Herpes-simplex virus type 1 (HSV-1) infected fibroblasts prior to BMT were shown to be more likely to develop GVH disease (138). Patients with low levels of NK activity showed no signs of the disease. Since no relationship was observed between the level of NK activity and the severity of the disease, it was considered unlikely that the severity of GVH disease was attributable to an NK effector mechanism.

In other studies, no association was observed between either donor NK activity or pre- and post-transplant NK levels and the subsequent development of GVH disease (139-141). Post-transplant peripheral blood NK levels against NK-sensitive tumour cells and virally infected target cells also were unrelated to the severity of GVH disease. However, these studies disclosed a close association between the appearance

of augmented NK activity early in the post-transplant period and the development of GVH disease. For example, in one of the studies, BM transplant patients that demonstrated augmented NK activity less than one month after transplantation were at high risk for GVH disease (141).

In one study, NK cell activity was shown to be elevated whether or not overt GVH disease was present (140). It is possible that NK cell activity in patients without overt GVH disease was elevated because of other factors, such as, infections. In a recent study significant levels of NK activity were detected in leukemic patients within 2 - 3 weeks of receiving a T cell-depleted BMT (142,143). The reconstitution of NK activity in the absence of mature T cells or clinically detectable GVH disease suggested that the NK cells may have become activated in response to viral and/or tumor-associated antigens.

In summary, the development of augmented NK cell activity in animals and patients with ongoing GVH reactions has suggested that NK cells play an important part in the pathogenesis of acute GVH disease. This idea has been further propagated by studies in animal models that have demonstrated increased survival times for 1) recipients of grafts prepared from donors with defective NK cell activity and 2) when NK cell activity had been depleted with anti-$ASGM_1$ and complement. However, because neither $ASGM_1$ cell-surface marker expression nor the mutation affecting NK activity in

beige mice is restricted solely to cells with an NK cell phenotype, no definite interpretation supporting a role for NK cells role in acute GVH disease can be made from these results. The question of NK cells in GVH disease has been further complicated by the demonstration that certain T cells are capable of non-MHC-restricted killing. As yet, no attempt has been made to identify these NK-like cells or to measure their contribution in mediating the pathogenesis of GVH disease.

1.4.3 Role of cytokines in GVH disease

1.4.3.1 Interleukin-2 (IL-2)

The observation that antigen-specific T cells could be cloned and maintained indefinitely when grown in medium supplemented with supernatant from PHA-treated PBL clearly illustrated that antigen by itself was not required for sustaining the proliferation of these cells in culture (144,145). Subsequent experiments revealed that a 15 kDa polypeptide secreted by T cells was responsible for the growth-promoting effect on T cells and that this factor was required for establishing cytolytic T cell lines (CTLL). This factor was originally called T cell growth factor (TCGF), but is now called interleukin-2 (IL-2).

T cell receptor-ligand interactions not only stimulate IL-2 secretion from activated T cells bearing a TH1 phenotype,

but also induce IL-2 receptor expression on both TH1 cells and CTL. The induction of IL-2 receptor on Con A-stimulated, class II-restricted (but not class I-restricted T cells) requires the presence of accessory cells (146). The up-regulation of IL-2 receptor expression is associated with the transition from G_0 into G_1 of the cell cycle. Once sufficient numbers of IL-2 receptors are occupied cells progress fom G_1 to S phase (147). Early studies of the IL-2-IL-2 receptor (IL-2R) interactions identified 3 different isoforms capable of binding IL-2: a 55 kDa low affinity binding α subunit (TAC antigen (CD25)), a 75 kDa intermediate affinity binding ß subunit and a high affinity bi-molecular complex resulting from the non-covalent association of an α subunit and a ß subunit (148). The rapid rate of IL-2 association to the α subunit together with its slow rate of disassociation from the ß subunit allows for the formation of a high-affinity receptor. Modulation of antigen receptors on T and B cells induces expression of the α subunit (148,149). B cells and NK cells, in comparison to T cells, constitutively express low levels of IL-2Rß subunit; however, incubation with IL-2 induces ß subunit expression (149,150). Most of the ß chains expressed on resting NK cells do not bind IL-2. Treatment of NK cells with IL-2 induces intermediate-affinity IL-2Rß subunit expression (150). This change in binding affinity in the absence of IL-2Rα subunit is now known to be regulated by the non-covalent association of a 64 kDa polypeptide chain

referred to as the IL-2Rγ subunit (150). The γ subunit also associates with the the IL-2Rα/ß complex following IL-2 stimulation (151). Intracellular signal trandsuction is triggered by the intracytoplasmic chain of the IL-2Rß resulting in the activation of one of a family of src protein tyrosine kinases; $p56^{lck}$ PTK in T cells and NK cells (152) and $p53/p56^{lyn}$ in B cells (153).

While IL-2 is more commonly known for its proliferative effect on lymphocytes (144,145,154-156), primarily T cells, it also has been shown to regulate a variety of cellular functions. For example, IL-2 augments NK cell and macrophage cytolytic activity (157,158). Lymphocytes cultured in the presence of IL-2 develop cytotoxicity against both NK-resistant and NK-sensitive tumor targets and have been used experimentally to treat cancer *in vivo* (159). These cells have been termed lymphokine-activated killer cells (LAK). If spleen cells are stimulated *in vitro* with rIL-2 and then injected into syngeneic recipients, which then continue to receive injections of IL-2, there is a significant decrease in the metastatic capacity of intravenously injected sarcomas (160). LAK activity has been shown to be inducible *in vivo* with rIL-2 but not with IFNα (161). LAK activity against the NK-resistant tumor target MCA-102 was generated by giving rIL-2 two times a day for 7 days, but when IFNα was used, there was no effect. However, treatment with both rIL-2 and IFNα was shown to increase the activity to levels 2-3 times than

produced by IL-2 alone, and to maintain the activity for a longer period of time. This increase in LAK activity was shown to correspond with an increase in the number of $ASGM_1^+$ and $NK1.1^+$ cells.

While certain lectins readily stimulate IL-2 production by lymphocytes isolated from healthy animals, the same cannot be said for lymphocytes removed from animals with GVH reactions. Spleen cells removed from BAF_1-hybrid mice experiencing chronic GVH reactions on day 7 and 43-90 days post-induction were unable to produce IL-2 after Con A stimulation (46,162,163). Normal cells mixed with spleen cells taken on day 7 from mice with acute GVH reactions failed to restore IL-2 secretion in these cultures suggesting that a suppressor cell mechanism was involved (162). The authors of this study suggested that changes in the thymus in the latter stages of the reaction resulted in an arrest of T cell maturation which accounted for the lack of IL-2 production at this time. In a subsequent study it was revealed that one of the results of arrested T cell development appears to be the inability to confer cortisone resistance (164). Mice with chronic GVH reactions exhibit a reduction in the numbers of $CD4^+$, $CD8^-$ thymocytes and that the depletion of this subset from the thymus is mediated by corticosteroids. The authors speculate that, since normal numbers of this thymocyte subset are present in adrenalectomized mice with GVH reactions, the thymus is capable of supporting the development of this

thymocyte population. However, because the altered thymic environment in the GVH animal is unable to confer cortisone resistance so these cells are eliminated. The consequence of this is believed to be too few IL-2-producing cells in periphery and a diminished production of IL-2 in response to mitogenic stimulus. It is worthy to note that a similar deficiency in IL-2 production by lectin-stimulated lymphocytes also has been observed in BMT patients with either acute or chronic GVH disease (165).

The importance of IL-2 in the pathogenesis of GVH disease has been studied in experiments involving the administration of either rIL-2 or MoAb against IL-2 receptor to mice with GVH reactions (104,166). In one study, murine radiation chimeras injected with rIL-2 three times a week for five weeks exhibited a marked increase in the rate at which animals die (166). The heightened effect on mortality by rIL-2 was thought to be due to an effect on Thy-1$^+$ cells since the identical treatment did not have a similar effect on recipients of T cell-depleted grafts. Paradoxically, lethally-irradiated B10 recipients injected with A/J BM-spleen cells demonstrated a marked reduction in mortality if recipients were treated with rIL-2 during the first five days of the reaction (167). The time at which IL-2 was administered appeared to be crucial factor to the outcome since treatment with IL-2 beginning 7 days into the reaction resulted in an acceleration in mortality. It was suggested

that the protective effect provided by IL-2 early in the reaction maybe mediated by a $CD3^+$, $CD4^-$, $CD8^-$, $Thy\text{-}1^+$, $NK1.1^+$ suppressor cell (168).

It has been suggested that some of the clinical features associated with GVH disease may be due to excessive IL-2 production (169,170). It has been observed that cancer patients receiving high doses of rIL-2 to induce LAK activity develop side effects resembling features of GVH disease (fever, rash, interstitial pulmonary edema, diarrhea, increased liver enzyme levels and lymphocytic infiltrates in the liver). Similarly, the injection of IL-2 into mice was shown to stimulate the proliferation of large mononuclear lymphocytes in the BM, lymph nodes, spleen, lung, liver and the epithelium of the gut and skin (171). These large mononuclear lymphocytes were $Thy\text{-}1^+$, $ASGM_1^+$, $L3T4^-$ and were able to lyse both YAC-1 and P815 tumor targets. However, when these cells were grafted into recipients they were 100 times less effective than lymph node cells in inducing GVH disease.

Finally, it has been observed that BDF_1-hybrid mice injected in the footpad with DBA/2 spleen cells fail to generate a significant increase in the size of the popliteal lymph node if they were also treated with AMT-13, an anti-IL-2 receptor antibody (172).

1.4.3.2 Interferon (IFN)

It had been well established that cells exposed to one virus became resistant to concomitant infection by a second virus (173). For example, chorioallantoic membrane removed from 10-11 day fertilized hens' eggs demonstrated resistance to infection by New Castle disease virus (NDV) if the membrane had been incubated previously with heat-inactivated NDV. This phenomenon, referred to as viral interference, was first shown by Isaacs and Lindenmann (174) to be mediated by a soluble factor which they called interferon. Three subtypes of IFN have been characterized to date; IFNα, IFNß and IFNγ. IFNα and IFNß, also referred to as type I IFN, are produced by macrophages, B cells and fibroblasts in response to viruses while IFNß is produced after stimulation with poly-ribonuceoltides such as poly I:C (175). IFNα is encoded by a multi-gene family giving rise to 23 known species of IFNα, while IFNß is encoded by a single gene that shares 40 - 50 % homology with IFNα genes (176). Both IFNα and IFNß are monomeric glycoproteins with apparent molecular weights of 20 kDa and 33 kDa, respectively (175). Murine cells are able to produce both IFN subtypes which has lead to the single designation IFNα/ß (177). IFNγ, also called immune or type II IFN, is produced by T lymphocytes following activation and, like NK cells, in response to stimulation with IL-2 (175). IFNγ, a monomeric glycoprotein of 15 kDa, shares no homology with IFNα/ß. In reciprocal competition binding studies IFNα

and IFNß compete for the same receptor, a 65 kda polypeptide, while IFNγ does not (178,179). The expression of cloned murine IFNγ receptor in human cells has identified a 90 kDa transmembrane glycoprotein as the high-affinity receptor for IFNγ (180).

In addition to mediating protection against viral infection, a multitude of other functional properties have been ascribed to the different types of IFN. Both types of IFN augment NK activity (discussed in section 1.5.1.2), either alone or synergistically with other cytokines such as IL-2 and TNFα. Both stimulate macrophages to increase their phagocytic activity and induce TNFα production. Interferons have anti-proliferative effects on a variety of cells including T cells and some tumor cell lines (176,181). IFNα/ß is known to induce MHC class I expression while IFNγ, either alone or synergistically with TNFα, is able to increase both class I and class II antigens (182). IFNγ release by TH1 cells regulates immunoglobulin isotype heavy chain switching by preferentially selecting Ig2a antibody responses and suppressing IgE and IgG1 antibody formation (183,184).

Because of its important immunoregulatory functions several investigations have focused on the detection and characterization of IFN produced in mice with GVH reactions. It has been observed that serum levels of IFN in mice with acute GVH disease (C57BL/6 → irrad. DBA/2) are maximal by day 5-6 of the reaction (185). Typing of IFN with antisera has

shown that IFNα/ß is the major species produced. Similar findings also were reported when the donor-host combinations A/J → B6AF$_1$-hybrid mice and B10.D2 → BALB/c, were used to produce GVH reactions (186). Spleen cell cultures prepared from mice with GVH reactions exhibited spontaneous IFN production. This has not been observed in cultures of spleen cells from normal mice or mice injected with syngeneic cells. Again, type-specific antibody neutralization techniques demonstrated that all the IFN activity could be attributed to IFNα/ß but not anti-IFNγ. In a subsequent study, IFNα/ß production was augmented by adding IL-2 or GM-CSF to spleen cell cultures prepared from B10.D2 → BALB/c graft recipients (187). This study showed that splenocytes from mice with GVH reactions could suppress the proliferative response of B10.D2 spleen cells in a one-way MLR against irradiated B6 cells. This effect could be abrogated by anti-IFNß antibody. It was thus concluded IFNß may be associated with the regulation of suppressor cells activated during GVH reactions.

Cytoplasmic immunofluorescence staining of cyto-centrifuged spleen cells taken from BALB/c mice with chronic GVH reactions have demonstrated that IFNγ is also produced (188). IFNγ production has been localized to the periarterial sheath in the spleen, which indicates that it is of T and/or NK cell origin, while IFNα/ß$^+$ cells are present throughout the spleen, suggesting that IFNα/ß is produced by macrophages. Although IFNγ was not detected *in vitro*, separation of the

spleen cell cultures on Percoll and then treatment of the different cell fractions with Con A identified a cell fraction that was able to produce IFNγ. The data suggested that mixing of the cell populations *in vitro* suppressed IFNγ production. Later it was shown that when mice with chronic GVH reactions (B10.D2 → BALB/c) were treated with anti-IFNγ, but not anti-IFNß, there was a reduction in immunosuppression and lymphoid hypoplasia associated with GVH disease. Spontaneous IFNß production in spleen cell cultures established from these mice was also prevented (189). These experiments suggested that IFNγ was required for the activation of cells that produced IFNα/ß during the reaction.

It was observed that when recipient F_1-hybrid mice were injected with the IFN-inducer poly I:C prior to induction, there was a significant reduction in the immune suppression and pathology associated with acute GVH disease (190). The reason for this observation is not known, but the authors of this study suggest several possible mechanisms. First, IFNα/ß itself is directly immunosuppressive. Second, IFNα/ß activates a suppressor cell which minimizes alloreactivity. Third, IFNα/ß activates NK cells in the recipient. NK cells have been implicated in hybrid resistance, a phenomenon associated with allograft rejection. However, if poly I:C is administered after the reaction has been established (1 - 2 days after transplant) there is an exacerbation of GVH disease, with more severe immunosuppression, a worsening of

GVH-related intestinal pathology (increased crypt cell hyperplasia and villus length followed by villus atrophy and more severe crypt cell necrosis) and increased mortality (105,191). In one study, the effect of poly I:C was diminished if the treatment was given after day 5 of the reaction (191). It was believed that the ability of IFN to exacerbate GVH disease was limited to a point in the reaction before immune suppression was maximal. It was also suggested that poly I:C might have had less of an effect on the cytokines or cells that are present in the later stages of the reaction.

In human studies, higher than normal IFN levels have been observed in the serum of BMT patients (192,193). Still higher levels of IFNγ were detected in those patients with GVH disease. However, these levels did not differ significantly from those of allogeneic BMT recipients without GVH disease (192). Because of its antiviral and anti-proliferative properties, IFNα has been given to BMT patients suffering from hemopoietic malignancies to prevent cytomegalovirus infection and to eliminate any residual tumor cells (194). Although this treatment was successful in reducing the relapse rate, the authors of this study suggested that because IFN can upregulate class I expression cells not normally sensitive to recognition may become targets of the reaction and lead to a worsening of the GVH disease (193).

1.4.3.3 Tumor Necrosis Factor α (TNFα)

One of the most striking features of acute GVH disease is cachexia. This severe wasting disorder is thought to be mediated by TNFα. After a GVH reaction is induced, mice begin to lose weight 11 to 12 days after induction (6). It has been reported recently that of ten parameters examined in mice with acute GVH disease, only two, plasma lipase levels and weight loss of greater than 10 % by day 20, were reliable indicators of acute GVH reactions (195). In another study, cachexia was shown to be preventable if the recipients were given 2 mg of rabbit anti-TNFα IgG after the first week of the reaction (196). Recipients that had received the anti-TNFα exhibited a diminished mononuclear cell infiltrate into the skin and gut epithelium and failed to demonstrate any of the GVH disease-associated lesions in these tissues when compared to day 16-18 GVH control mice. Anti-TNFα treatment also markedly reduced the mortality from acute GVH disease. Anti-TNFα was also shown to prevent the intrapulmonary bleeding that is seen in conjunction with alveolitis encountered in severe acute GVH reactions (197). However, this treatment had no effect on other features of the alveolitis, nor on the development of chronic (after day 25) pneumopathies. Increased TNFα mRNA expression in lung tissue was shown also to coincide with the alveolitis. The authors concluded that TNFα was, in part, responsible for tissue injury and speculated that the cells most likely responsible for TNFα production in GVH disease are

macrophages and LGL. Both are known to secrete TNFα (198).

Macrophage secretion of TNFα requires two stages. The first involves macrophage priming by IFN (199) and the second, triggering of TNFα release by LPS (200). While IFNγ increases TNF gene transcription (201), it does not induce measurable TNF secretion (200). It was reported that peritoneal macrophages isolated from F_1-hybrid mice with acute GVH reactions secreted TNFα in response to LPS alone, indicating that priming of the macrophages by IFN had already occurred (202). Macrophage priming was shown to be cell dose dependent since macrophages isolated from recipients of a graft insufficient to cause mortality (30×10^6 cells) produced less TNFα when compared with mice with acute GVH reactions. Although this data suggested that the amount of IFN generated in a GVH reaction was related to the size of the graft, this was not confirmed because IFN levels for each cell dosage were never determined. Priming in F_1-hybrids with ongoing GVH reactions rendered the recipients hyper-responsive to endotoxin since these animals succumbed within 36 hrs of injecting sublethal doses (10 μg) of LPS. The same animals also developed some of the pathological features of GVH disease (hunched posture, piloerection and diarrhea) within a 2-6 hr period following the injection of LPS. The authors of this study speculated that LPS enters the body from the gut and, via the portal circulation finds its way to the liver. Once the capacity of the liver to clear LPS from the

circulation is overwhelmed, LPS then enters the systemic circulation and interacts with primed macrophages causing the release of TNFα, which mediates the cachexia and/or mortality associated with acute GVH disease.

The view that TNFα is important in the pathogenesis of acute GVH disease has not gone unnoticed by bone marrow transplanters who observed TNFα in the serum of BMT patients with GVH disease (203,204). In one of these studies, the severity of GVH disease was closely related to the amount of TNFα in the serum (204). In murine models of acute GVH disease TNFα has not been detectable in the serum (196,202) leading some to speculate that in mice, TNFα is secreted by cells infiltrating the target tissues (196). It has also been suggested that TNFα is rapidly cleared from the circulation by tissues, thus making what TNFα that there is present, undetectable (202).

1.4.3.4 Interleukin-1 (IL-1)

Interleukin-1 (IL-1) is a soluble monomeric polypeptide of 17-18 kDa which is produced primarily by macrophages in response to infection, injury or antigen recognition (59). Two isoforms of IL-1 have been identified; IL-1α and IL-1ß. Although preference in IL-1ß production has been demonstrated, both isoforms of IL-1 exhibit the same biologic effects on a variety of cell types after binding to specific membrane receptors. The IL-1 receptor (IL-1R) on T cells, fibroblasts

and endothelial cells has been shown to be an 87 kDa monomeric transmembrane protein possessing an extracellular portion characteristic of the immunoglobulin gene superfamily (205,206). In contrast, B cells and myelomonocytic cells express a serologically distinct polypeptide of 66 kDa (207). The higher molecular weight receptor has been designated IL-1RI and the lower molecular weight receptor IL-1RII.

Ferrara has shown that mRNA specific for IL-1α is detectable in the skin of mice with acute GVH reactions but not in control animals (208). Furthermore, the injection of an IL-1R antagonist (IL-1ra), which possesses no agonistic activity, blocks the development immunosuppression and significantly reduces the mortality associated with acute GVH disease. The authors speculated that because of the similarities between TNFα and IL-1, these two cytokines, together, may play important roles in the pathogenesis of acute GVH disease. Also, because neutralization of either factor markedly reduces but does not prevent entirely the mortality from GVH disease, they postulated that anti-TNFα and anti-IL-1 agents used together may synergize to reduce mortality even further.

1.5 Cells Mediating Non-MHC-Restricted Cytotoxicity

Cell-mediated cytotoxicity is a property of several lymphoid cell populations. The most widely studied is the cytotoxic T lymphocyte (CTL). CTL are generated in response

to specific cell surface antigenic determinants (eg. viral-encoded) or non-self (allo-) antigens presented on APC in association with self MHC-encoded antigens. This phenomenon is known as MHC-restriction and was first demonstrated by Zinkernagel and Doherty (209) who observed that L cells infected with lymphocytic choriomeningitis (LCM) virus were lysed only by preparations of immune T cells which shared H-2 antigens with the target cell.

MHC-restriction is not shared by all cytotoxic cells since some cells will lyse allogeneic target cells. Non-MHC-restricted killing is mediated by at least two separate lymphoid cell populations, NK cells and a subset of T cells termed "NK-like" cells. The characteristics of these two cytotoxic effector cell subsets has been summarized in Table 1.1. Several phenotypic characteristics appear to differentiate between these two populations (210). These include the presence of TCR and the TCR-associated molecule, CD3. In contrast to mature T cells, neither mouse or human NK cells express transcripts of TCR chains (α, ß, γ, δ) (211-213). Also, NK cells do not express the ε, δ, or γ chains of the CD3 molecule, although the zeta-chain of CD3 has been detected on the surface of these cells (213-215). Human and mouse NK cells express FcγRIII (CD16) receptor and can mediate antibody-dependent cellular cytotoxicity against antibody sensitized target cells (216). CD56 (Leu-19 or NKH1), a cell-surface marker expressed on all human NK cells and on some T

Table 1.1 Characteristics of murine non-MHC-restricted cytotoxic effector cell populations

	NK	NK-like	Ref.#
Cytolytic Activity			
Endogenous	+	-	210,223,278
NK-sensitive	+	-	210,240
NK-resistant	-	-	210,240
LAK activity	+	+	168,210,277
NK-sensitive	+	+	168,210,277
NK-resistant	+	+	168,210,277
Surface Markers			
NK-associated			
NK1.1	+	-[1]	243
$ASGM_1$	+	+	134,234-236
CD16	+	+/-	216,233
T cell-associated			
Thy-1	+/-	+	230,233,238-240
CD4	-	-	285
CD8	-	-[2]	279,285
CD3	-[3]	+	213,215
TCR			
- α/β	-	+	213,215
- γ/δ	-	+	213,215
Morphology	LGL	LGL	225,277,284

[1]Induced on some $CD3^+$ cells with IL-2 (219) and constituitively expressed on 10 % of splenic $NK1.1^+$ cells (351).
[2]Expressed on $TCR\gamma/\delta^+$ cells as a homodimer of the α chain (279).
[3]$CD3^-$ NK cells express the zeta chain of the CD3 complex (214).

cells (in particular those T cells mediating non-MHC-restricted killing), has been shown to be an isoform of a neural cell adhesion molecule (N-CAM) (217). In mice, NK1.1 and NK2.1 were originally believed to be cell-surface markers unique to NK cells. However, a subset of TCRα/β^+, $CD3^+$ murine T cells has also been shown to express NK1.1 and FcγRIII receptor. These cells also demonstrated NK-like cytotoxic activity (218). Cells with this phenotype have been implicated as the effector cells responsible for rejection of BM allografts and and for the phenomenon of hybrid resistance (26). In studies in which $CD3^+$, $CD4^-$, $CD8^-$, $Thy\text{-}1^+$, $NK1.1^+$ cells were cloned in IL-2 supplemented medium from MAC-1-depleted bone marrow cells it was shown that these cell lines were able to suppress the generation of alloreactive CTL in mixed-lymphocyte cultures and could mediate lysis of NK-sensitive and NK-resistant tumor targets (168). One particular cell line, H3, prevented GVH disease associated mortality when injected into lethally-irradiated syngeneic recipients along with a bone marrow-spleen cell allograft. It was shown that the graft survived in 30-40% of the surviving recipients. The authors attributed the increased survival to the suppressor activity of these cells. However, the low rate of reconstitution by allogeneic cells in this study also suggested that rejection of the graft might also be involved. The origin of $CD3^+$, $NK1.1^+$ cells has recently been addressed in a study showing that $CD3^+$, $CD4^-$, $CD8^-$, $NK1.1^-$ spleen cells

cultured in IL-2 for 6 days become NK1.1$^+$ (219). These results suggest that precursors of splenic NK-like cells become NK1.1$^+$ following activation.

It should be noted that the term "LAK" merely describes the functional state of a cell in response to IL-2, and not a particular cell type (210). NK cells express endogenous cytolytic activity which can be augmented by stimulation with IL-2. By definition the higher levels of killing following IL-2 stimulation is LAK activity even though the cells mediating the killing are still NK cells. Also included in the LAK cell definition are non-MHC-restricted T cells which become activated in response to IL-2.

1.5.1 Natural Killer Cells

NK cell activity was first described in human studies of CTL-mediated lysis of tumour targets (220-222). In these experiments normal lymphocytes were shown to be equally or more effective in lysing tumour targets than lymphocytes pre-sensitized to tumour antigens. Since these studies, NK activity has been observed *in vitro* against a wide variety of tumour targets. NK cell activity can be elicited in the absence of prior sensitization with target cell antigen. NK-target cell interaction is neither MHC-restricted, nor clonally specific. NK cells do not produce memory cells after exposure to NK-sensitive targets, nor do they require antibody or complement to effect cytotoxicity (223).

Enriched populations of NK cells have been isolated from the non-adherent cell fraction of human peripheral blood lymphocytes by target cell adsorption and elution or by density fractionation on discontinuous Percoll gradients (224-226). The low density Percoll fraction containing the NK activity can be further enriched (>95 %) by removing the E-rosette-forming cells (227). Morphologic studies have shown that cells from this Percoll fraction are comprised of a homogeneous population of cells containing azurophilic granules, having an abundant cytoplasm and a reniform nucleus (225). The term LGL (large granular lymphocyte) has been applied to these cells (225). NK activity in mice is also associated with LGL (228,229). Studies of the tissue distribution of LGL have shown that the level of NK activity in the tissue was related directly with its content of LGL (228). Very low levels of activity were detected in the lymph nodes, whereas no NK activity was detected in the thymus. The number of LGL in the spleen, and consequently its level of NK activity appears to vary with the mouse strain (229). C57BL/6 display high levels of NK lytic activity whereas A/J strain mice show low levels.

1.5.1.1 Cell-Surface Markers

Despite the apparent homogeneity of purified LGL, cells within this population have been shown to be heterogeneous with respect to cell surface markers. This has made the

phenotypic characterization of NK precursors and effectors difficult.

NK cells are distinct from B cells in that they do not possess B cell-specific surface markers such as IgM and Lyb-2 (230,231) nor do they adhere to nylon wool (232). It has been observed that when preparations of SRBC, coated with monoclonal anti-SRBC of the different subclasses, are mixed with non-adherent CBA spleen cells NK-mediated killing of YAC-1 tumour targets is depleted. Since this effect was only observed with SRBC preparations carrying anti-SRBC of the $IgG2_b$ subclass it was concluded that NK cells expressed FcγRIII (CD16) on their cell surface (233).

The lytic activity of both BCG-induced peritoneal exudate NK cells (234) and NK cells isolated from the spleens of different inbred strains (235,236) can be abolished after incubating the effector cells with anti-ganglio-N-tetraosyl ceramide (asialo-GM1; $ASGM_1$) antiserum and complement. Allo-reactive CTL activity, generated from spleen cells during MLR against allogeneic tumour or spleen stimulator cells, was unaffected by anti-$ASGM_1$ (234-236). It was concluded that $ASGM_1$ is expressed on NK cells but not mature cytotoxic T cells. However, these results contrast with the observation that the cytotoxic activity of 7 out of 8 cloned CTL lines tested could be removed with anti-$ASGM_1$ antiserum and complement (129). Furthermore, Beck and co-workers (134) have since shown that depletion of $ASGM_1^+$ spleen cells before the

MLR is initiated does abrogate CTL activity. In their study the absence of CTL activity was accompanied by a decreased ability to produce IL-2 in response to mitogen and that the addition of exogenous IL-2 could not generate CTL activity. The authors thus concluded that $ASGM_1$ is expressed on the precursors of allo-reactive CTL and T helper cells as well as NK cells.

Thy-1 is a T cell-specific differentiation antigen which appears on the cell surface during the thymic phase of T cell maturation and is present on all T cell subsets. Thy-1 is also expressed on brain cells, epithelial cells and fibroblasts, but not on B cells, macrophages or hemopoietic stem cells (237). At least two subpopulations of NK cells exist, one is $Thy\text{-}1^+$, the other $Thy\text{-}1^-$. Treatment of spleen cells with anti-Thy-1 and complement results in a substantial reduction (50 - 60 %) in NK activity (230,238,239). These results have been confirmed by immunohistochemical studies which showed that 60 % of splenic LGL are $Thy\text{-}1^+$ (233).

$Thy\text{-}1^+$ and $Thy\text{-}1^-$ NK cell populations are heterogeneous with respect to target specificity and function (240). $Thy\text{-}1^+$ NK cells are able to lyse both the NK-sensitive tumor cell lines, such as YAC-1, as well as the NK-resistant tumor cell lines, such as P815. In the same study, it was also observed that only the $Thy\text{-}1^-$ NK cell population suppressed *in vitro* PFC responses to SRBC. The authors suggested that a $Thy\text{-}1^+$ NK cells mediated this effect either by direct suppression or by

elimination of antigen-presenting cells. These results agree with previous work which showed that "large" Thy-1$^+$ cells from the spleens of nude mice were able to suppress the LPS mitogen response when these cells were mixed with mitogen-responding B cells (241).

The cell surface marker NK-1, originally described by Koo and co-workers (230,242), is closely associated with NK cells. This marker was first demonstrated in antibody and complement depletion experiments using (C3H x BALB/c) anti-CE antiserum. It was shown that splenic NK activity from C57BL/6 mice could be depleted with this antiserum. Later, two allelic forms of this surface marker were later found, NK-1^a and NK-1^b (225).

Monoclonal antibodies to this determinant displayed identical patterns of reactivity to the NK-1 antisera used previously (243). Experiments were done to demonstrate that NK1.1 is present on NK cells that lyse the tumour target cell line, YAC-1. Further analysis of NK1.1$^+$ cells showed that over 90 % of these cells are ASGM$_1^+$, NK2.1$^+$, and Qa-5$^+$. Only 60 % of the cells are Thy-1$^+$ (244).

NK1.1$^+$ cells are LGL and occur in the same Percoll fraction. The injection of anti-NK1.1 into mice at weekly intervals appears effective in ablating NK activity *in vivo* without affecting either T cell or B cell functions or the frequency of L3T4$^+$, Lyt-2 and SIg$^+$ cells (245,246). Similarly, *in vitro* treatment with anti-NK1.1 antisera and complement has no effect on the viability of polymophonuclear leukocytes, and

in functional studies, does not affect T or B cell proliferative responses nor diminish the effector response of CTL or activated macrophages (247,248). *In vitro* culturing of either bone marrow or spleen cells from NK1.1$^-$ mice in the presence of IL-2 restores NK activity, suggesting that NK precursors are NK1.1$^-$. This result agrees also with previous studies which showed that NK progenitors are NK2.1$^-$ (249). Recent cloning of the NK1.1 gene has identified one of the 3 known gene loci (gene 40) encoding for the murine homologue of NKR-P1 as the gene locus for NK1.1 (250). NKR-P1 genes encode for homodimeric 70 - 80 kDa (glycosylated form) type II transmembrane C-type lectin carbohydrate binding proteins (250,251). Although, the function of NK1.1 is still unknown, it is not believed to be involved in target cell binding since anti-NK1.1 does not inhibit effector-target-cell conjugate formation (250). However, it has been suggested that ligand interaction with NK1.1 may provide signals for directing the granules and microtubule organizing center towards the target cell (250).

The presence of several T cell markers on NK cells and the absence of B cell and myeloid cell markers has led to the idea that NK cells and T cells share a common precursor cell early in ontogeny. However, the presence of NK-1 on spleen cells from nude mice, and the high level of NK activity in these spleens indicates that at some point, T cell and NK cell developmental pathways diverge (242).

1.5.1.2 Cytokine Regulation

Although NK cells express a basal level of lytic activity, this can be increased by several cytokines. One of the most potent is IFN. All three species of IFN (α, ß, and γ) enhance NK activity; however, the effectiveness of IFNγ varies with the source of NK cells and with the time of exposure. Incubation of murine spleen cells overnight with recombinant human IFNα, but not recombinant mouse IFNγ, has been shown to increase NK activity. In humans, all three IFN species can augment NK cell activity in peripheral blood lymphocytes (PBL). In this instance, a longer incubation period (24 hr) with IFNγ is required to achieve the same levels of NK activity observed after only a 3 hr exposure to IFNα and IFNß.

IFN increases NK cell activity through several mechanisms. First, IFN increases the number of NK cells by stimulating the transformation of non-binding LGL into LGL that can bind to tumor cells and by inducing non-lytic LGL that are able to bind targets to become cytolytic (252,253). Second, IFN can induce blastogenesis and proliferation of NK cells *in vivo* (254). It should be noted that the proliferation of NK cells in response to poly I:C is insensitive to inhibition by cyclosporin and therefore is not mediated indirectly by IL-2 (255). Third, IFN increases the rate of lysis. It has been shown that almost all target cells conjugated with IFN-treated NK effector cells are lysed within

30 minutes of binding (versus 3 hr in controls) (252). Finally, effector cells stimulated with IFN recycle continuously while untreated effectors must go through a 2 hr refractory period before they are able to bind target cells (252).

A variety of stimuli are known to induce the release of IFN. These include viruses, some tumor cell lines, pyran, LPS, manganese and poly I:C (256,257). Poly I:C, a synthetic analog of viral double-stranded RNA (dsRNA), injected into mice or incubated *in vitro* with lymphocytes from the either the spleen or lymph nodes markedly enhances NK cell activity (257). Dose-response and time course studies have shown that for *in vivo* stimulation a dose of 100µg injected intraperitonealy is optimal. The effector cells are harvested 18 hr later. The augmentation of NK cell activity is related directly with IFN production and can be inhibited with anti-IFNß (257). Removal of macrophages by adherence, or inactivation of the macrophage population with either silica or carrageenan before poly I:C treatment significantly depresses the response of NK activity to poly I:C (258), thus indicating that macrophages are the primary source of poly I:C-induced IFN.

It has been reported that IFN is unable to induce non-MHC-restricted killing in T cells (259). In these experiments the removal of T cells by E-rosetting also failed to diminish IFN-augmented killing of tumor targets; however, cytolytic

activity was depletable by EA-rosetting with IgG-sensitized red cells. In contrast, non-MHC-restricted killing has been induced in $CD3^+$, $CD4^-$, $CD8^-$, $WT31^-$ (marker for $\alpha/\beta TCR^+$ cells) T cell clones following incubation with either IL-2 or rIFNß (260). It has been suggested that the spectrum of target cells lysed by IFN-activated cells is the same as that of unstimulated cells (223). However, IFN-induction with poly I:C has been shown to not only augment killing of the NK-sensitive tumor target YAC-1, but also to activate a $Thy\text{-}1^+$, $ASGM_1^+$ population of non-MHC-restricted cytotoxic cells that are able to lyse the NK-resistant tumor target P815 (240).

The augmentation of NK cell cytotoxic activity by IFN is transient and generally followed by a period during which NK activity is suppressed (223). This has also been observed in patients given rIFNα (261,262).

IL-2 also increases NK cell activity. Although, IL-2-induced NK cell activity peaks within a 24 hr period (157,263-265), this lytic activity could be augmented further by the addition of IFN (157,263,264). While a short term exposure to IL-2 augments NK cell activity against NK-sensitive targets, it does not induce either NK or NK-like cell cytotoxic activity against NK-resistant tumor targets (266). However, human PBL cultured for 3 or more days with high levels of rIL-2 (100-1000 U/ml) generate cytotoxic cells that are able to kill both NK-sensitive and NK-resistant tumor target cells (266,267). It should be noted that while most of the LAK

activity in these studies was mediated by $CD3^-$ cells with NK-associated markers (CD56), cells with the same lytic capacity can also be developed from lymphoid organs devoid of NK cell activity. For example, $CD3^+$, $CD56^+$ non-MHC-restricted cytotoxic cells can be generated from thymocytes after culturing in IL-2 (267).

Pre-incubation of non-adherent cells from human PBL for 18 hr with TNFα has been shown to augment NK cell activity against both NK-sensitive and NK-resistant tumor targets (268). This activity could be increased still further by the addition of IL-2 to the culture. It has also been shown that LAK cell activity could be generated from $CD3^-$, plastic non-adherent LGL isolated from human PBL and cultured for 3 days with suboptimal concentrations of IL-2 if TNFα had also been added (269).

Pre-incubation of PBL with IL-1 for 18 hr has been shown to augment NK cell activity (270). Interestingly though, incubation of purified LGL was not found to have any effect on NK cell cytotoxicity. Because anti-IL-2 antibodies were able to inhibit the augmented NK cell activity induced by IL-1, the authors suggest that IL-1 increases NK cell activity indirectly by stimulating the secretion of IL-2. IL-1 is also known to synergize with IL-2 and IFN to augment NK cell activity and to increase target cell binding (59).

More recently, natural killer cell stimulatory factor (NKSF, IL-12), a 70 kDa heterodimeric (30 kDa and 45 kDa)

soluble factor produced by macrophages and B cells, has been shown to have multiple biological effects on NK cells. IL-12 augments NK endogenous cytotoxicity, synergizes with IL-2 to induce LAK activity, induces IFNγ production by NK cells, is directly mitogenic for activated NK cells and enhances IL-2 production by PBL (271,272). Interestingly, at high doses of IL-2, IL-12 inhibits IL-2-mediated proliferation of NK cells by inducing TNFα release (273). This inhibitory mechanism was also described for TCRγ/δ cells but not for T cells expressing TCRα/ß.

1.5.2 γ/δ T cells

All mature T cells express either the α/ß or γ/δ CD3-associated heterodimeric polypeptide TCR on their surface. Many of the classical functions associated with T cells, such as MHC-restricted cytotoxicity, alloreactivity and T-dependent B cell help, are mediated by T cells expressing TCRα/ß. While a few of these functions have been demonstrated with γ/δ T cell clones, there are several features of γ/δ T cells which distinguish them from α/ß T cells (274-276). First, the vast majority of γ/δ T cells possess a potential for non-MHC-restricted cytolytic activity. Analysis of a large panel of human γ/δ T cells clones showed that most are able to lyse K562 as well as NK-resistant fresh uncultured melanoma cells. These clones also killed P815 target cells after exposure to the lectin PHA (277). Fresh γ/δ T cells, unlike NK cells, do

not exhibit endogenous cytotoxic activity against either NK-sensitive or NK-resistant tumor targets. However, once activated with either IL-2 or IFN, these cells acquire the capacity to lyse these tumor cell targets (278). This cytolytic activity can also be induced with antibodies directed against both CD3 and CD2, the receptor for sheep erythrocytes. It has also been shown that $CD3^+$, $CD4^-$, $CD8^-$, $TCR\gamma/\delta^+$ lymphocytes incubated with immobilized anti-CD3 or with PHA secrete a variety of cytokines including IFNγ, IL-2, IL-3, TNF and GM-CSF (279-281). Some anti-CD3 (UCHT1) and anti-TCR MoAb are able to block the cytotoxicity of γ/δ T cell lines (282,283). Paradoxically, with certain tumor targets, such as the monocytic cell line U937, the addition of anti-CD3 causes an enhancement of lytic activity (283). Because this effect was blocked by aggregated human IgG, it was thought to be related to IgG Fc receptor expression on the target cells which caused redirected target cell lysis.

Morphologically, virtually all resting γ/δ T cells isolated from human peripheral blood are LGL (277,284). Incubation of γ/δ T cells with IL-2 has been shown to markedly increase cytoplasmic granularity. On the other hand, only $CD8^+$ $\alpha/ß$ T cells are LGL.

In general, γ/δ T cells share many of the cell surface markers expressed on $\alpha/ß$ T cells. However, while the vast majority of mature $\alpha/ß$ T cells express either CD4 or CD8, most γ/δ T cells do not express either. Phenotyping studies done

on mouse lymphocytes isolated from the thymus, spleen and lymph nodes have shown that the majority of γ/δ T cells are $CD3^+$, $CD4^-$, CD8-. This subset of γ/δ T cells comprise 5% of all $Thy\text{-}1^+$ cells in the spleen and lymph nodes (285). In mice, most IEL γ/δ T cells express CD8. However, CD8 on γ/δ T cells is a homodimer of the α chain (279). Peripheral blood lymphocytes taken from normal donors have been used also to demonstrate the expression of NK-related surface markers γ/δ T cells. CD11b, the complement receptor for C3bi, has been shown on the surface of 50% of γ/δ T cells, while 10% have been shown to express CD16 (286). A similar phenotypic profile exists for human γ/δ T cells.

In contrast to α/ß T cells, γ/δ T cells comprise only a small fraction of the cells in lymphoid tissue. However, in the mouse, almost the entire IEL population consists of γ/δ T cells (ie., 50-100% of the lymphocytes in the epidermis and IEL in the gut and lungs) (287). What makes the tissue distribution of γ/δ T cells even more intriguing is the association of certain Vγ Vδ chains with a particular tissue. Most of the γ/δ T cells in the epidermis express Vγ3-Vδ1 while the majority of those in the lung express Vγ2-Vδ5/6 (288). The propensity of γ/δ T cells to accumulate in epithelial tissue seems restricted to mice. In man, for instance, no γ/δ T cells have been demonstrated in the epidermis, a site dominated by α/ß T cells (286). Furthermore, γ/δ T cells constitute only 10-13% of human intestinal IEL (286).

While α and ß TCR gene expression is responsible for the recognition of a wide range of antigens in the context of self-MHC, γ/δ T cells have a much more limited TCR repertoire. This is has been related to: a smaller number of γ and δ V germline gene segments; a reduced junctional diversity demonstrated among epithelial γ/δ T cell clones; and restricted γ chain and δ chain pairings. Because of their anatomic localization, it has been suggested that one of the functions of γ/δ T cells is immune surveillance. Also, because the γ/δ TCR repertoire is limited, it is unlikely that they are able to recognize foreign or altered antigenic determinants on host cells to the same extent as α/ß T cells. This idea is further supported by the experiments which have demonstrated that TCRγ/δ$^+$ cells are able to recognize antigen presented in the context of non-conventional class I antigens which have little allelic polymorphism. For example, a γ/δ T cell clone and a γ/δ T cell hybridoma have been shown to recognize allogeneic determinants encoded by genes which map into the TLa region (289,290). Also, a γ/δ T cell hybridoma specific for the GT copolymer in association with a class I molecule encoded by the gene *QA-1* also has been identified (291). Thus, it has been proposed that some TCRγ/δ$^+$ cells bind and kill autologous cells which express one of a limited number of microbial-derived products and/or endogenous antigens induced in response to infection or transformation (292,293). The recognition of non-conventional class I gene

products and HSP by γ/δ T cells suggests that these ligands represent some of the autologous target molecules induced in response to infection or stress (294,295). It also has been suggested that the presence of a particular γ/δ TCR subset may coincide with induction of a particular endogenous antigen in specific tissue or organs (287); however, the hypothesis could be extended to include microbial products commonly encountered in particular tissue sites.

1.5.3 Mechanisms of Cell-mediated Cytotoxicity

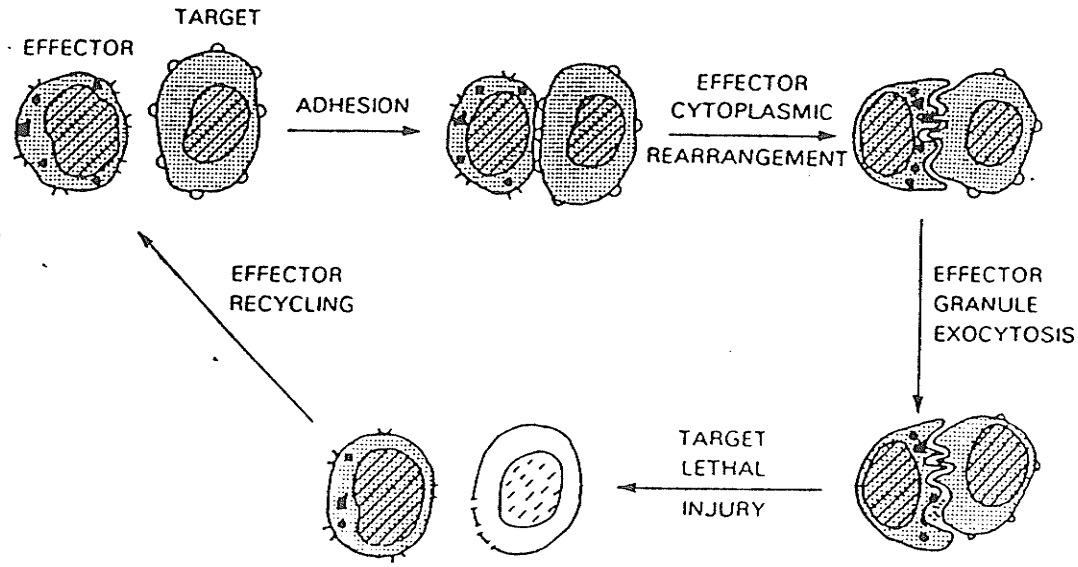
Cell-mediated cytotoxicity is a multi-step process involving both recognition and binding of the target cell by an effector cell (conjugate formation), programming for cell lysis or delivery of the "lethal hit", followed by dissociation of the effector cell from the target cell. This process culminates with the recycling of the effector cell so that it can kill again (Figure 1.3, 296).

$CD4^+$ and $CD8^+$ CTL recognize and bind antigen associated with self class II and class I molecules, respectively, through antigen-specific T-cell receptor interaction. Recognition structures used by non-MHC-restricted cytotoxic cells such as NK cells and non-MHC-restricted T cells and the target cell determinants to which they bind have yet to identified. Recently, MoAb that are able to inhibit tumor target lysis by non-specific cytotoxic cells isolated from teleost fish were found to prevent killing of K562 tumor

target by human NK cells and IL-2 activated T cells (297). The reduction of lytic activity was associated with decreased effector-target cell binding. Similar experiments with anti-CD3 failed to reduce the number of NK cell and T cell conjugates and thus indicated that the recognition structure was distinct from the CD3/TCR complex (298). Biochemical studies have shown that the determinant recognized by this MoAb to be located on a novel evolutionarily conserved structure, referred to as FAM (function-associated molecule). FAM has some homology with vimentin and is distinct from the T cell receptor. This suggests that both NK cells and IL-2-activated T cells possessing NK-like activity, may express this determinant.

As mentioned earlier, most cytotoxic lymphocytes are LGL. The possibility that the cytoplasmic granules may be involved in target cell killing was originally proposed by Granger (299) and supported by biochemical and ultrastructural experiments which demonstrated that lysosomal enzymes such as acid phosphotase (300) and other granule-associated components (301) are released (degranulation) into the intercellular space formed as a result of effector-target cell binding (296). While a number of molecules released from the granules have been characterized, only one, perforin (also known as cytolysin) (302), is cytolytic (303). Although normally hydrophillic, in the presence of Ca^{2+}, the 70 kDa monomeric form of perforin is thought to become amphipathic on release,

Figure 1.3 The granule exocytosis model of cell-mediated cytotoxicity. Taken from Henkart, P.A. Mechanism of lymphocyte-mediated cytotoxicity. Ann. Rev. 3:31, 1985 (296).


TARGET
EFFECTOR
ADHESION
EFFECTOR
CYTOPLASMIC
REARRANGEMENT
EFFECTOR
GRANULE
EXOCYTOSIS
TARGET
LETHAL
INJURY
EFFECTOR
RECYCLING

thus allowing it to interact with the cell membrane while polymerizing with other perforin molecules. This results in the formation of pores in the target cell membrane causing lysis of the target cell (304).

A family of seven serine esterases (designated as granzyme A to G) have also been described. These comprise approximately 90% of the secretory granule proteins of NK cells and CTL (305). Purified granzymes are non-cytolytic, since inhibition of their activity does not prevent lysis (306). However, elevated levels of granzyme mRNA in activated NK cells, activated mature T cells and IL-2 cultured thymocytes appears to correlate with increased cytolytic activity (307,308). It has been suggested that while granzymes are not directly cytolytic, they are intimately associated with those cellular functions leading to cytolysis (308). For example, granzymes may act on membrane proteins, and facilitate perforin insertion. Also, they may aid in the detachment process following the delivery of the lethal hit enabling recycling of the cytolytic effector cell (309). While the specific roles of the granzymes in cytolysis have yet to be elucidated, much is already known. For example, inhibitors of granzyme A have been shown to reduce nuclear DNA release as indicated by the decreased measurement of ^{125}I-DNA from Triton X-100-treated target cells (310). While purified perforin is effective in causing release of ^{51}Cr from target cells, granzyme A is required for a significant release of

DNA. These results suggest a role for granzyme A in the degradation of target cell DNA (apoptosis).

More recently, two serine proteases, RNKP-1 and fragmentin, have been identified. Both of which possess a high degree of homology with granzyme B (311,312). Northern blot analysis of normal rat splenocytes have failed to detect constitutive expression of RNKP-1 mRNA. However, lectin-stimulation induces RNKP-1 mRNA expression, which coincides with maximal NK and LAK activity (311). Fragmentin has been shown to induce DNA fragmentation and apoptosis (312). In the presence of Ca^{2+} and perforin, purified fragmentin causes a rapid cleavage of tumor target DNA. No DNA damage was observed in the absence of perforin. It is of interest to note that membrane damage was also observed at fragmentin concentrations too low to induce DNA damage, thus indicating that fragmentin likely has more than one mode of action.

Another major granule-associated molecule is chondroitin sulphate-A proteoglycans. Because of their acidic nature, proteoglycans are believed to bind the more basic proteins in the granules, thereby function in the packaging of granzymes and/or perforin (305).

While the exocytosis model of lymphocyte-mediated lysis is supported by a number of experimental observations, this model, as yet, fails to explain some data. For example, target cell lysis can be initiated by CTL effector cells which lack granules or occur in the absence of granule release

(313,314). Moreover, some CTL clones have been shown to lyse tumor targets in the presence of a Ca^{2+} chelating agent (313). An alternative model of cell-mediated lysis has been proposed to explain these observations. Lymphocyte-triggered internal target cell disintegration presupposes that binding of effector cell recognition structures with determinants on the target cell causes a redistribution of cell-surface proteins which in turn alters the membrane permeability of the cell to Ca^{2+} (315). Elevation of intracellular Ca^{2+} is believed to activate autolytic cellular mechanisms that can initiate apoptosis and lead to cell death. The increase of intracellular Ca^{2+} in the presence of a chelating agent suggested that intracellular Ca^{2+} stores, such as in the mitochondria and endoplasmic reticulum may act as a source of second messenger to initiate the autolytic cascade.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

Female A/J ($H\text{-}2^{k/d}$), (C57BL/6 x A/J)F_1-hybrids ($B6AF_1$), and CBA ($H\text{-}2^{k}$) mice were purchased from Jackson Laboratory, Bar Harbor, ME. Male C3H ($H\text{-}2^{k}$) and female BALB/c ($H\text{-}2^{d}$), C57BL/6 (B6, $H\text{-}2^{b}$) and (C57BL/6 x DBA/2)F_1-hybrids (BDF_1, $H\text{-}2^{b/d}$) were obtained from Charles River, Wilmington, MA. We bred (CBA x A/J)F_1-hybrid (BAF_1) and (BALB/c x C3H)F_1-hybrid mice in our animal care facility. Mice were used when they were between 12 - 14 weeks old, except when making ascites at which time the animals were 8 weeks or older.

2.2 Cell Lines

2.2.1 Cell lines

CTLL-2 ($H\text{-}2^{b}$), an IL-2-dependent cytotoxic T cell clone (133) and EL-4 ($H\text{-}2^{b}$), a mouse thymoma, were a gift from Dr. D. Rayner, Department of Pathology, University of Manitoba, Winnipeg, MB. The murine fibroblast cell line, L929, was received from Dr. P. Roth, Department of Medical Microbiology, University of Manitoba.

2.2.2 Tumor target cells

The mastocytoma P815 ($H\text{-}2^{d}$) and the Moloney leukemia virus-induced T cell lymphoma YAC-1 ($H\text{-}2^{a}$) were obtained from American Type Culture Collection (ATCC), Rockville, MD. BW-1100.129.237 (BW1100), an α^{-},$ß^{-}$ TCR/gene-loss variant developed from the AKR/J ($H\text{-}2^{k}$) T cell lymphoma BW5147.G.1.4 OUA^{r}.1, was

the generous gift of Dr. P. Marrack (316).

2.2.3 Hybridomas

The murine hybridoma, PK136, which secretes cytotoxic IgG_{2a} directed against the strain-specific ($H\text{-}2^b$) NK-cell surface marker NK1.1 (243), the heterohybridoma R4-6A2, which secretes monoclonal antibodies directed against murine IFNγ, and the murine hybridomas 28-13-3s and 34-4-21S, which secrete monoclonal anti-$H\text{-}2K^b$ and anti-$H\text{-}2D^d$ antibody, respectively, were obtained from ATCC. The heterohybridoma 145-2C11, which secretes a non-cytotoxic hamster monoclonal IgG antibody specific for the ε chain of the murine CD3 molecule, was obtained from Dr. J. Bluestone (317).

All cell lines, except L929 and CTLL-2, were maintained in RPMI 1640 (RPMI) medium (Gibco, Grand Island, NY) supplemented with either 10 or, in the case of PK136, 15 % FCS, glutamine (200 mM; Gibco), sodium pyruvate (100 mM; Gibco), penicillin-streptomycin (5000 mg/ml) and Hepes (4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid, 10 mM; Mallinckrodt, Paris, KY) (appendix 2.13.2). L929 and CTLL-2 which were grown in RPMI-5 % FCS. CTLL-2 medium supplement (appendix 2.13.1) and 2 % phorbol myristate acetate (PMA)-induced EL-4 supernatant, produced as described below in section 2.4, were added to the medium reserved for CTLL-2 cells.

2.3 Antibodies

Cytotoxic monoclonal antibodies to Thy-1.2, L3T4 (CD4, clone YTS 191.1) and Lyt-2.2 (CD8) were obtained from Cedarlane Laboratories, Hornby, ON. Rabbit anti-$ASGM_1$ was received from Wako Fine Chemicals, Dallas, TX. Culture supernatants from 28-13-3s and 34-4-21S were used as a source of cytotoxic monoclonal anti-H-$2K^b$ and anti-H-$2D^d$, respectively. Culture supernatant from R4-6A2 was used as a source of rat MoAb (IgG_1) directed against murine IFNγ. 145-2C11 culture supernatant was used as a source of hamster IgG monoclonal antibodies specific for murine CD3. Anti-γ/δ TCR, a hamster IgG MoAb secreted by the clone GL3 that reacts with all T cells bearing γ/δ TCR but not with those T cells expressing the $\alpha/ß$ TCR (318), was obtained from Pharmingen, San Diego, CA. Rabbit antiserum reactive with hamster IgG was obtained from Jackson ImmunoResearch Laboratories West Grove, PA. Ascites fluid containing anti-NK1.1 was harvested from (BALB/c x C3H)F_1 mice as described below in section 2.6. FITC-conjugated anti-Thy-1.2 (clone 30-H12) and anti-Lyt-2.2 (clone 53-6.7) as well as streptavidin-conjugated anti-L3T4 (GK1.5) were obtained from Becton Dickinson, Mississauga, ON.

2.4 Sources of IL-2

Human recombinant IL-2 (rIL-2) was obtained from Boerhinger-Mannhiem, Laval, PQ and murine rIL-2 was received from Genzyme Corp., Boston, MA. IL-2 containing culture

medium was prepared from EL-4 cells stimulated with PMA as previously described (319). Briefly, EL-4 cells (1.0×10^6 cells/ml) were cultured in 100 ml of RPMI-5 % FCS and PMA (10 ng/ml) for 40 - 48 hr at 37 °C, 5 % CO_2. A stock solution of PMA was prepared by dissolving PMA (Sigma) in absolute ethanol to a concentration of 100 µg/ml. The cells were removed by centrifugation at 200 x g for 5 min in 50 ml culture tubes (Corning Labware, Corning, NY) and the supernatant was filter sterilized and stored at -70 °C in 2 ml aliquots.

2.5 Preparation of encephalomyocarditis (EMC) virus stock

L929 cells were grown until confluent in a T75 flask (Corning) containing 20 ml of RPMI-5 % FCS. The medium was removed and 10 ml of fresh medium containing 0.5 ml EMC virus stock (obtained from Dr. R. Warrington, Department of Immunology, University of Manitoba) was added. After 24 hr the supernatant was removed, centrifuged at 350 x g for 10 min in a 15 ml sterile culture tube (Corning) and 500 µl aliquots dispensed into sterile 1.5 ml centrifuge tubes (Fisher Scientific, Edmonton, AB). The new virus stock was stored at -70 °C.

2.6 Preparation of anti-NK1.1 ascites

Generally, B-cell hybridomas propagated as a tumor in the peritoneum of mice generate ascites fluid containing high titre antibody as compared to *in vitro* culturing (320,321). PK136 was grown in (BALB/c x C3H)F_1-hybrid mice to generate ascites fluid possessing high titre anti-NK1.1 using a previously described method (321). Briefly, F_1-hybrids (8 weeks or older) were injected with 0.5 ml of pristane (2,6,10,14-tetramethyl-pentadecane; Sigma). After a period of 10 to 14 days, 1.0 - 2.0 x 10^6 PK136 cells in Hank's buffered salt solution (HBSS; see Appendix 2.13.3 for formulation) were injected into the peritoneal cavity of each mouse. The mice were rested for a week after which they were monitored daily for peritoneal swelling. Ascites fluid was drained through an 18 gauge needle held over a 15 ml culture tube. The ascites was centrifuged at 350 x g for 10 min to remove cells and then passaged through a layer of gauze to remove any fibrous material; the ascites was stored at -20 °C. To ensure anti-NK1.1 was present in the ascites, each sample was tested for their capacity to deplete NK cell activity against YAC-1 exhibited by spleen cells isolated form poly I:C-treated C57BL/6 or BDF_1 mice at antibody dilutions of 1/10, 1/100 and 1/1000.

2.7 Induction of GVH reactions

2.7.1 Preparation of the graft

Acute GVH reactions were induced in F_1-hybrid recipients by injecting parental A/J strain cells into $B6AF_1$ mice and by injecting parental C57BL/6 strain cells into BDF_1 mice. Chronic GVH reactions were induced in BAF_1 mice by injecting parental A/J strain cells. Methods used for the preparation of donor cell suspensions were the same for all GVH reactions and have been previously described (117). Briefly, donors were killed by cervical dislocation and their spleen and lymph nodes (inguinal, cervical, brachial and axillary) placed into a petri dish containing fresh HBSS. In some experiments the donors had been injected intraperitoneally with 100 µl of poly I:C (1 mg/ml; Sigma) in HBSS 18 hr prior to removing the spleen and lymph nodes. Cell suspensions were prepared by pressing the tissue into a #60 mesh stainless steel screen, suspended over a 60 x 15 mm culture dish (Becton Dickinson) of HBSS, with a stainless steel spatula. The cells were washed thoroughly in HBSS by centrifugation, 350 x g for 10 min, to prevent clotting. Using a pasteur pipette the cell suspension (10 - 15 ml in HBSS) was then passaged through a double layer of gauze to remove any clumps. The cell suspension was washed and resuspended to a known cell volume and the viable cell number determined by trypan blue dye exclusion. The cells were then resuspended to a final cell concentration of 1.67×10^8 cells/ml. Using a 26 gauge needle and a 1 ml syringe each

recipient was injected with 300 µl of the donor cell suspension (5.0×10^7 viable cells) via the tail vein.

2.7.2 Treatment of the graft with anti-NK1.1

In some experiments involving the parental-F_1-hybrid combination C57BL/6 → BDF_1 the graft prepared from the donors, both untreated and poly I:C-treated, was treated *ex vivo* with anti-NK1.1 and complement or complement alone. In these experiments a portion of the graft was resuspended in cytotoxicity medium (Cedarlane) containing anti-NK1.1 ascites (1/20) to a cell concentration of 2.0×10^7 cells/ml. The cell suspensions (no more than 25 ml in 50 ml conical centrifuge tubes) were incubated at 4 °C for 1 hr after which the cells were washed by adding 25 ml of cold HBSS to each tube and then centrifuging at 350 x g, 4 °C for 10 min. The cells were then resuspended in 25 ml cytotoxicity medium containing a 1/8 dilution of Low-Tox rabbit complement and incubated at 37 °C for 1 hr. The cells were than washed and the antibody treatment repeated a second time. After the second treatment the viable cells were counted, resuspended in HBSS to 1.67×10^8 cells/ml and injected into recipients as already described for untreated grafts. It should be noted that while the viability of the graft after the antibody treatment is high (>90 %), cell loss can range from 30 - 50 %.

2.8 Mixed-lymphocyte reactivity (MLR) of NK1.1-depleted and untreated C57BL/6 grafts

NK1.1-depleted and non-depleted grafts prepared from poly I:C-stimulated donors were used a source of responder cells. Spleen cells from sex-matched BDF_1 mice were used as a stimulator cell population. Stimulator cells were prepared by adding 25 µg of mitomycin C (Sigma; 50 µl of 0.5 mg/ml in HBSS) to 1-6 x 10^7 cells/ml and incubating for 20 min at 37 °C in the dark. The mitomycin C-treated stimulator cells were washed three times with HBSS, resuspended in RPMI-5 % FCS and plated (5.0 x 10^5 cells/ml) into triplicate wells of a 96 well u-bottom plates (Linbro, Flow Laboratories, Mississauga, ON) with and without an equal number of responder cells. The cultures were pulsed for 16 hr with ^{3}H-thymidine (Amersham, Oakville, ON) and the plates processed at this point in an identical manner described below in section 2.9.2

2.9 Monitoring of GVH reactions

2.9.1 Splenomegaly

The spleen and body weight was measured for each of the killed mice and the weights used to estimate the degree of the GVH reaction at each time interval following the induction so as to verify the presence of a GVH reaction. The degree of the GVH reaction was expressed as a Spleen Index (S.I.) defined as:

$$S.I. = \frac{\text{Spleen weight. of Expt.}}{\text{Body weight. of Expt.}} \div \frac{\text{Spleen weight. of Cont.}}{\text{Body weight. of Cont.}}$$

where Expt. refers to the experimental animal and Cont. to the control animal. The control animals used were normal, sex-matched, non-injected F_1-hybrid littermates. To correct for variability amongst control animals the value calculated from control animals is the mean of no less than 3 control animals.

2.9.2 Immune suppression (mitogen non-responsiveness)

Diminished proliferative responsiveness to mitogens is a characteristic feature of mice with GVH reactions (117). The mitogen responsiveness of splenic lymphocytes, from control and GVH animals, was measured by ^{3}H-thymidine incorporation. Spleens were aseptically removed from GVH and control mice and each placed into a 60 x 15 mm culture dish containing sterile HBSS. Cell suspensions made from each spleen were prepared by pressing the spleen into a sterile #60 mesh screen as described above in the induction procedure (section 2.7). The cell suspensions were washed once by centrifugation, 350 x g for 10 min, resuspended in 5 ml of RPMI-5 % FCS and the viable cell concentration determined by trypan blue dye exclusion. A sample containing 9.0×10^6 cells was removed from the cell suspension, at which time the remaining cells were used to assay for cytotoxic cell activity. The sample was washed once in RPMI-5 % FCS, 350 x g for 10 min, and resuspended in 3 ml

of RPMI-5 % FCS in a sterile 12 x 75 mm borosilicate glass culture tube (Baxter Healthcare, McGraw Park. IL) to give a final cell concentration of 3.0 x 10^6 cells/ml. The 3 ml sample was evenly divided over four 12 x 75 mm tubes by aliquoting 750 µl into each of three tubes, one tube for each of the mitogens ConA (Sigma), LPS (Sigma) and PHA (Wellmark Diagnostics, Guelph, ON), and retaining the original sample tube as the no treatment tube. A 1/50 dilution of mitogen stock solutions was prepared in RPMI-5 % FCS to give a final concentration of 2 µg/ml ConA, 20 µg/ml LPS and 2 µg/ml PHA. An 80 µl aliquot of each was added to the corresponding tube containing spleen cells while 80 µl of RPMI-5 % FCS was added to the no treatment tube. A volume of 200 µl was plated, in triplicate for each treatment, into U-bottomed 96-well tissue culture plates (Linbro). The plates were incubated for 48 hr at 37 °C, 5 % CO_2 and then pulsed with 1 µCi of ^{3}H-thymidine (Amersham) in HBSS (20 µl/well) for 16 hr. The cells were harvested onto glass fibre filter discs (Mandel Scientific, Rockwood, Ont., Can.) with a cell harvester (Skatron Inc., Sterling, VA.). The degree of mitogen responsiveness for each treatment was expressed as the mean decay per minute (Dpm) x 10^{-3} ± S.E. of triplicate samples.

2.9.3 Histopathology

In mice with acute GVH reactions histopathologic changes in lung, liver, thymus and salivary-gland tissue were also

examined. Tissue samples from grafted and ungrafted F_1-hybrid mice were collected at the time of killing and fixed in 10 % phosphate-buffered (ph 7.2) formalin. The tissue samples from each mouse were loaded into a cassette and sent to the Department of Pathology, Health Sciences Centre, Winnipeg, MB. Slides were prepared by embedding the tissue in parrafin from which sections of 0.75 μ were cut and stained with hematoxylin and eosin. We then examined the slides by light microscopy for histopathology.

2.9.4 Cachexia

Cachexia was monitored in BDF_1 graft recipients and normal littermates by measuring their body weight (g) at fairly regular intervals over the course of the study; in particular day 20 since a 10 % decrease in body weight by this time of the reaction has been shown to strongly correlate with acute GVH disease (195).

2.10 Cytokine Studies

2.10.1 Culture conditions for IL-2 production

IL-2 production was measured from lymphocyte cultures prepared from normal (untreated) F_1-hybrid mice, syngeneic graft recipients and allograft recipients on different days after induction. The spleen of each mouse was removed aseptically and a cell suspension prepared as described above in section 2.7 and 2.9.2. A viable cell count of each cell

suspension was determined and lymphocytes from each cell suspension were plated either in triplicate into 96-well flat bottom plates (Linbro) at a density of 5.0×10^5 cells/well in 200 µl RPMI-10% FCS or into a single well of a 24-well plates at a cell density of 5.0×10^6 cells/well in 2 ml RPMI-10% FCS and cultured at 37 °C, 5% CO_2. Supernatants from the cultures were harvested after 72 hr and assayed for the presence of IL-2.

2.10.2 IL-2 quantitation

A rapid colorimetric biological assay, described previously by Mosmann (322), was used to test for the presence of IL-2 in culture supernatants by the ability of the test supernatants to maintain the growth of the IL-2-dependent T cell clone CTLL-2. Briefly, 24 and 72 hr culture supernatant (50 µl) from each cell suspension was sampled into two sets of triplicate wells occupying the first two rows of a sterile 96-well flat-bottom tissue culture plate (Linbro). The samples in the second triplicate (row 2) were then titrated by doubling dilution with CTLL-2 medium without IL-2 (50 µl) to the bottom of the plate (row 8). CTLL-2 cells were washed by centrifugation and resuspended in IL-2-free CTLL-2 medium. A 50 µl volume of this cell suspension (2.0×10^5 cells/ml) was added to each well. Therefore, the first triplicate comprised of equal parts test supernatant and medium containing cells represents a 1/2 dilution while the triplicate sample in row

8 made from doubling dilutions of the second row represents a 1/256 dilution. The plates were then incubated for 17 hr at 37 °C in 5 % CO_2. A solution of the tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma), was prepared in HBBS (5 mg/ml) and 10 µl was pipetted into each well. The plates were incubated for 4 hr after which 200 µl of MTT developing solution (Appendix 2.13.4) was added to each well. The absorbance was measured using a Biotek EL308 microplate reader (Mandel Scientific, Rockwood, ON.) with a test and reference wavelength of 590 and 630 nm, respectively. To correct for inter-assay variability a standard curve was constructed by culturing the CTLL-2 cells in the presence of titrated human rIL-2 (Boerhinger-Mannhiem) or PMA-stimulated EL-4 supernatant each time samples were tested. IL-2 activity was calculated by probit analysis and expressed as half maximum units per ml (HMU/ml) of supernatant (145) where 1 HMU is defined as the dilution giving 50 % maximal activity and the number of HMU/ml is expressed as the reciprocal of that dilution.

2.10.3 Assay for IFN

Culture supernatants were assayed for IFN activity using a cytopathic effect (CPE) reduction assay (323). Briefly, supernatants (100 µl) sampled from 72 hr spleen cell cultures were titrated in 96 well flat-bottom tissue culture plates (Corning) in duplicate by doubling dilutions in RPMI-5 % FCS

using the same method described for IL-2 (sections 2.10.1 and 2.10.2). A volume of 100 µl of RPMI-5 % FCS containing 2.0 x 10^4 L929 cells was plated into each well to give a final volume of 200 µl. After an incubation of 24 hr at 37 °C, 5 % CO_2 the plates were inverted over a waste container to remove the medium from the wells and patted dry on sterile guaze pads. The plates were washed twice by carefully dispensing 100 µl of HBSS into each well. EMC virus was prepared by diluting an aliquot of virus stock in medium to obtain the minimal concentration necessary to achieve maximal CPE; in our experiments this was achieved with a 1 in 7.0 x 10^5 dilution of virus stock. A 25 µl volume of diluted virus stock was placed into each well and the plates were incubated for 24 hr. After the incubation period the plates were inverted and the virus-containing medium dumped into a container of 10 % hypochlorite solution. Reduction in CPE was observed by staining the viable cells with the vital dye crystal violet (0.5 % w/v in 20 % methanol; 50 µl/well) for 10 min at room temperature. The plates were then washed of the excess stain by rinsing the plates in cold tap water and then allowed to air dry. Wells exhibiting maximal viral CPE (no protection) and no CPE (no virus) were also included. Each test plate contained a row of serially diluted mouse IFNα/ß standard (2,400 IU/ml in the first well; Sigma) to provide a standard curve against which unknown levels of IFN could be quantitated.

2.10.4 Characterization of IFN

Murine IFNγ was differentiated from IFNα/ß by neutralization of the antiviral activity with a rat anti-murine IFNγ MoAb using a previously described method (324). Briefly, culture supernatants, titrated by doubling dilution, were added 1:1 with 50 µl of R4-6A2 culture supernatant for 1 hr at 37 °C in 96 well flat-bottom tissue culture plates (Linbro). With this procedure we found that 1 ml of culture supernatant was able to neutralize up to 5000 U/ml of rIFNγ (ICN Immunobiologicals, Lisle, IL) in an equal volume of medium. To identify IFNγ, culture supernatants from cell suspensions were first heated to 56 °C for 1 hr in a waterbath before testing for antiviral activity (325).

2.11 Cell-mediated cytotoxicity assay

2.11.1 Isolation of effectors

Methods for isolation of cytotoxic effector cell populations, their treatment with cytotoxic antibodies and complement, and subsequent assay for lytic activity has been described in detail elsewhere (117). Pooled cell suspensions of different lymphoid organs were prepared from three or more mice in each group using the same methods already described (2.7 and 2.10.2). If cell suspensions had been prepared under sterile conditions for the purpose of other studies, for example cytokine determinations, then cell suspensions of the same lymphoid organs taken from mice of the same test group

were pooled and handled under non-sterile conditions. Cell suspensions were fractionated on nylon wool columns to isolate the non-adherent cell fraction using the method of Julius (326). Cells were washed by centrifugation, 350 x g for 10 min, and resuspended to a volume necessary to give 4 ml for each column; to prevent overloading of the column more than one column was used for each cell suspension. For example, cells from no more than two GVH spleens were added to each column. Nylon wool columns were prepared in advance by soaking 0.6 g of nylon wool (Baxter) in RPMI-5 % FCS at 37 °C, 5 % CO_2, for 30 min. The nylon wool was then placed into the barrels of 20 ml syringes affixed with 22 gauge needles stoppered with cork. Excess medium was removed from the top of each column prior to the addition of the cell suspension. Cell suspensions were added to the column and the columns were unstoppered to allow approximately half of the cell suspension to run into the nylon wool. The column was re-stoppered and the nylon wool gently teased up to make full contact with the remaining portion of the cell suspension. The columns were then incubated for 1 hr at 37 °C, 5 % CO_2. After the incubation, the columns were suspended over 50 ml culture tubes (Corning) and the non-adherent cell population collected by eluting each column with 50 ml of RPMI-5 % FCS. The non-adherent cells were pelleted by centrifugation, 350 x g for 10 min, and then resuspended in 4 ml RPMI-5 % FCS. The cell suspension was then carefully layered onto 4 ml of Lympholyte-

M (Cedarlane) to remove red cells. In those situations where the cell pellet was large the cells were diluted to an appropriate volume and split among several tubes to prevent overloading of the gradients. The tubes were centrifuged at 600 x g for 25 min and the cells removed from the interface. The cells were washed three times in RPMI-5 % FCS and then stored as a pellet in the last wash medium overnight at 4 C in a capped tube. The number of viable effector cells in each test group was then determined by trypan dye exclusion. Once the cell counts were known the size of the assay could be determined and the necessary number of ^{51}Cr-labelled target cells determined.

2.11.2 Treatment of effector cells with antibody and complement.

The cell-surface phenotype of cytotoxic effector cells was characterized by depleting those cells that expressmarkers recognized by a panel of cytotoxic antibodies and complement. Thus, absence of lytic activity against the definitive tumor target denotes marker expression by the effector cell. The monoclonal antibodies, antisera and their respective dilutions used to treat the effector cells are summarized in Table 2.1. All antibodies were diluted in ice-cold cytotoxicity medium (Cedarlane). Aliquots of effector cells (7.0×10^6 cells for each tumor target cell population) were sampled into 12 x 75 mm borosilicate tubes (Baxter); one tube for each antibody

Table 2.1 Summary of antibodies used to deplete effector cells.

Antibody	Source	Specificity	Dilution
5-A8	ascites	Thy-1.2	1/500
YTS191.1	ascites	CD4 (L3T4)	1/500
AD4	ascites	CD8 (Lyt-2.2)	1/500
Rab. α $ASGM_1$	antiserum	N/A[1]	1/100
PK136	ascites	NK1.1	1/20
28-13-3s	supernatant	$H-2D^k$	dilute to 2.0 x 10^7 cell/ml
34-4-21s	supernatant	$H-2D^d$	dilute to 2.0 x 10^7 cell/ml
145-2C11	supernatant	CD3	dilute to 1.0 x 10^7 cell/ml
GL3	buffer[2]	γ/δ	1/25
Rab. α Ham.IgG	antiserum	H + L chain	1/25

1. Not applicable.

2. Affinity-purified from culture supernatant.

treatment. Except where indicated in Table 2.1, a volume of diluted antibody required to give a final cell concentration of 2.0 x 10^7 cells/ml was added to each tube. The cells where resuspended by quickly vortexing and placed into the refrigerator (4 °C) or held on ice for 1 hr. Because neither of the hamster IgG antibodies, anti-CD3 and anti-γ/δ, mediated complement-directed lysis a second antibody step involving rabbit anti-hamster IgG antiserum was added to deplete effector cells by indirect complement-mediated lysis. The cells were washed with ice-cold HBSS (4 ml) and then centrifuged at 350 x g, 4 °C for 10 min. The supernatant was removed by suction and complement (1/8 in ice-cold cytotoxicity medium) was added to each tube at a cell concentration of 2.0 x 10^7 cells/ml. The tubes were incubated for 1 hr at 37 °C after which the cells were washed with HBSS as already described. The supernatant was suctioned off and the cells resuspended to a cell concentration of 1.0 x 10^7 cells/ml. Control tubes consisted of cells resuspended only in cytotoxicity medium (no treatment), and cells which received complement alone. The effector cells were then serially diluted over the effector:target cell (E:T) ratio range of 100:1 to 12.5:1. Each dilution was plated in triplicate into 96 well v-bottom plates (Corning).

2.11.3 Preparation of the tumor targets

A cell count was determined for each of the tumor targets

and the appropriate number of target cells were sampled into 15 ml conical capped culture tubes (Corning). The cells were pelleted by centrifugation, 350 x g for 10 min, and the supernatant aspirated off. 100 µl of ^{51}Cr (Day 0) was added directly to the pellet; an additional 50 µl was added for each million cells over 2 x 10^6. The cells were resuspended by gently tapping the bottom of the tube and incubated for 1 hr at 37 °C in a shaker waterbath. The cells were then washed once with 15 ml HBSS and twice with 15 ml RPMI-5 % FCS. After the third wash the target cells were counted and resuspended in a volume of RPMI-5 % FCS to give a final cell concentration of 1 x 10^5 cells/ml.

2.11.4 Cytotoxicity assay

A standard 4 hr ^{51}Chromium-release assay, similar to that originally described by Brunner et al. (327) was used to measure cytotoxic cell activity in the nylon wool non-adherent fraction of lymphoid cell suspensions. Effector cells were serially diluted to obtain E:T ratios ranging from 100:1 to 12.5:1. 100 µl of the target cell suspension was plated into each well containing effector cells and into wells designated for minimal and maximal ^{51}Cr release. The plates were incubated for 4 hr at 37 °C, 5 % CO_2. The plates were centrifuged at 350 x g for 5 min to pellet the cells and 100 µl of supernatant transferred from each well into rimshot tubes (Fisher). In the wells specified for maximal release

the cells were resuspended in the well and 100 µl sampled. The amount of ^{51}Cr release was measured using a LKB 1272 Clinigamma (Wallac, Turku, Finland). Percent lysis was calculated as follows:

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

where $CPM_{Expt.}$ is the counts per min. from each test well, $CPM_{Min.}$ is the mean minimal or spontaneous release from the triplicate wells and $CPM_{Max.}$ is the mean maximal release from the triplicate wells. The mean percent lysis for each dilution was calculated from the average of the triplicate wells. Results were expressed either as a percent cytotoxicity for a given E:T ratio ± S.E. or as lytic units (LU) which were calculated using a previously described method (328). Briefly, dose response curves were generated from percent lysis data over the 4 E:T ratios and LU per 10^7 effector cells calculated using exponential fit. One LU was defined as the number of effector cells required to achieve 10% cytotoxicity.

2.12 Flow cytometry

Cells suspensions were treated with fluorescently-labelled antibodies directed against cell-surface markers and analyzed by flow cytometry. Aliquots containing 1.0×10^6 cells were sampled into a 12 x 125 mm plastic disposable tubes

(Fisher), centrifuged and supernatant removed by suction. Fluorescein isothiocyanate (FITC)-conjugated antibodies, anti-Thy-1.2 and Lyt-2.2 (Becton Dickinson), were deaggregated by centrifuging a 30 µl sample of the antibodies in cellulose propionate centrifuge tubes (Beckman) for 10 min at 100,000 x g (20 psi) in a Beckman airfuge. The cells were resuspended in 95 µl of HBSS-2 % FCS and 5 µl of deaggregated antibody added. After incubating the samples for 30 - 45 min at 4 °C in the dark the cells were washed with 2 ml ice-cold HBSS-2 % FCS by centrifuging for 15 min at 350 x g, 5 °C, resuspended in 0.5 ml HBSS-2 % FCS and held on ice for flow cytometry. Control tubes consisted of cells resuspended in antibody diluent without antibody. To label L3T4-positive cells the cell pellets were resuspended with 45 µl of HBSS-2 % FCS plus 5 µl of biotinylated anti-L3T4 (Becton Dickinson). After incubating for 30 min on ice the cell suspensions were washed as already described and the cell pellets resuspended in 80 µl HBSS-2 % FCS plus 20 µl of streptavidin-conjugated phycoerythrin (Becton Dickinson). Control tubes consisted of cells minus biotinylated anti-L3T4 plus streptavidin-conjugated phycoerythrin. The cells were incubated for 30 min on ice, washed with 2 ml HBSS-2 % FCS and then resuspended to 0.5 ml in HBSS-2 % FCS for flow cytometric analysis. If the labelled cells were not be analyzed on the same day as the labelling the cell suspensions were washed 2-3 times with HBSS to remove FCS and fixed with 0.5 ml 2 % paraformaldehyde w/v

in 0.85 % saline. Cells treated in this manner can be stored at 4 °C for up to a week.

2.13 Appendix to Materials and Methods

2.13.1 CTLL-2 medium supplement

Materials

1. Hepes..... 25.70 g
2. L-Glutamine (200 mM)..... 90.00 ml
3. 2-ß-Mercaptoethanol (12 M)..... 35.00 µl
4. Sodium Pyruvate (11,004 mg/l)..... 100.00 ml
5. Penicillin-Streptomycin (5000 mg/ml). 100.00 ml

The final volume is made up to 300 ml with ddH_2O, dispensed in 30 ml aliquots into non-sterile 50 ml tubes and stored at -20 °C. One litre of CTLL-2 medium is made by adding 30 ml of the CTLL-2 supplement to 920 ml RPMI 1640 plus 2.0 g $NaHCO_3$, pH to 7.2 and filter-sterilized.

2.13.2 RPMI 1640 medium

1. RPMI 1640 (with L-glutamine)..... 10.44 g
2. $NaHCO_3$..... 2.00 g
3. Hepes..... 2.60 g
4. Penicillin-Streptomycin (5000 mg/ml). 20.00 ml
5. ddH_2O..... 800.00 ml

Once the ingredients are in solution the medium is pH to 7.1, brought up to a final volume of 880 ml, filter-

sterilized and stored at 4 °C. To make RPMI-10 % FCS, 50 ml of heat-inactivated (56 °C for 1 hr) FCS plus 5 ml Sodium Pyruvate (100 mM) is added to 440 ml of RPMI medium. If the RPMI medium has been stored for longer than 2 weeks, 5 ml of L-Glutamine (200 mM) is added.

2.13.3 Hank's buffered salt solution (HBSS)

1. Hank's 10X..... 100.00 ml
   NaCl..... 40.00 g
   KCl..... 2.00 g
   Na_2HPO_4.... 0.50 g
   KH_2PO_4..... 0.50 g
   Dissolve ingredients in 500 ml ddH_2O.

2. Tris(hydroxymethyl)aminomethane (1 M).. 27.50 ml
   Tris (F.W. 121.1)... 24.20 g
   Dissolve in ddH_2O to a final volume
   of 200 ml, pH 7.3.

3. $MgSO_4$ (0.4 M)..... 2.00 ml
   $MgSO_4.7H_{20}$..... 4.95 g
   Dissolve in 50 ml ddH_2O.

4. $CaCl_2$..... 1.65 ml
   Anhydrous $CaCl_2$..... 6.10 g

Dissolve in 50 ml ddH_2O.

5. Dextrose..... 2.20 g

6. $NaHCO_3$..... 1.40 g

7. Hepes..... 2.60 g

8. ddH_2O..... 869.00 ml

The solution is pH to 7.2, brought up to 1 L, filter-sterilized and stored in 50 ml volumes at 4 °C.

2.13.4 MTT developing solution

Isopropyl alcohol..... 375.00 ml
1 N HCl..... 15.00 ml
ddH_2O..... 110.00 ml

The ingredients make 500 ml of MTT developing solution, which can be stored at room temperature indefinitely.

CHAPTER 3

NATURAL KILLER (NK) CELL ACTIVITY IN MICE WITH ACUTE GRAFT-VERSUS-HOST REACTIONS: CORRELATION OF CYTOKINE PRODUCTION WITH THE AUGMENTATION OF NK CELL ACTIVITY AND THE ACTIVATION OF NK-LIKE CELL CYTOTOXIC ACTIVITY

3.1 Abstract

Previously studies from this laboratory have shown that acute GVH reactions produced in the parental-F_1 hybrid combination, A/J → (C57BL/6 x A/J)F_1, result in the activation of two $CD4^-$, $CD8^-$cytotoxic cell populations: a $Thy\text{-}1^{+/-}$ NK cell with a lytic spectrum confined to YAC-1 targets, and a $Thy\text{-}1^+$ NK-like cell that has the ability to lyse target cells that are normally insensitive to lysis by NK cells. Cold-target inhibition (CTI) experiments showed that the greater range of target cell killing exhibited by the NK-like population is mediated by a single effector cell with a broadened lytic repertoire. Percoll density fractionation studies revealed that NK and NK-like cells co-fractionate, indicating that both are large granular lymphocytes.

Results from this study demonstrate a close temporal relationship between elevated levels of IL-2 secretion by spleen cell cultures from mice with GVH disease and the subsequent emergence of augmented NK activity in both acute (A/J → (C57BL/6 x A/J)F_1) and chronic (A/J → CBA x A/J)F_1 GVH reactions. It was also noted that, despite high levels of IL-2 secretion, mice with chronic GVH reactions do not generate NK-like activity. IFN measurements showed that, although increased IFN activity can be detected in both acute and chronic models, a preponderance of IFNα/β and some IFNγ is produced in the acute reaction, whereas only IFNγ can be

detected in the chronic model. These results suggest that, although IL-2 may participate in augmenting conventional NK activity, this cytokine by itself does not generate NK-like activity. It is suggested that IFNα/ß, either alone or in concert with IL-2, triggers the NK-like cell response.

The NK-like cell described in this study resembles a phenotypically similar, donor-derived LGL, identified by others, in close proximity to dead or dying epithelial cells in mice with GVH disease (329). It has been suggested that these cells may mediate tissue injury. If in fact these graft-derived NK-like cells are involved in the pathogenesis of acute GVH disease, the findings of this investigation suggest that they must first be activated by an appropriate complement of cytokines that must include IFNα/ß. Thus, whether or not a GVH reaction is to pursue an acute, rapidly lethal, course depends not only on the cohort of effector cells in the graft, but also on the stimulatory cytokines released during the early, lymphoproliferative phase of the reaction.

3.2 Introduction

GVH reactions are severe and often fatal complications of bone marrow transplantation in man. Although cytotoxic T cells are believed to be the principal cellular effectors of the lesions that characterize GVH disease (98,99), there is a growing body of evidence suggesting that NK cells may play a significant, but as yet undefined, role in the pathogenesis of tissue injury (103,112,113,117,123,135,141,329-331).

In previous studies of pulmonary NK cell activity in a murine parent $\rightarrow$ F_1-hybrid model (P $\rightarrow$ F_1) of acute GVH disease (332), it was found that two phenotypically distinct types of non-MHC-restricted cytotoxic cells were activated: a population of conventional NK cells that kill only YAC-1 targets, and a population of "NK-like" cells that were Thy-1^+, and had the ability to lyse not only YAC-1 cells, but also P815 targets, cells that are normally resistant to NK cell lysis. Both NK and NK-like cells also expressed the $ASGM_1$ cell-surface marker, but did not express either Ly-1 or Lyt-2. It was suggested that, because of their ability to lyse NK-resistant targets and the fact that they were Thy-1^+, NK-like cells represented LAK cells that emerged concomitantly with elevations of IL-2 secreted during the early, lymphoproliferative phase of acute GVH reactions.

The purpose of this study was to determine what cytokines were involved in the activation of NK and NK-like cytotoxicity. Since it had been postulated that NK-like cell

activity might be inducible with IL-2, experiments were conducted to determine whether NK-like activity appeared co-extensively with elevations of IL-2 secretion, or whether some other cytokine such as interferon was involved.

3.3 Experimental Design

Two models of the GVH reaction were studied: A/J $\rightarrow$ B6AF$_1$ which causes an acute form of GVH disease, and A/J $\rightarrow$ BAF$_1$ which produces chronic GVH reactions. B6AF$_1$ recipients exhibit histo-incompatibility with A/J strain mice across the entire MHC while BAF$_1$ recipients differ from A/J strain mice at the H-2D locus (*k* versus *d*). Because of the fewer differences in MHC-encoded antigens existing between donor and recipient in the chronic GVH model it was postulated that the degree of alloreactivity in the chronic model would be less than that observed in the acute model. It was further speculated that minimal alloreactivity would lead to a reduction of the cytokines being produced and therefore lower or a complete absence of NK-like activity. Following induction, groups of mice were sacrificed at intervals and the time course of NK and NK-like activity in the spleen was assayed. On each of these days an aliquot of each of the spleen cell suspensions from each individual mouse was cultured and the level of IL-2 and IFN in the supernatant measured. The type of interferon being produced was also determined. Changes with time in the level of cytokine

production was compared with changes in the level of cytotoxicity to YAC-1 and P815 targets.

3.4 Results

3.4.1 Splenomegaly and suppression of mitogen responses in mice with GVH reactions

These are consistent features of GVH reactions in mice (9). Splenomegaly was followed over the course of all GVH reactions to confirm successful induction. Figure 3.1 shows the 5-6 fold increase in spleen index in mice acute GVH reactions and the 2-3 fold increase in mice with chronic reactions. No splenic enlargement was seen in syngeneic controls. Immunosuppression was demonstrated by measuring the mitogenic response to ConA, LPS and PHA (Table 3.1). Representative data obtained from $B6AF_1$ and BAF_1 mice with GVH reactions are shown in Table 3.I. Results indicate that spleen cells from $B6AF_1$ and BAF_1 controls showed a marked proliferative response to all three mitogens. In contrast, spleen cells from F_1-hybrid mice with GVH reactions were completely non-responsive. Mice with acute reactions also demonstrated weight loss, runting, diarrhea, and 100% mortality before day 35. Mice with chronic reactions showed minimal weight loss and all survival beyond 50 days. Survival in chronic GVH mice was normally in excess of 100 days.

Figure 3.1 Changes in spleen weight with time, expressed as a mean spleen index, in $B6AF_1$ (▲-▲) and BAF_1 (•-•) mice after the injection of 50 million parental (A/J) spleen and lymph node cells. Splenic indices from $B6AF_1$ and BAF_1 syngeneic control experiments are indicated by ■-■ and o-o, respectively. Error bars indicate the standard error (S.E.) of the mean spleen index calculated from a minimum of 3 mice. In those instances where error bars are not visible, the S.E. is smaller than the size of the symbol.

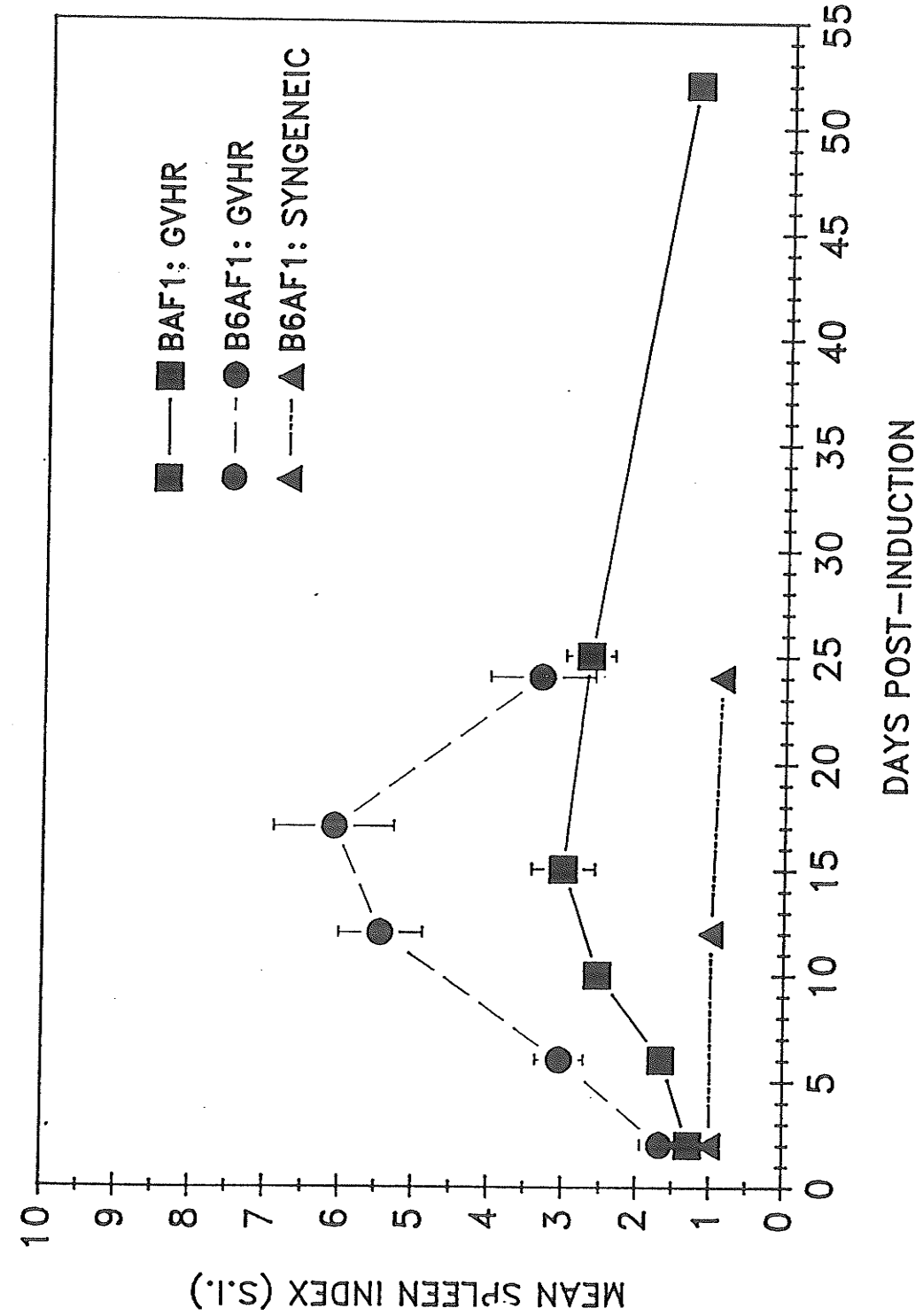

BAF1: GVHR
B6AF1: GVHR
B6AF1: SYNGENEIC
MEAN SPLEEN INDEX (S.I.)
DAYS POST-INDUCTION
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
25
30
35
40
45
50
55

Table 3.1 Mitogen non-responsiveness by spleen cells isolated from mice with either acute or chronic GVH reactions

	Treatment (Dpm x 10^{-3} +/- SE)			
Mice	NS[a]	ConA	LPS	PHA
$B6AF_1$[b]	9.3 +/- 1.6	100.3 +/- 35.5	52.8 +/- 7.3	195.6 +/- 62.9
$A/J \rightarrow B6AF_1$	8.7 +/- 5.6	10.4 +/- 7.4	3.8 +/- 2.8	9.2 +/- 6.1
		(p<0.1)	(p<0.01)	(p<0.05)
BAF_1[b]	40.2 +/- 8.7	182.6 +/- 6.9	162.2 +/- 30.8	264.4 +/- 9.1
$A/J \rightarrow BAF_1$	17.3 +/- 0.3	30.0 +/- 12.0	16.2 +/- 8.2	18.1 +/- 0.3
		(p<0.001)	(p<0.02)	(p<0.001)

[a]No stimulation. [b]Normal F_1-hybrid mice age- and sex-matched to F_1-hybrid mice with GVH reactions. Spleen cell suspensions were prepared on day 15 and 17 from a minimum of 3 mice with either chronic or acute GVH reactions, respectively. A significant difference in the proliferative response to all mitogens was demonstrated by F_1-hybrid mice with GVH reactions as compared to normal controls (Student's t-test; p values at 4 d.f. are in brackets).

3.4.2 Time course of IL-2 production and its relationship to NK and NK-like activity in acute and chronic GVH reactions

Figure 3.2A shows changes with time in IL-2 production by spleen cells from B6AF$_1$ mice with acute GVH reactions. The data indicate that there was a transient increase in IL-2 production which peaked on day 2 and then declined to control levels by day 12. Although the kinetics of both NK activity and IL-2 production were roughly parallel (Figure 3.2A and 3.2B), maximal IL-2 secretion preceded the peak in anti-YAC-1 activity by only a few days. The anti-P815 response (Figure 3.2C) lagged well behind IL-2 production and, indeed, this NK-like activity was generally highest when IL-2 levels had already returned to control values. IL-2 production in spleen cell cultures from BAF$_1$ mice with chronic GVH reactions (Figure 3.3A) peaked on day 2, and then sharply decreased to levels that were slightly above control values for the remainder of the reaction. Maximal lysis of YAC-1 targets (Figure 3.3B) occurred on day 2 and then quickly declined to below control levels. This peak in splenic NK activity coincided exactly with maximum levels of IL-2 secretion. No lytic activity against P815 (Figure 3.3C) cells was observed in these mice despite the marked elevation of IL-2 secretion levels. No significant amount of IL-2 was ever observed in spleen cell cultures from normal control mice (Figure 3.2A and 3.3A) or F$_1$-hybrids that had received syngeneic cells (data

Figure 3.2 Representative curves comparing the kinetics of *in vitro* IL-2 production (A) by spleen cells isolated from a minimum of 3 $B6AF_1$ mice with acute GVH reactions with the kinetics of cytotoxicity to YAC-1 (B) and P815 (C) target cells. Error bars indicate the standard error of the mean HMU/ml in triplicate cultures. In those instances where error bars are not visible, the S.E. is smaller than the size of the symbol.

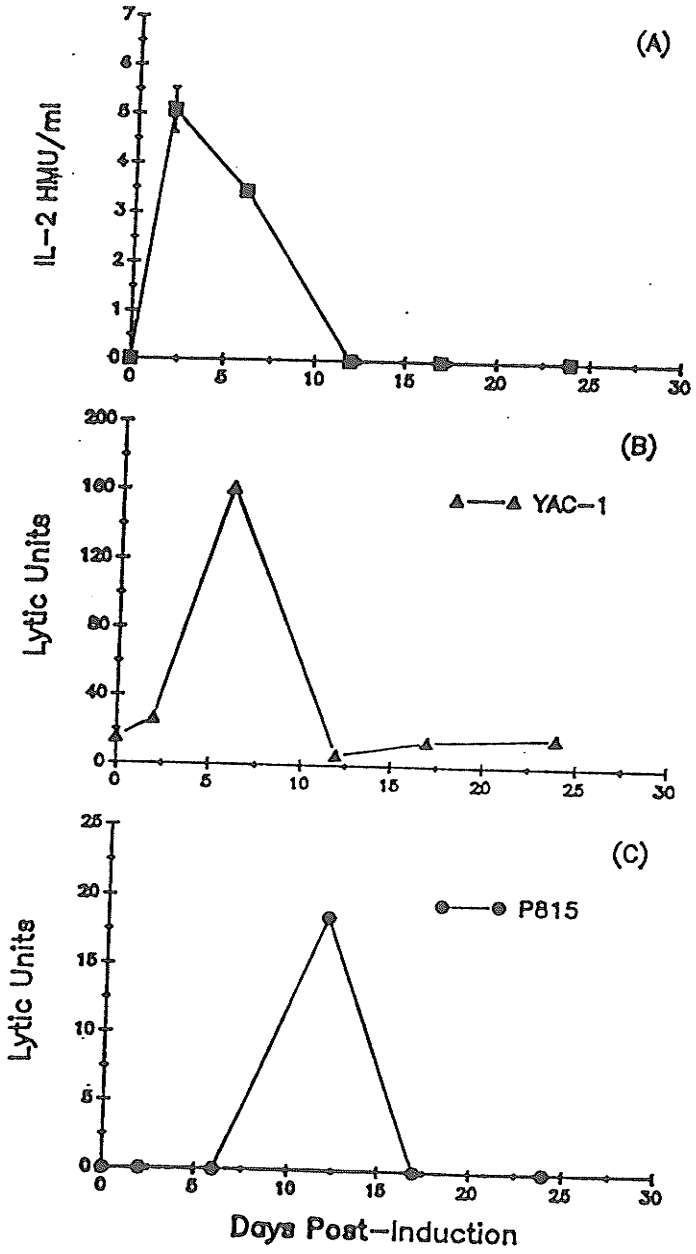

(A)
IL-2 HMU/ml
(B)
YAC-1
Lytic Units
(C)
P815
Lytic Units
Days Post-Induction

Figure 3.3 Representative curves comparing the kinetics of *in vitro* IL-2 production (A) by spleen cells isolated from a minimum of 3 BAF_1 mice with chronic GVH reactions with the kinetics of cytotoxicity to YAC-1 (B) and P815 (C) target cells. Error bars indicate the S.E. of the mean HMU/ml in triplicate cultures. In those instances where error bars are not visible, the S.E. is smaller than the size of the symbol.

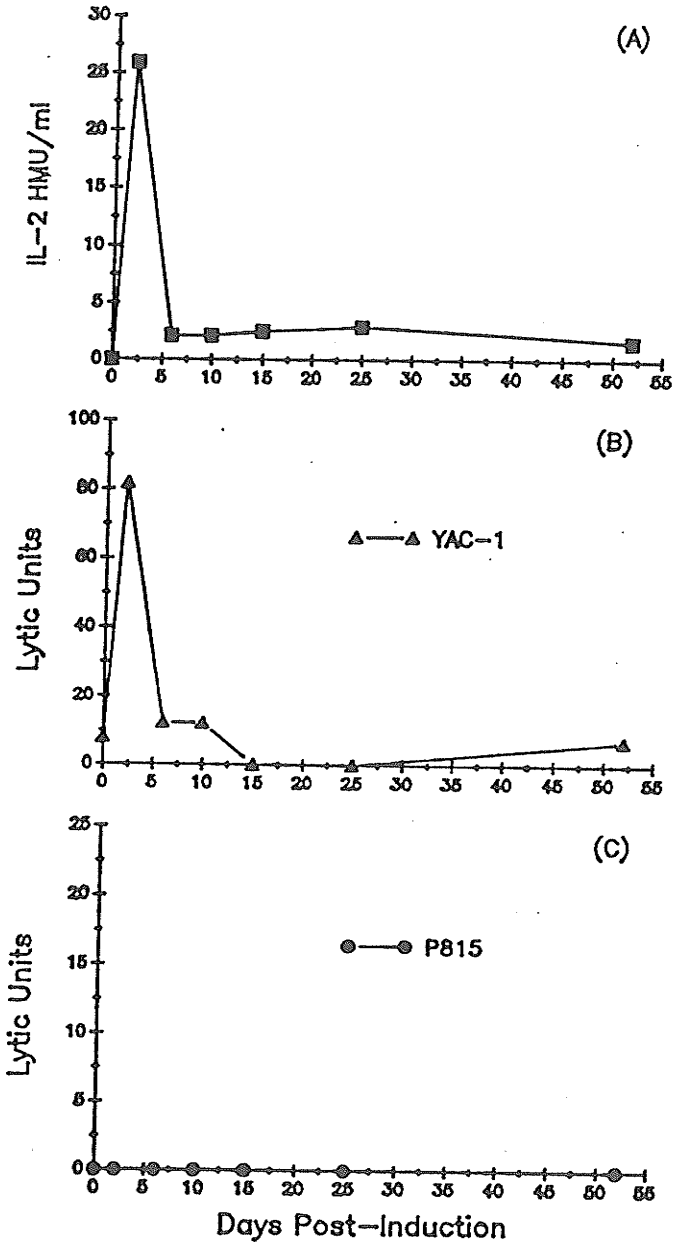

(A)
IL-2 HMU/ml
(B)
Lytic Units
YAC-1
(C)
Lytic Units
P815
Days Post-Induction

Table 3.2 A comparison of spleen cytotoxic cell activity against YAC-1 and P815 between mice with either acute or chronic GVH reaction

	Lytic activity ($LU_{10}/10^7$ cells)	
$P \rightarrow F_1$	YAC-1[a]	P815
$A/J \rightarrow B6AF_1$	181.94 +/- 22.29[b]	18.74 +/- 1.78
$A/J \rightarrow BAF_1$	106.00 +/- 9.47	0

[a]Figures presented are the mean maximal LU +/- S.E. observed against YAC-1 and P815 during acute (3 experiments) and chronic (2 experiments) GVH reactions.

[b]Statistical significance; $p<0.05$ (d.f.=3). The endogenous level of cytotoxic activity against YAC-1 in $B6AF_1$ and BAF_1 mice is 29.06 +/- 9.36 (n=3) and 15.6 +/- 5.3 (n=3) LU, respectively. No lytic activity was measured against P815 in normal F_1-hybrid mice.

not shown).

The two GVH models were also shown to differ in the maximal levels of cytotoxic activity produced against the two tumor targets. Activity generated against YAC-1 and P815 in the spleen of $B6AF_1$ mice with GVH reactions was shown to be consistently higher than that observed in BAF_1 mice with GVH disease. A comparison of the mean maximal lytic units (LU) generated in the spleen during GVH reactions in both models is shown in Table 3.2 These results show the maximal lytic activity observed in the spleen against YAC-1 and P815 to be significantly higher in the acute GVH reactions as determined by Student's t test analysis.

3.4.3 IFN production in mice with acute and chronic GVH reactions

IFN secretion by spleen cell cultures was determined by measuring the ability of spleen cell culture supernatants to protect L929 fibroblasts from viral-mediated lysis in a CPE reduction assay. Data shown in Tables 3.3 and 3.4 indicate that IFN activity could be detected in spleen cell cultures established from mice with either acute or chronic GVH reactions, respectively. In acute reactions, IFN activity was first evident on day 2 and was highest on days 6 - 12. In contrast, IFN levels in chronic GVH mice generally peaked very early in the reaction (days 1-2). There were significant differences in the type of IFN produced during the two

Table 3.3 Kinetics and characterization of interferon activity in mice with acute GVH reactions

	Day Post-induction						
Treatment	N	1	2	6	12	16	24
NT[a]	<19	75[b]	100	125	125	75	50
Ab	<19	75	75	ND[c]	75	75	50
H.I.[d]	<19	<19	38	75	50	<19	<19

[a]No treatment. [b]Values indicate mean units of IFN/ml from a minimum of 3 mice. [c]Not determined. [d]Heat-inactivated. The minimum amount of IFN detectable was 19 U/ml.

Table 3.4 Kinetics and characterization of interferon activity in mice with chronic GVH reactions

	Day Post-induction					
Treatment	N	1	2	5	10	15	25
NT[a]	<19	300[b]	125	75	75	50	50
Ab	<19	<19	<19	<19	<19	ND[c]	ND
H.I.[d]	<19	300	125	75	75	ND	ND

[a]No treatment. [b]Values indicate mean units of IFN/ml from a minimum of 3 mice. [c]Not determined. [d]Heat-inactivated. The minimum amount of IFN detectable was 19 U/ml.

reactions. IFN activity in acute GVH mice was only partially sensitive to neutralization by R4-6A2, whereas the same antibody completely abrogated IFN activity in supernatants from mice with chronic reactions. Conversely, heat treatment had no effect on IFN activity in supernatants from chronic GVH reactions, whereas it eliminated most, and on some days, all of the IFN activity from mice with acute reactions. These results indicate that only IFNγ is produced in the chronic model, whereas IFNα/ß predominates in the acute reaction. Culture supernatants from normal BAF_1 controls and BAF_1 mice given syngeneic grafts (data not shown) did not show IFN activity.

3.5 Discussion

The results of this study verify previous findings that acute GVH reactions in $B6AF_1$ mice result in the activation of two phenotypically distinct NK cell populations: a Thy-$1^{+/-}$ NK cell with a lytic spectrum confined to YAC-1 targets, and a Thy-1^{+} NK-like cell that has the ability to lyse target cells that are normally insensitive to lysis by NK cells.

These experiments have shown that BAF_1-hybrid mice with chronic GVH reactions demonstrate a transient elevation of conventional NK activity early in the reaction, but they do not generate an anti-P815 response. Why NK-like activity should be absent in this model may lie in the results of the cytokine measurements. It was found that there is a close

temporal relationship between elevated levels of IL-2 secretion in spleen cell cultures and the subsequent emergence of splenic NK activity in both acute and chronic models. This is not surprising since IL-2 is well known to augment conventional NK activity both *in vitro* (157) and *in vivo* (156). On the other hand, no such relationship appears to exist for NK-like activity. In the acute model, anti-P815 activity appeared well after IL-2 is at its highest level and in the chronic model no splenic NK-like activity is generated despite very high levels of IL-2 production. IFN measurements in spleen cell culture supernatants from GVH mice have shown that although increased IFN activity can be detected in both acute and chronic models, a preponderance of IFNα/β and some IFNγ is secreted in the acute model whereas in the chronic model, only IFNγ is produced. Although these results are descriptive and essentially correlative, they nevertheless suggest that even though IL-2 may play a role augmenting conventional NK activity, it alone is insufficient for the activation of NK-like activity and that another cytokine is probably involved. Results from these experiments strongly suggest that IFNα/β may be the putative factor.

There is ample evidence in the literature implicating IFN as a potent activator of NK-cell activity (section 1.5.1.2). It is known that if recombinant IFNα, but not IFNγ, is added either alone, or in combination with IL-2 to murine lymphocyte cultures, NK activity is increased (263,333). Thus, it

possible that, in the acute model, IFNα/ß may act synergistically with IL-2 to generate comparatively higher levels of NK activity than occur in the chronic reaction. Conversely, the absence of IFNα/ß secretion in the chronic model might account for the significantly lower levels of NK activity seen in this reaction, despite relatively greater IL-2 production. The suggestion that IFNα/ß is involved in activating NK-like cells is supported by other studies showing that a Thy-1$^+$ effector cell that has the morphologic and phenotypic characteristics of NK cells and the ability to lyse P815 targets, can be recovered from spleen of mice injected with the IFN-inducer poly I:C (240,334).

The fact that increased IFN activity in GVH reactions could be demonstrated is by no means unprecedented. Increased IFNα/ß has been found in spleen cell cultures from lethally-irradiated DBA/2 mice with allogeneic acute GVH disease (185). More recently, both IFNα/ß and IFNγ have been detected using immunohistochemical techniques in the spleen of BALB/c mice with GVH disease (188). The same group has also reported that IFNα/ß is the predominant species produced in an A/J → B6AF$_1$ model of GVH disease, the same model employed in this study (186).

The role played by NK and NK-like cells play in the pathogenesis of GVH disease remains unresolved. Clinical studies have so far been equivocal since some have shown that there is a correlation between NK cell activity and the

development of GVH disease (138,141), while others have disclosed no such association (139). Results from animal studies have been more compelling, but still not definitive. It has been shown that GVH disease can be prevented by treating recipients with anti-$ASGM_1$ antiserum to abrogate NK-cell activity *in vivo* (123). Experiments using beige mice have revealed that characteristic pathological changes do not develop if donor mice are homozygous beige, i.e. NK cell deficient (117). Other studies have suggested that because histopathologic changes of acute GVH disease first appear in the liver and pancreas when NK activity is maximal in the spleen, NK cells may be responsible for the tissue damage produced by the reaction in non-lymphoid organs (113).

Using electron microcopy and immunohistochemistry to characterize mononuclear cells infiltrating the skin, liver and colon of mice with GVH reactions, Ferrara and co-workers (329) have identified donor-derived LGL in close proximity to dead or dying epithelial cells. These cells are Thy-1^+, $ASGM_1^+$, Mac-1^+, Ly-1^-, Lyt-2^- and Ia^+ and it has been suggested that they may be γ/δ T cells. They have postulated that these LGL mediate tissue injury by recognizing and lysing host epithelial cells expressing allogeneic Class I MHC antigens. The similarity of this cell to the donor-derived NK-like cell whose characteristics has been described in this and other studies (331) suggests that they are one in the same. Results from this study suggest that simply the presence of these

cells in a bone marrow allograft may be insufficient to produce acute GVH disease and that these cells must be stimulated by an appropriate cytokine (IL-2 and IFNα/β) in order to become effective. In other words, whether or not a GVH reaction is to pursue an acute, rapidly lethal, course may depend not only on the cohort of effector cells in the graft, but also on the complement of stimulatory cytokines released during the early, lymphoproliferative phase of the reaction.

3.6 Appendix

The experiments presented in this chapter have been published in an article entitled "Natural killer (NK) cell activity in F_1-hybrid mice with acute graft-vs-host reactions: Characterization of a Thy-1^+ NK cell with a broadened spectrum of lytic activity in the spleen and lymph nodes". MacDonald G.C. and Gartner J.G. Scand J Immunol 33:553, 1991. All of the cytokine studies were the sole work of the candidate. However, technical assistance with some of the cyotoxicity assays was given by Mrs. Veronica Sanders.

CHAPTER 4

THE HOST/DONOR ORIGIN OF CELLS MEDIATING NK AND NK-LIKE CYTOTOXIC ACTIVITY IN F_1-HYBRID MICE WITH ACUTE GRAFT-VERSUS-HOST REACTIONS

4.1 Abstract

NK cells and NK-like cells represent two functionally and phenotypically distinct non-MHC-restricted cytotoxic lymphocyte populations that are activated in F_1-hybrid mice with acute GVH reactions. In these experiments spleen cells isolated from $B6AF_1$ mice with acute GVH reactions at different times post-induction were treated with 28-13-3s culture supernatant (anti-H-$2K^b$) and complement or complement alone. Results showed that augmented NK activity was susceptible to depletion with the host-strain specific antibody. In contrast, most of the NK-like cell activity was resistant to depletion. These results indicate that the augmented NK cell activity observed in F_1-hybrid recipients with acute GVH reactions is host-derived, while NK-like activity is derived primarily from the donor. The broader lytic capacity of NK-like cells versus NK cells and the donor origin of NK-like cells suggests a role for NK-like cells in the pathogenesis of acute GVH disease.

4.2 Introduction

The augmentation of NK cell activity against a variety of tumor cell targets, as well as, virally-infected cells has been observed in both experimental animals and human bone marrow transplant recipients with GVH disease (112,138,331). The association of increased non-MHC-restricted killing with the development of GVH disease has lead to the proposition that NK cells have some role in the induction of the immune response and/or as an effector cell mechanism in GVH reactions.

It has recently been demonstrated that another type of non-MHC-restricted cytotoxic cell is activated during GVH reactions produced in a murine parent $\rightarrow$ F_1-hybrid ($P \rightarrow F_1$) model (332). This nylon wool non-adherent cell population differs from typical NK cells in its ability to kill the NK-resistant mastocytoma cell line P815. The activation of this cell appears to depend not solely on IL-2, but also the presence of IFNα/β during the allogeneic phase of the acute GVH reaction (Chapter 3,335). For this reason this cell population has been referred to as NK-like rather than LAK which presupposes IL-2-dependent activation. The surface markers of these cells resemble those of conventional NK cells in they are ASGM1$^+$, CD4$^-$ and CD8$^-$ (332,335, Chapter 3). On the other hand, anti-Thy-1 and complement completely eliminates NK-like cytotoxicity activated during GVH

reactions, whereas classic NK cell activity is only partially sensitive to depletion with this antibody.

Little is known about the host/donor origin of the augmented NK cell activity seen during GVH reactions. Studies that have addressed this issue have provided conflicting conclusions. For example, a host cell origin was suggested because depletion of the donor inoculum with anti-$ASGM_1$ and complement failed to prevent GVH disease (123,127,336), while treatment of the recipient abrogated the reaction (123,126,336). In another, depletion of the donor with anti-$ASGM_1$ 4 - 6 hr after pre-sensitization with host-strain lymphoid cells appeared to prevent GVH disease. The authors argued that these results meant that the NK effector-cell involved in the pathogenic mechanism of GVH disease was donor-derived (127). In another report, elevated NK activity in BDF_1-hybrid neonatal mice with acute GVH disease produced with maternal spleen cells was shown to be exclusively of donor origin (337). It is possible that the absence of a host-derived contribution to augmented NK cell activity in this model may have been due to the absence of functional NK cells in very young mice. This model probably does not reflect what is occurring in a system in which immunologically mature recipients are employed, a situation that more closely resembles the setting of human bone marrow transplantation.

The purpose of this study was to examine the extent to

which host and/or donor cells contribute to the expression of NK and NK-like cell activity in F_1-hybrid mice with acute GVH reactions.

4.3 Experimental Design

Acute GVH reactions were produced in an adult parental (C57BL/6 x A/J)F_1-hybrid (H-$2^{a,b}$) mice by intravenously injecting 50 x 10^6 spleen and lymph node cells from age- and sex-matched A/J (H-$2^{a(k/d)}$) donors. Groups of 3 or more mice were sacrificed on different days after induction, their spleens removed and pooled, and a single cell suspension prepared. Spleen cell suspensions were treated with 28-13-3s culture supernatant, which contains monoclonal anti-H-2^b, and complement, and the splenic NK activity measured using a standard 4 hr ^{51}chromium-release assay. YAC-1 and P815 target cells were radiolabelled and cytotoxicity was measured at 4 E:T ratios (100, 50, 25, and 12.5:1). Dose response curves were generated from percent lysis data over the 4 E:T ratios and LU per 10^7 effector cells calculated using exponential fit. Because antibody secreted by the hybridoma 28-13-3s is specific for cells of the H-2^b haplotype, cells of host origin would be susceptible to complement-mediated lysis while cells of donor origin would be resistant. Thus, a decrease in LU activity against one of the tumor targets would indicate host-derived cytolytic effector cell activity while resistance to depletion would

indicate that the cytolytic effector cell originated from the donor.

Previous studies in the candidate's laboratory have shown that neither NK nor NK-like cells express the T cell-associated cell surface markers CD4 or CD8 (332,335). To confirm this phenotype spleen cells from GVH mice were incubated with either anti-CD4 or anti-CD8 and complement and assayed for cytolytic activity against YAC-1 and P815.

4.4 Results

Spleen cells were isolated from A/J and B6AF$_1$ mice and treated with culture supernatant from 28-13-3s and complement to confirm that antibody secreted was specific only for cells of the H-2^b haplotype (Table 4.1). This control experiment revealed that this supernatant could deplete augmented NK cell activity in spleen cell suspensions derived from (C57BL/6 x A/J)F$_1$-hybrids injected with poly I:C, but had no effect on NK cell activity derived from the spleen of similarly treated A/J mice. Therefore the supernatant contained antibody specific for the H-2^b haplotype and that this reagent was not non-specifically cytotoxic.

In a separate experiment, the CD8$^-$, CD4$^-$ phenotype of NK and NK-like cells was verified by negative selection with complement and the cytotoxic antibodies anti-Lyt-2.2 and anti-L3T4, respectively, using previously described

Table 4.1 Haplotype specificity of clone 28-13-3s

	Treatment	
Strain	Complement (C)[1]	anti-H-2K^b + C
A/J	19.06 +/- 0.42	20.14 +/- 0.65
B6AF$_1$	21.25 +/- 0.33	4.31 +/- 0.35

[1]Values indicate the mean percent lysis +/- S.E. of YAC-1 tumor targets at a 50:1 E:T ratio. Treatment with anti-H2-K^b + C did not significantly deplete A/J strain spleen cell cytotoxic activity (Student's t-test, t=1.395554 at 4 d.f.) but did significantly deplete B6AF$_1$ spleen cell activity ($p<0.0001$). A minimum of 3 mice were used for each sample.

procedures (332,335). The results (Table 4.2) indicated that cytotoxic activity to both tumor target cells could not be depleted by either anti-Lyt-2.2 or anti-L3T4. The anti-YAC-1 cytotoxicity was partly sensitive to anti-Thy-1.2 and completely eliminated by anti-ASGM1. The anti-P815 response (NK-like activity) was completely abolished by both anti-Thy-1.2 and anti-ASGM1.

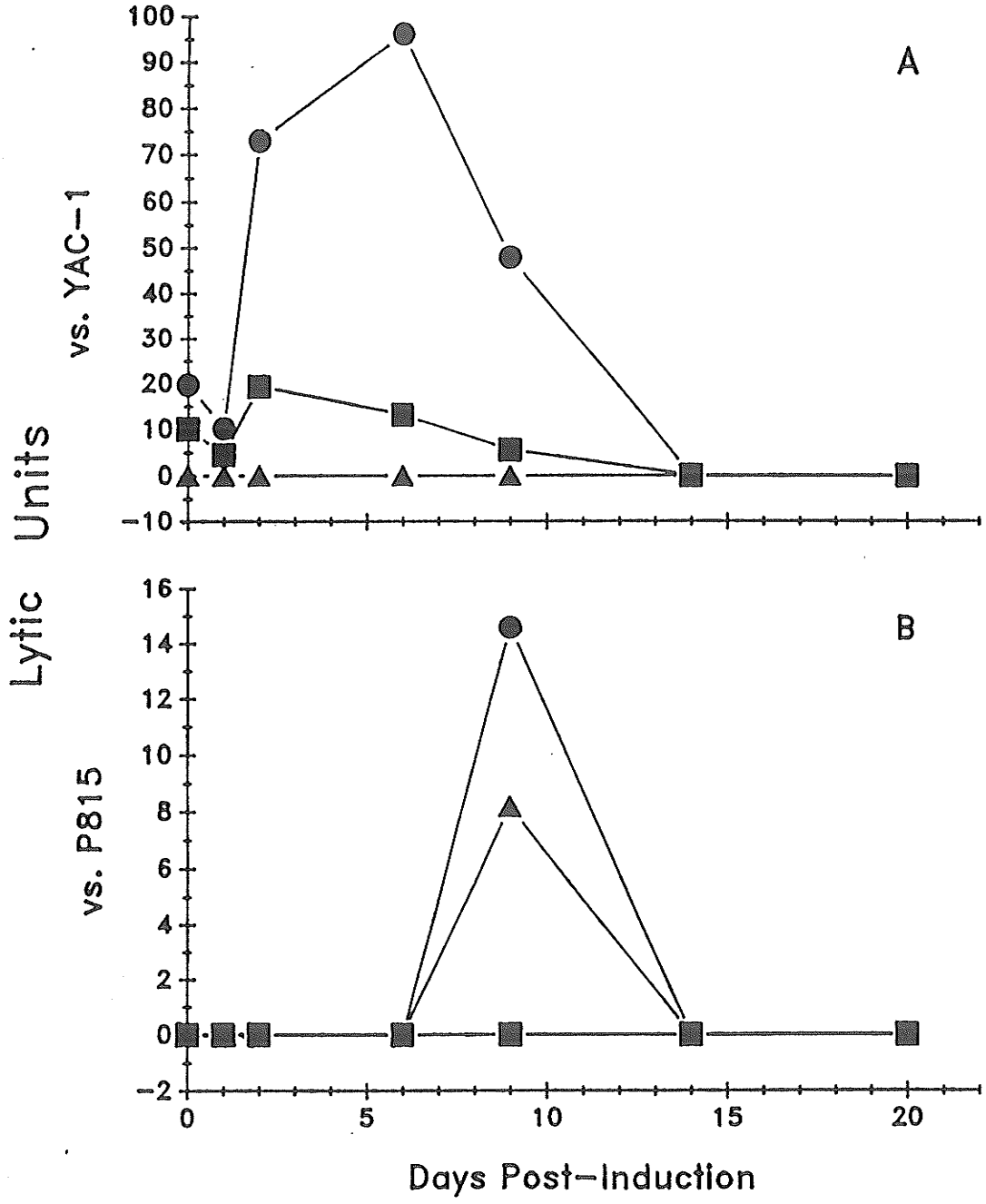
In Figure 4.1 representative data from one of three separate experiments is shown. The curves show changes with time in the level of cytotoxic activity directed at YAC-1 and P815 target cells in mice with acute GVH reactions, and the differential effects of the cytotoxic antibodies anti-Thy-1.2 and anti-K^b on this activity. Lytic activity against YAC-1 was increased above control values on days 2, 6 and 9 of the reaction. Much of this activity was depletable with anti-Thy-1.2 and complement (Figure 4.1A). The data also demonstrate that anti-K^b completely eliminated anti-YAC-1 NK cell activity from effector cell suspensions derived from both control mice (day 0) and mice with GVH reactions. Lytic activity directed against P815 targets was detected only on day 9 of the reaction (14.48 $LU_{10}/10^7$ cells) and could only be partly eliminated by anti-K^b and complement, with most of the activity (57 %) resistant to the treatment (Figure 1B). The donor cell contribution to the anti-P815 response in the two other experiments was

Table 4.2. Cell surface phenotype of the cells mediating NK and NK-like activity

		Target ($LU_{10}/10^7$ cells)	
Ab	Complement	YAC-1	P815
-	-	35.00	24.31
-	+	38.21	33.32
anti-Lyt-2.2	+	39.02	32.72
anti-L3T4	+	32.60	36.01
anti-Thy-1.2	+	11.13	0
anti-$ASGM_1$	+	0	0

Values shown are the LU activity measured on day 5 against YAC-1 and on day 11 against P815 from a single experiment. Normal $B6AF_1$ mice demonstrated 5.36 and 0 LU activity against YAC-1 and P815, respectively.

Figure 4.1 Representative curve showing the effects of different cytotoxic antibodies and complement on NK and NK-like lytic activity in mice with acute GVH reactions. Effector cells were treated with either complement alone (•–•), anti-Thy-1.2 + complement (■–■), or anti-K^b + complement (▲–▲). Treatment with anti-$ASGM_1$ and complement completely removed lytic activity against both tumour targets on all days activity was measured (data not shown). Each point indicates the mean spleen index +/- S.E. calculated from a minimum of 3 mice. Error bars are not visible because the S.E. is smaller than the size of the symbol.


A
B
Lytic Units
vs. YAC-1
vs. P815
100
90
80
70
60
50
40
30
20
10
0
-10
16
14
12
10
8
6
4
2
0
-2
0
5
10
15
20
Days Post-Induction

shown to be 56 % and 95 %, respectively (data not shown). Anti-Thy-1.2 and complement completely eliminated the anti-P815 response.

4.5 Discussion

These findings show that in GVH reactions produced in a $P \rightarrow F_1$ model, augmented conventional anti-YAC-1 NK activity originates from the host. We have obtained identical results in another $P \rightarrow F_1$ combination, C57BL/6 ($H\text{-}2^b$) $\rightarrow$ (C57BL/6 x DBA/2)F_1 ($H\text{-}2^{b/d}$) (Chapter 5). Results similar to these have been reported by others using $P \rightarrow F_1$ strain combination CBA $\rightarrow$ (CBA x BALB/c)F_1 (114). These authors also suggested that donor cells were partly responsible for YAC-1 killing, since alloantiserum specific for the host was incapable of depleting all NK activity. However, the low levels of lytic activity remaining after antibody depletion and the absence of a cell dosage effect for the two E:T ratios shown in their paper would suggest that this was not a significant level of cytotoxicity.

These findings also show that NK-like activity was mediated largely by donor-derived cells. This observation is consistent with findings of Ferrara *et al* who identified donor-derived, Thy-1^+, ASGM1^+, Mac-1^+, Lyt-1^-, Lyt-2^- LGL at the site of tissue injury in mice with acute GVH disease (329).

The activation of donor-derived cytotoxic cells in mice

with GVH reactions is not particularly surprising; however, the demonstration of host-derived effector cells is somewhat puzzling. In $P \rightarrow F_1$ model of GVH disease, the recipient shares MHC antigens with the donor and is deemed to be immunologically incapable of responding to the graft. Despite this restriction, activation of a variety of cell types originating from the host has been demonstrated. For example, activated host-derived peritoneal macrophages capable of lysing tumor targets of host genotype have been isolated from F_1-hybrid mice with acute GVH reactions (338). Similarly, B cells stimulated to proliferate in the popliteal lymph node of F_1-hybrid mice after the injection of parental-strain lymphocytes into the footpad have been shown to be mainly host-derived (37). Non-specific activation of these cells by cytokines released from alloreactive donor cells is believed to be the most likely mechanism by which host cells become activated in the $P \rightarrow F_1$ model (38). It has been observed, for example, that when mitomycin-treated parental lymphoid cells are mixed with F_1-hybrid lymphocytes and cultured *in vitro*, although parental strain cells cannot proliferate, they can still release cytokines into the medium and thereby non-specifically stimulate proliferation and activation of F_1-hybrid cells (339). It has also been shown that augmented NK activity in F_1-hybrid mice with GVH reactions is preceded by an anti-host delayed-type hypersensitivity response that is

associated with the production of IFNγ (114). Previous work from the candidate's laboratory has revealed that the augmentation of NK activity in mice with either acute or chronic GVH reactions occurs concomitantly with elevated levels of IL-2 production (Chapter 3,335). The much higher levels of NK activity seen in mice with acute reactions appears to be related to the production of IFNα/ß during these reactions, and not IFNγ as suggested by others (114). It has been shown also that, although NK-like activity in mice with acute GVH reactions is temporally related to IL-2 production, the presence of this cytokine alone is insufficient, and it appears that IFNα/ß production is required for activation of NK-like cells (Chapter 3,305).

In summary, these experiments have shown that augmented NK cell activity in F_1-hybrid mice with acute GVH reactions is mediated by effector cells originating from the host, whereas NK-like cell activity is derived from both host and donor with most of the activity originating from the latter. It is suggested that the donor derived, Thy-1$^+$, CD8$^-$, CD4$^-$, NK-like cell may be the functional counterpart of an LGL bearing a similar phenotype described by others in the cellular infiltrates associated with GVH disease (329), and may be a cell that is involved in mediating injury to the host.

4.6 Appendix

The material presented in this chapter has been published as a brief communication entitled "The host/donor origin of cells mediating NK and NK-like cytotoxic activity in F_1-hybrid mice with acute graft-versus-host reactions", MacDonald G.C. and Gartner J.G., Transplantation 52:141, 1991. All of the experiments were conducted by the candidate with some technical assistance from Mrs. Veronica Sanders.

CHAPTER 5

PREVENTION OF ACUTE LETHAL GRAFT-VERSUS-HOST DISEASE IN F_1-HYBRID MICE BY PRE-TREATMENT OF THE GRAFT WITH ANTI-NK-1.1 AND COMPLEMENT

5.1 Abstract

The effect of depleting NK1.1$^+$ cells from an allograft of lymph node and spleen cells was measured on the outcome of GVH disease in the parent → F_1-hybrid combination C57BL/6 → (C57BL/6 x DBA/2)F_1. Four treatment groups were established: Group I mice were transplanted with an unmodified graft from normal donors. Group II mice were transplanted with an NK1.1-depleted graft that had been harvested from normal donors. Group III mice received grafts from donors that had been injected with poly I:C (100 μg IP) 18 hr prior to harvesting. These grafts were incubated with complement, but no antibody. Group IV mice were transplanted with depleted grafts harvested from donors that had received poly I:C. Recipients in each group were monitored for splenomegaly, mitogen responsiveness, NK and CTL activity, histopathology, weight loss, and mortality. Results showed that recipients in all four groups developed splenomegaly and unresponsiveness to LPS, PHA and ConA by day 12. Augmented host-derived NK activity and graft-derived anti-host CTL activity was also demonstrated. Group IV showed little or no weight loss, minimal histopathological changes and a marked reduction in mortality. Recipients in all other groups (I-III) developed features characteristic of GVH disease and exhibited a steady decline in body weight beginning by day 12. Mortality generally began on day 18 and reached 75-90% by day 60. It is postulated that anti-NK1.1 depletes cells from the graft intimately connected with the

effector mechanism in acute GVH disease.

5.2 Introduction

GVH disease is well recognized as a major impediment to the therapeutic success of allogeneic bone marrow transplantation. There is a growing body of evidence suggesting that natural killer (NK) cells may be involved in the pathogenesis of GVH disease either by being directly cytotoxic to the host or by participating in the activation of other non-specific cellular effector mechanisms (117,138,32). The most compelling evidence for this idea has come from studies of murine GVH reactions in which the effect of NK cells has been removed either by antibody and complement depletion or by the use of mice that are congenitally NK cell deficient. For example, GVH disease can be prevented by treating recipient mice with anti-$ASGM_1$ antiserum (123). Other experiments employing NK cell deficient beige mice have demonstrated that pathological changes characteristic of GVH disease do not occur when donor mice are homozygous beige (117,118). These studies are nevertheless inconclusive since, in such approaches, cells other than NK cells are also functionally deficient (118,129).

In previous studies of NK cell activity in F_1-hybrid mice with acute GVH disease it was demonstrated that two distinct kinds of non-MHC-restricted cytotoxic cells

appeared in the spleen, lymph nodes and lung: a conventional NK cell with a spectrum of cytotoxicity limited to NK-sensitive tumor targets such as YAC-1, an a second "NK-like" cell with a lytic repertoire that includes target cells resistant to lysis by conventional NK cells (Chapter 3,335). The surface phenotype of this "NK-like" cell is $ASGM_1^+$, Thy-1^+, Lyt-2^-, L3T4$^-$. It is of donor origin (Chapter 4,340), and is activated during acute lethal, but not chronic, GVH reactions (Chapter 3,335). The activation of NK-like cells appears to depend upon the secretion of IFNα/β (Chapter 3,335).

In the experiments reported in this chapter, the effect of removing NK and NK-like cells from a parental donor graft of lymph node and spleen cells is explored in an F_1-hybrid model of GVH disease. The MoAb anti-NK1.1, which recognizes an allotypic determinant on NK cells from H-2^b mice was used (243). Results showed that depletion of NK1.1^+ cells from the graft effectively prevented acute, lethal GVH disease if, and only if, the donors had been injected with the IFN inducer, poly I:C 18 hr before the grafts were harvested. Previous studies have shown that poly I:C can augment endogenous NK and induce "NK-like" activity (240).

5.3 Experimental Design

The objective of these experiments was to determine whether removal of NK1.1^+ cells from a lymph node and spleen

cell allograft harvested from normal or poly I:C-stimulated parental donor mice had any effect on the outcome of the resulting GVH reaction. Four treatment groups were therefore established: Group I mice were transplanted with an unmodified graft from normal donors; Group II mice were transplanted with an NK1.1-depleted graft that had been harvested from normal donors; Group III mice received grafts from donors that had been injected with poly I:C (100 μg IP) 18 hr prior to harvesting (these grafts were incubated with complement, but no antibody); Group IV mice were transplanted with depleted grafts harvested from donors that had received poly I:C. The GVH reactions were studied by sacrificing at least three randomly-selected mice from each of the four treatment groups at several time points during the reaction and measuring splenomegaly, immunosuppression and splenic cytotoxic cell activity. Immunosuppression was monitored by measuring the proliferative response of spleen cells to ConA, PHA, and LPS in a ^{3}H-thymidine incorporation assay performed as previously described (297). Liver tissue, salivary gland and thymus were placed in 10% phosphate-buffered formalin for subsequent histological study. Mice from each treatment group were set aside, weighed at regular intervals, and monitored for mortality from GVH disease. Mice succumbing to the disease were autopsied and tissue samples were collected for histopathological study.

5.4 Results

5.4.1 Depletion of NK and NK-like activity from the graft with anti-NK1.1 and complement.

The effectiveness with which anti-NK1.1 and complement could deplete the donor inoculum of cytotoxic activity against the YAC-1 and P815 tumour targets is shown in Table 5.1. Significantly augmented lytic activity to both YAC-1 and P815 target cells was seen in donor mice that had been injected with poly I:C (148.3 and 20.1 LU respectively) when compared with untreated donors (8.1 and 0 LU). Treatment of the grafts with anti-NK1.1 and complement depleted 88% of NK (anti-YAC-1), and all of the NK-like (anti-P815) cytotoxicity. Complement alone produced no reduction in cytotoxicity.

5.4.2 The effect of anti-NK1.1 and complement on T cell numbers and mitogen responsiveness in the graft.

To exclude the possibility that the depletion process removed other lymphocyte populations from the graft we determined the relative number of T cells in the graft, as well as the mitogenic response to ConA, LPS and PHA. We found that the depletion procedure did not decrease either the percentage of Thy-1$^+$, CD4$^+$ or CD8$^+$ cells in the graft (Table 5.2) or alter the graft's proliferative response to ConA, LPS or PHA (Table 5.3).

Table 5.1 Depletion of NK and NK-like activity from the graft with anti-NK1.1 and complement[a]

Treatment Groups	$LU_{10}/10^7$ cells	
	YAC-1	P815
I	8.1	0
II	0	0
III	148.3	20.1
IV	17.6	0

[a] 5×10^7 cells were removed from each donor inoculum before and after depletion. The nylon-wool non-adherent cell fraction of each sample was recovered and the cytotoxic activity measured in triplicate cultures using a standard ^{51}Cr-release assay.

Table 5.2 The affect of anti-NK1.1 depletion on T-cell surface marker expression in the graft

	Ab (% labelled cells)		
Treatment	Thy-1.2	Lyt2.2	L3T4
NT	32.8	8.2	25.8
complement (C)	32.3	8.3	20.2
α-NK1.1 + C	33.2	8.3	19.7

Results shown are from a single experiment. Cells for grafting were prepared from poly I:C-treated B6 mice. NT, not determined.

Table 5.3 Mitogen responsiveness of B6 donor spleen cells before and after treatment with anti-NK1.1 and complement

		Treatment			
poly I:C	Ab	NT	ConA	LPS	PHA
+	-	1.44	84.79	16.91	135.47
		+/-0.29	+/-7.28	+/-1.00	+/-18.26
+	+	3.45	205.39	65.79	374.45
		+/-0.25	+/-7.48	+/-2.03	+/-9.66

Figures indicate the mean DPM (1×10^{-3}) of a triplicate sample. Treatment of the graft prepared from non-poly I:C-treated donors with anti-NK1.1 and C did not significantly diminish the proliferative response when compared with non-depleted controls (data not shown). A minimum of 3 mice were used in each group. Ab, treatment with anti-NK1.1 antibody and complement (C).

5.4.3 Splenomegaly and immunosuppression in mice with GVH reactions.

Mice in all four treatment groups showed a similar pattern of splenomegaly (Figure 5.1). Spleen weights increased steadily early in the reaction, peaked around day 12 and declined thereafter. Recipients of NK1.1-depleted lymphoid cells from poly I:C-treated donor mice (Group IV) still demonstrated some degree of splenomegaly (spleen index of 1.46 +/- 0.09) on day 56 of the reaction. The proliferative response of spleen cells to ConA, PHA and LPS was profoundly suppressed in all four treatment groups by day 12 of the reaction (Figure 5.2). This immunosuppression was permanent in Groups I-III. Mice in Group IV regained their responsiveness to ConA and LPS by day 56, but the PHA response remained lower than control values by 25 %.

Figure 5.1 The change in spleen weight with time observed in each of the four treatment Groups. Error bars indicate the SE of the mean spleen index calculated from a minimum of 3 mice.

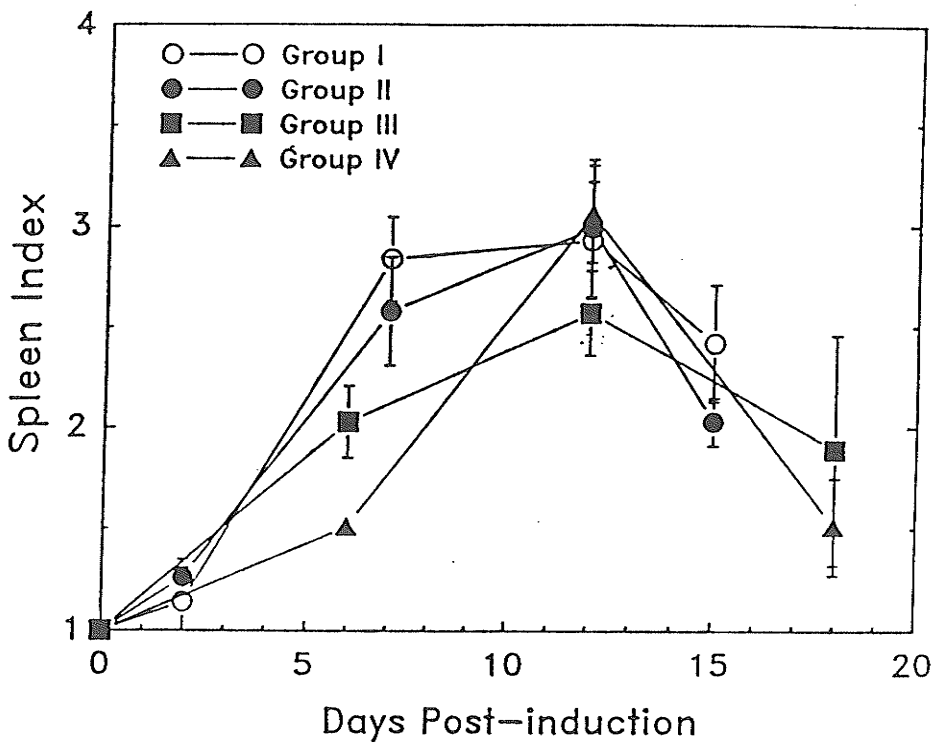
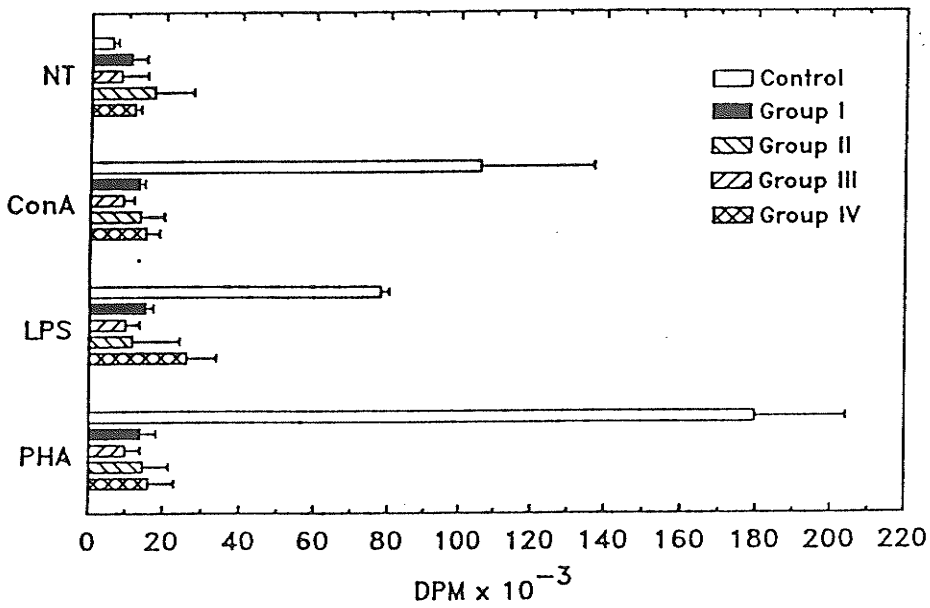

Group I
Group II
Group III
Group IV
Spleen Index
4
3
2
1
0
5
10
15
20
Days Post-induction

Figure 5.2 Mitogen responsiveness of spleen cells isolated from each of the four treatment Groups on day 12 of the reaction. Error bars indicate the SE of the mean DPM calculated from triplicate cultures.


NT
ConA
LPS
PHA
Control
Group I
Group II
Group III
Group IV
0
20
40
60
80
100
120
140
160
180
200
220
DPM x 10^-3

5.4.4 Comparison of weight loss and mortality from GVH disease in the four treatment groups.

BDF_1-hybrid recipients in Groups I-III developed characteristic features of acute GVH disease, such as ruffled fur, hunched posture (hind legs are drawn under the body and the tail rests on the surface), wasting and diarrhea before succumbing to the disease. Weight loss in these three groups began in the second week of the reaction and continued until death. Mortality from GVH disease was generally first observed around day 18 and was generally 75-90 % by day 60. Figure 5.3 shows data pooled from 4 separate experiments which demonstrate differences in mean body weight between the four treatment groups at day 20 and normal, uninjected control mice. Groups I-III exhibited a day 20 mean body weight (20.4 +/- 0.49) that was 17.5 % below that of uninjected control mice whereas Group IV showed a weight loss of only 2.5 % (24.14 +/- 0.43). A 10% decrease in body weight by day 20 has been shown to be a reliable indicator of the severity of acute GVH disease in mice (195). There was a marked reduction in mortality from GVH disease in Group IV. The overall mortality by day 80 in the four experiments was 8.7%. In two experiments all Group IV mice survived beyond day 300. Cumulative mortality curves for the four experiments are shown in Figure 5.5.

Figure 5.3 Graph showing body weight of control mice (n=13), Groups I-III mice combined (n=25), and Group IV (n=23) mice on day 20 after induction. The data have been combined from 4 separate experiments, with each data point indicating the weight of an individual mouse. There was no significant difference between the mean weight (line) of Controls (24.76 +/- 0.43) and Group IV (24.14 +/- 0.43). The difference between the mean weight of Groups I-III (20.43 +/- 0.49) and Controls and between Groups I-III and Group IV were both statistically significant (Student's t-test, $p<0.001$).

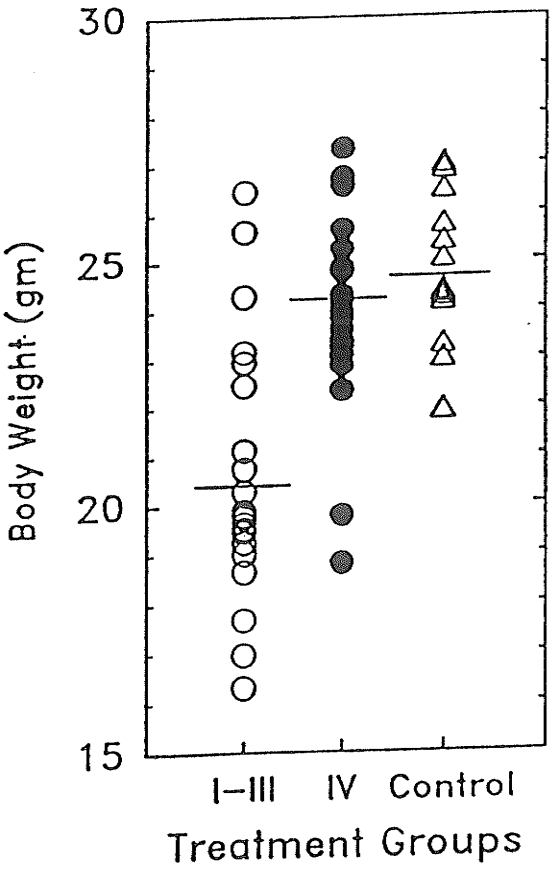
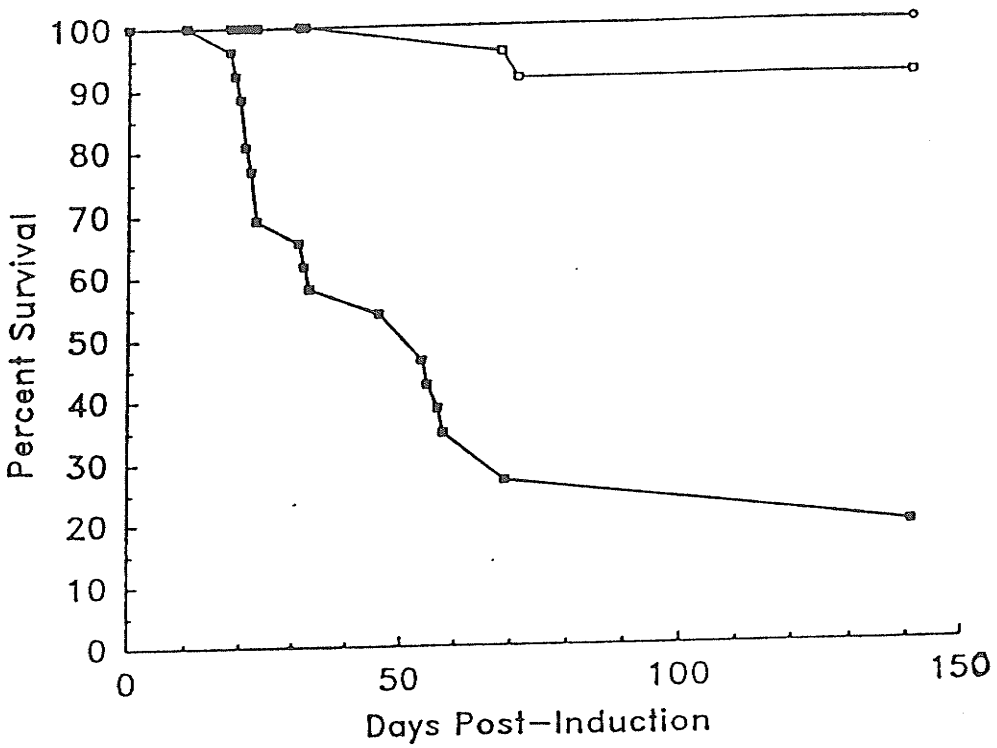

30
25
20
15
Body Weight (gm)
I–III
IV
Control
Treatment Groups

Figure 5.4 Graph showing mortality/survival curves of normal F_1-hybrid controls (O, starting n=13), mice in Groups I-III combined (■, starting n=27), and mice in Group IV (□, starting n=23). The curves have been constructed from pooled data from 4 separate experiments. The difference in mortality/survival between Group IV and Groups I-III was statistically significant (chi-square analysis, $p<0.005$)


100
90
80
70
60
50
40
30
20
10
0
Percent Survival
0
50
100
150
Days Post-Induction

5.4.5 Generation of splenic cytotoxic effector cells in BDF_1 graft recipients.

Increased NK cell activity directed against YAC-1 tumor target cells was seen early (days 2-6) in the reaction in all four treatment groups. Recipients in groups III and IV generally developed their highest level of YAC-1 lysis (40 - 60 LU) on day 6, in contrast to Groups I and II in which this response was maximal (80 - 120 LU) on day 2 (data not shown). In Groups I-III, this activity declined over the course of the reaction and was usually non-detectable by day 15. Augmented NK cell activity in Group IV recipients also declined gradually, but never completely disappeared. Indeed, anti-YAC-1 activity approximating that seen in controls could be found in Group IV mice (8 - 10 LU) as late as day 56 after induction (data not shown). Cell surface phenotyping by negative selection (Table 5.4) using a panel of cytotoxic antibodies and complement demonstrated that anti-YAC-1 cytotoxicity was depletable with anti-NK1.1, anti-$ASGM_1$ and the host-specific MoAb, anti-H-$2D^d$. This activity could be partially removed by anti-Thy-1.2, but was unaffected by anti-Lyt2.2. Lytic activity against P815 target cells was never detectable during the first week in any of the treatment groups, but it did appear by day 12 and then decreased. By day 18 it was 50% or less of its maximal value in all four groups. Immunophenotyping studies (Table 5.5) revealed that anti-P815 activity was resistant to

Table 5.4 Phenotypic characterization of effector cells mediating lysis of YAC-1 tumour targets

B6 Donor Cells	C	Ab + C				
		NK1.1	Thy-1.2	Lyt-2.2	$ASGM_1$	$H\text{-}2D^d$
-	66.9	0	42.1	51.8	ND	ND
α-NK1.1	100.1	0	56.7	98.5	ND	ND
poly I:C	29.1	6.7	21.3	29.3	0	0
poly I:C + α-NK1.1	20.2	5.1	14.3	21.8	0	0

Values presented are $LU_{10}/10^7$ spleen cells calculated from a single experiment. Effector cells were prepared from BDF_1 recipients on day 2 and day 6 for non-poly I:C-treated and poly I:C-treated donors, respectively. Complement control mice had an LU_{10} of 14.4. ND, not determined; C, complement; Ab, antibody.

Table 5.5 Phenotypic characterization of effector cells mediating lysis of P815 tumour targets

B6 Donor Cells	C	Ab + C				
		NK1.1	Thy1.2	Lyt2.2	$ASGM_1$	$H\text{-}2D^d$
–	66.9	78.5	0	5.2	ND	ND
α-NK1.1	51.9	80.2	0	1.3	ND	ND
poly I:C	118.6	129.7	0	0	0	82.9
poly I:C + α-NK1.1	64.1	57.4	0	0	0	50.6

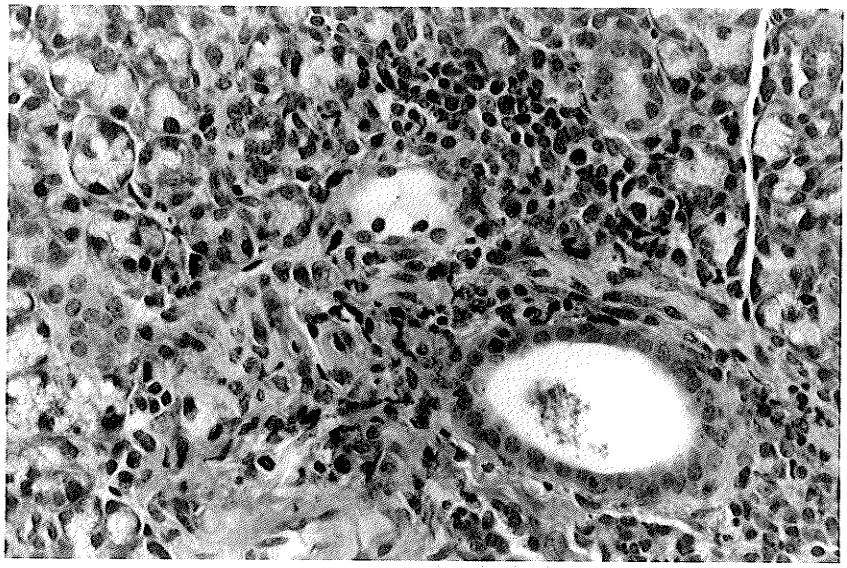
Values presented are $LU_{10}/10^7$ spleen cells calculated from a single experiment. Effector cells were prepared from BDF_1 recipients 12 days after induction. Control mice had an LU_{10} of 0. ND, not determined; C, complement; Ab, antibody.

depletion with anti-NK1.1 and completely depletable by anti-Thy-1.2, Lyt2.2 and anti-$ASGM_1$. Anti-H-$2D^d$ removed only a small amount of cytotoxic activity to P815 target cells. These results indicate that the cell mediating the anti-P815 response was a graft-derived cytotoxic ($CD8^+$) T cell. This conclusion is consistent with the fact that P815 target cells are H-2^d and that our GVH reaction was directed at the H-2^d haplotype.

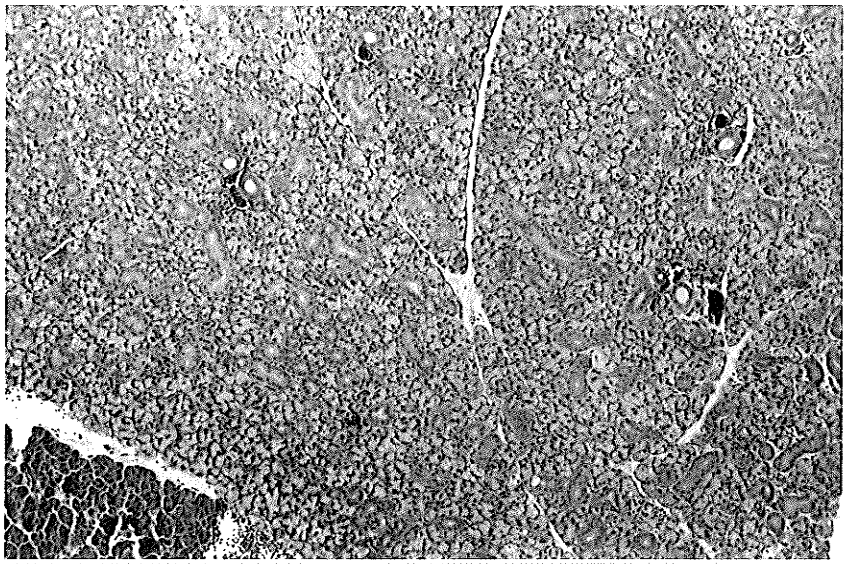
5.4.6 Histological findings.

Mice in Groups I-III developed periductal lymphocytic infiltrates in salivary gland by day 12 of the reaction (Figure 5.5A). These infiltrates increased in intensity over the course of the reaction. In contrast, lymphocytic infiltrates were not observed in salivary gland tissue taken from Group IV mice on day 12 (Figure 5.5B). In Groups I-III the thymus showed evidence of stress involution (decreased thymic mass, lymphocyte depletion) and GVH disease-associated injury (loss of corticomedullary demarcation) as early as day 12 in Groups I-III (Figure 5.6A). This also progressed so that by day 25 of the reaction the thymus was profoundly stress involuted (data not shown). Again, this contrasted with what was seen in Group IV. The thymus of Group IV was indistinguishable from normal thymic tissue (Figure 5.6B). Sections of liver taken from mice in Groups I-III on day 12 showed periductal lymphocytic infiltrates in

Figure 5.5 A comparison of salivary gland tissue taken on day 12 of the reaction from BDF_1-hybrid mice that had received untreated (A, 250x) and NK1.1-depleted (B, 100x) grafts from poly I:C-stimulated B6 donors.

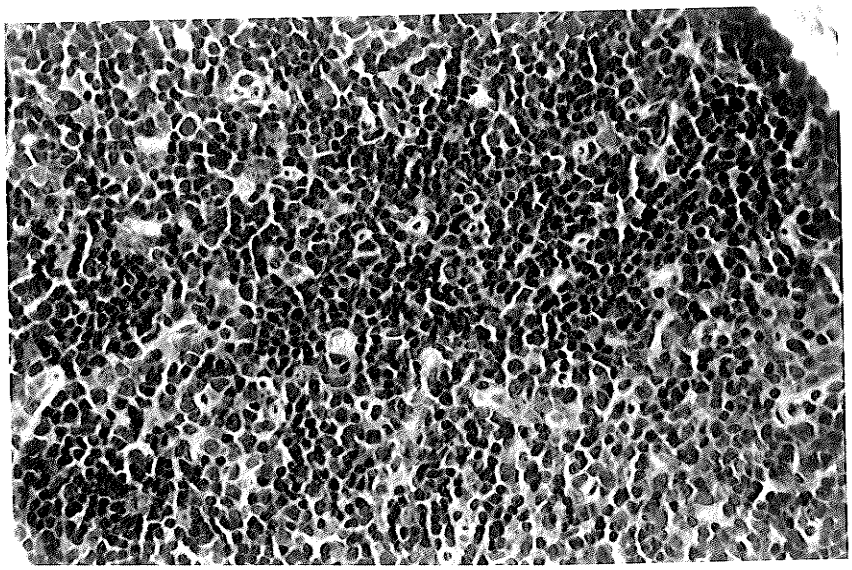


A

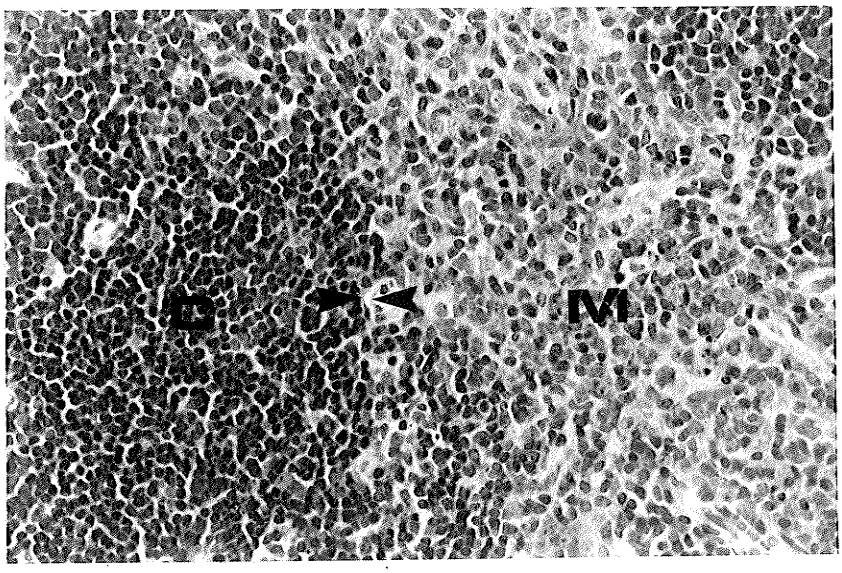


B

Figure 5.6 A comparison of thymic tissue taken on day 12 of the reaction from BDF_1-hybrid mice that had received untreated (A, 250x) and NK1.1-depleted (B, 250x) grafts from poly I:C-stimulated B6 donors. C indicates cortex and M indicates medulla. Arrows indicate cortico-medullary demarcation.



A



B

the portal areas of the liver and Kupffer cell hyperplasia (data not shown). By day 18-25, the liver showed lymphocytic infiltrates around central veins and in the sinusoids and liver cell cords; liver cell necrosis was also observed (data not shown). While lymphocytic infiltrates were present in the livers of mice from Groups I - III, infiltrates were not seen in Group IV mice on day 12. The liver from these animals (Group IV) showed isolated aggregates of lymphocytes in the liver cell cords adjacent to central veins (data not shown). There was no evidence of hepatocellular necrosis and portal ares were normal. Otherwise, it was difficult to distinguish between sections from Group IV and control mice.

5.5 Discussion

In these experiments it was shown that when F_1-hybrid mice are engrafted with lymph node and spleen cells harvested from parental donors injected 18 hr earlier with poly I:C, the severe wasting and high mortality associated with acute GVH disease is virtually eliminated if the graft is first depleted of NK1.1$^+$ cells. Although wasting and mortality are prevented by this pretreatment, these mice still develop splenomegaly and immunosuppression, two hallmarks of GVH reactivity (9). They also develop some of the histopathologic manifestations of GVH disease, but these lymphocytic infiltrates are not as extensive as those seen

in mice with the acute reactions, nor are they associated with injury to host tissue elements. These findings are consistent with those of Ghayur and co-workers (117) who showed that in GVH reactions induced with lymph node and spleen cells harvested from "beige" mice, acute, lethal GVH disease was prevented when donor mice were homozygous "beige", but immunosuppression and splenomegaly still developed. However, since T cells as well as NK cells are functionally defective in beige mice (118,129), the precise cellular defect responsible for preventing acute lethal GVH disease could not be stated conclusively.

These experiments also revealed that donor-derived CTL ($CD8^+$) activity directed at host MHC can still be detected in Group IV recipients, thus dissociating classical CTL activity from the wasting syndrome and high mortality that characterizes acute GVH disease. This does not imply that CTL have no part in the mechanism of GVH disease, but only that these cells are not exclusively responsible for the lethal effect of an acute GVH reaction. We also observed that mice in Group IV developed transient elevations in host-derived NK cell activity, suggesting that activation of these cells in the host is not instrumental in the pathogenesis of acute GVH disease.

These findings clearly suggest that a graft-derived $NK1.1^+$ effector cell has a fundamental role in the pathogenesis of acute GVH disease. Furthermore, in order

for this cell to be removed from the graft, the donor must be treated with the IFN inducer, poly I:C shortly before the graft is harvested. The way in which IFN interacts with this effector cell or its precursor to permit its removal from the graft by anti-NK1.1 and complement remains unknown. It is possible that IFN increases the density of NK1.1 on the surface of cells that constitutively express this marker, which would result in a more thorough depletion by antibody and complement. On the other hand, IFN might induce NK1.1 expression on a population of $NK1.1^-$ precursors, thereby permitting their elimination. Regardless of the mechanism, these results clearly show that simply removing endogenous NK cell activity from the graft does not prevent GVH disease. Similar observations have been published by Blazar and co-workers (135).

Although originally described as an NK-specific marker, NK1.1 has recently been shown to be co-expressed with CD3 and TCRα/ß on a small population of $CD4^-/CD8^-$ lymphocytes in the marrow, spleen and thymus (29,339). It is possible that the effector cell removed from the graft may be this type of T cell. Previous work from the candidates laboratory has suggested that a population of graft-derived "NK- like" cells may be involved in the pathogenesis of acute GVH disease. These cells are $CD4^-$, $CD8^-$, $Thy\text{-}1^+$, $ASGM_1^+$, and $NK1.1^+$, have the capacity to lyse both NK-sensitive and NK-resistant tumour cell targets, and are activated by IFNα/ß

in conjunction with IL-2 (Chapter 3,335). The activation of these cells during GVH reactions is closely associated with the lethality of GVH disease. Work by this candidate, as well as that of others (240), has shown that cells of this nature can be activated by poly I:C injection. Using immunohistochemical techniques Ferrara and co-workers (329) have demonstrated the presence of LGL in proximity to dead and dying epithelial cells in the skin and liver of mice with acute GVH disease. The cell surface markers of these cells were virtually identical to the "NK-like" cells described in our functional studies. It is thus possible that elimination of this effector cell population from the graft can prevent the tissue damage associated with acute GVH disease.

From the results of these experiments it cannot be determined conclusively whether NK1.1 depletion causes graft failure, as has sometimes been observed with T cell depletion in clinical bone marrow transplant recipients (84). These results suggest that there is at least short term survival of the graft since donor-derived anti-host CTL activity can be detected in those mice in which acute GVH disease has been prevented. The presence of splenomegaly in these mice is a further indicator of GVH alloreactivity.

In conclusion, it has been shown that by removing poly I:C-activated NK1.1$^+$ cells from a parental graft of lymph node and spleen cells, the lethal wasting syndrome and

mortality associated with acute GVH disease in a parent → F_1-hybrid model can be prevented. It appears that neither NK cells nor classical CTL are directly responsible for these effects. This depletion procedure does not hinder the development of the lympho-proliferative or immunosuppressive effects of GVH disease. It is postulated that the depletion procedure may be removing, or interfering with, a cellular mechanism that is involved in mediating injury to epithelial surfaces during the reaction and that the cell responsible may be an $NK1.1^+$, NK-like cell.

5.6 Appendix

The material presented in this chapter has been published as an article entitled "Prevention of acute lethal graft-versus-host disease in F_1-hybrid mice by pretreatment of the graft with anti-NK1.1 and complement", MacDonald G.C. and Gartner J.G., Transplantation 54:147, 1992. All of the experiments presented in this chapter were conducted soley by the candidate.

CHAPTER 6

THE IDENTIFICATION OF NK-LIKE TCRγ/δ T CELLS IN THE PATHOGENESIS OF ACUTE MURINE GVH DISEASE

6.1 Abstract

Previous studies have shown that two non-MHC-restricted cytotoxic cell populations are activated during acute GVH disease: a host-derived, Thy-1$^{+/-}$ NK cell capable of lysing NK-sensitive (YAC-1), but not NK-resistant (P815 and BW1100) tumor targets, and a donor-derived Thy-1^{+} NK-like cell that is able to kill both types of tumor targets. Furthermore, depletion of the graft (derived from donors pre-treated with poly I:C 18 hr before harvesting) with anti-NK1.1 and complement prevents the cachexia and mortality associated with GVH disease. The results presented in this chapter showed that splenic NK-like activity does not occur in recipients of NK1.1-depleted grafts, whereas recipients of untreated grafts developed maximal activity on day 8 of the reaction. The absence of NK-like activity is not related to a reduction in alloreactivity since NK1.1-depleted grafts show similar levels of MLR-induced proliferation when compared with non-depleted grafts. Also, the levels of IL-2 and IFN production measured in spleen cell cultures prepared from recipients of either untreated or depleted grafts were not significantly different. Further, negative selection experiments with specific MoAb and complement showed that most splenic NK-like activity is mediated by cells expressing TCRγ/δ and CD3. These results suggest that NK-like TCRγ/δ cells may have an important effector role in the pathogenesis of acute GVH disease.

6.2 Introduction

Two distinct non-MHC-restricted cytotoxic cell populations have been shown to be activated during acute GVH reactions. The first, a conventional NK cell population which is Thy-$1^{+/-}$, $CD4^-$, $CD8^-$ and $ASGM_1^+$, is capable of lysing only the NK-sensitive tumor target YAC-1. The second cell population, which has been referred to as NK-like, is Thy-1^+ and able to lyse a variety of NK-resistant targets (BW1100, P815 and DAUDI), in addition to NK-sensitive targets. It has been shown also that pretreatment of the graft prepared from poly I:C-stimulated C57BL/6 mice with anti-NK1.1 and complement can prevent the cachexia and mortality associated with acute GVH disease in (C57BL/6 x DBA/2)F_1-hybrid recipients. Interestingly though, it does not prevent host-derived augmented NK activity or donor-derived CTL activity. It has been postulated that if NK-like cells are involved in mediating acute GVH disease then anti-NK1.1 and complement may prevent mortality by eliminating these cells from the graft once they had been activated. To test this hypothesis, cytotoxicity against BW1100, an MHC-unrelated NK-like sensitive tumor target, was determined in spleen cell suspensions from recipients of NK1.1-depleted prepared from poly I:C-stimulated donors and compared to the level of cytotoxic activity measured in recipients of untreated grafts from the same donors. It was predicted that the survival

observed in recipients of NK1.1-depleted grafts would correlate with the absence of NK-like cell activity.

It has been proposed by others that the donor cells described in close association with dead or dying epithelial cells of the skin in mice with acute GVH reactions were TCRγ/δ T cells (329). Because of functional and phenotypic similarities observed between NK-like cells and TCRγ/δ T cells, phenotyping studies were performed on NK-like cells activated during acute GVH reactions for the expression of the TCR-associated molecule CD3 and the TCRγ/δ.

6.3 Experimental Design

The objective of these experiments was to determine whether NK-like cell activity was present in F_1-hybrid recipients of anti-NK1.1-depleted grafts and to further characterize the cell surface phenotype of NK-like cells activated during acute GVH disease. $B6AF_1$ recipients were transplanted with grafts, prepared from poly I:C-stimulated B6 donors, that had been treated with either anti-NK1.1 and complement or left untreated. NK-like cell activity was measured against BW1100 using a standard 4 hr ^{51}Cr-release assay at a time in the reaction when NK-like activity was known to be maximal. GVH reactions in both groups were monitored by measuring splenomegaly and weight loss at time intervals over the course of the study.

Several studies have reported that the prevention of

acute murine GVH disease by treating with anti-$ASGM_1$ antiserum and complement. Because others have described the expression of $ASGM_1$ on the surface of both CTL and alloreactive T cell precursors (129-134) it is possible that the increased survival observed in these experiments may have been the result of the removal of effector T cells or alloreactive T cells. To determine whether NK1.1 depletion may also affect the T cell function in the graft the *in vitro* MLR response of anti-NK1.1 depleted grafts was compared with non-depleted grafts. Experiments were also performed to determine whether recipients of NK1.1 depleted grafts had lower levels of cytokine production during the alloreactive phase of the reaction. IL-2 and IFN levels were measured in spleen cell cultures prepared from recipients of non-depleted and depleted grafts and the amount in each group compared with controls.

To determine whether NK-like cells expressed TCRγ/δ and CD3 experiments were performed using the MoAb 145-2c11 (anti-CD3) and GL3 (anti-TCRγ/δ). Because these were not cytotoxic, antiserum specific for the first antibody was used to effect indirect complement-mediated lysis. Cytotoxic activity remaining after the depletion was determined in ^{51}Cr-release assay using the tumor targets YAC-1 ($H\text{-}2^{k/d}$), P815 ($H\text{-}2^{d}$) and BW1100 ($H\text{-}2^{k}$).

6.4 Results

6.4.1 Monitoring of F_1-hybrid graft recipients.

Recipients were monitored at several time intervals post-induction for splenomegaly and weight loss. Recipients in both treatment groups demonstrated a 2 - 3 fold enlargement of the spleen by weight (data not shown) which is consistent with what has already been described for this model (see Chapter 5). Recipients of untreated grafts exhibited a steady decrease in body weight with time while recipients of NK1.1-depleted grafts maintained a body weight equivalent to that of non-injected littermates. For example, on day 20 the mean body weight of the group that had received untreated grafts was 22.8 % less than that of controls (t-test $p<0.001$, 11 d.f.) whereas no significant deviation from control values was observed in mice that had received NK1.1-depleted grafts (Figure 6.1). As discussed previously, a decrease in body weight of 10 % or greater has been shown to strongly correlate with the development of GVH disease (195). These results are identical with our earlier observations that while recipients of NK1.1-depleted grafts develop some of the features of GVH disease, such as splenomegaly, they do not develop the cachexia that is associated with the lethality of acute GVH disease.

6.4.2 Absence of splenic NK-like activity in recipients of NK1.1-depleted grafts.

Maximal activation of splenic NK-like activity in F_1-hybrid graft recipients with acute GVH disease has been previously shown to occur early in the second week of the reaction (Chapter 3, 332). In these present experiments cytolytic activity against BW1100, an MHC-unrelated NK-resistant tumor target, was measured in the nylon wool non-adherent spleen cell fraction. Data from a representative experiment showing NK-like activity against BW1100 in recipients of non-depleted grafts on day 8 of the reaction is displayed in Figure 6.2. Normal control BDF_1 mice did not demonstrate any lytic activity against BW1100 (data not shown). In three separate experiments the mean cytolytic activity against BW1100 on day 8 was 8.02 +/- 1.84 LU. No measurable level of cytolytic activity against BW1100 was detected in recipients of NK1.1-depleted grafts (Figure 6.2).

6.4.3 The effect of anti-NK1.1 and complement on MLR-induced proliferation.

In these experiments untreated, complement control and anti-NK1.1 and complement depleted spleen cells harvested from poly I:C- treated donors were cultured with mitomycin-treated BDF_1 spleen stimulator cells. Non-depleted and complement-control responder cells (in the presence of BDF_1 stimulator cells) exhibited a 5 - 6 fold increase in ^{3}H-thymidine

Figure 6.1 Body weights (g) of individual BDF_1 graft recipients (I and II) and control mice (III) on day 20 of the reaction. Graft recipients received either unaltered grafts (I) or grafts treated *ex vivo* with anti-NK1.1 plus complement (II). Horizontal lines indicate the mean body weight +/- S.E. of each group; group I, 18.43 +/- 0.77 (n=6); group II, 23.90 +/- 0.41 (n=5); group III, 23.88 +/- 0.67 (n=8). The mean body weight of group I was 22.8 % less than that of group III (t-test $p<0.001$, 11 d.f.) whereas no significant deviation from control values was observed in group II mice.

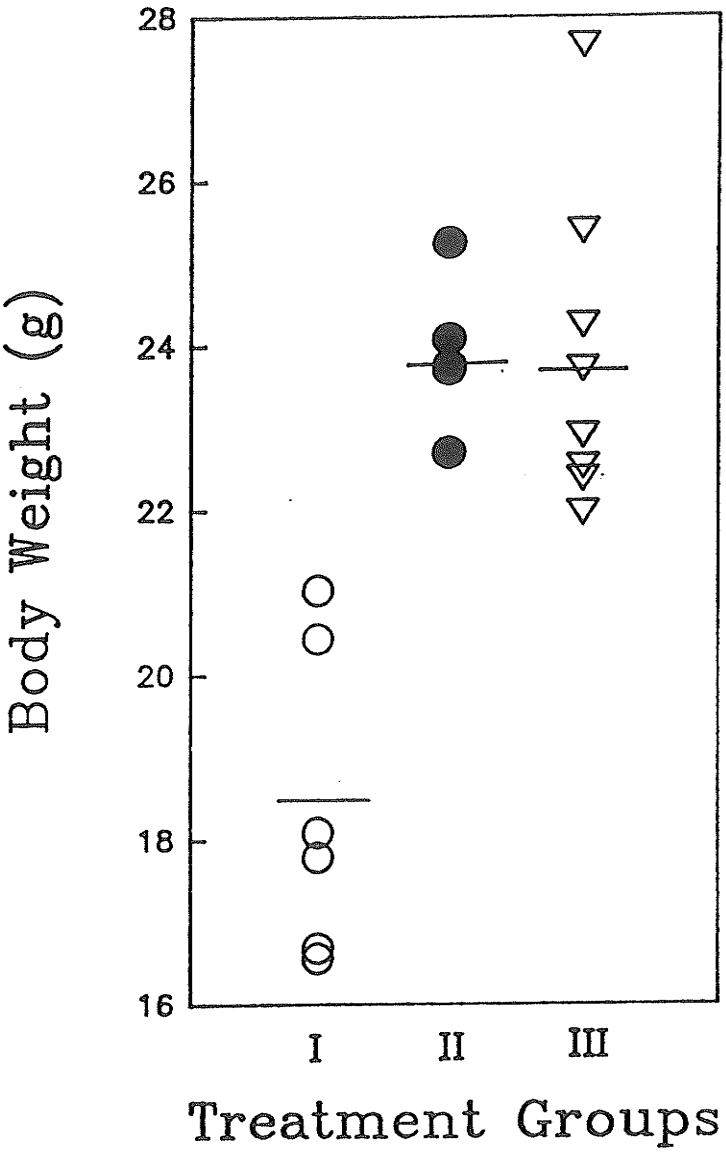
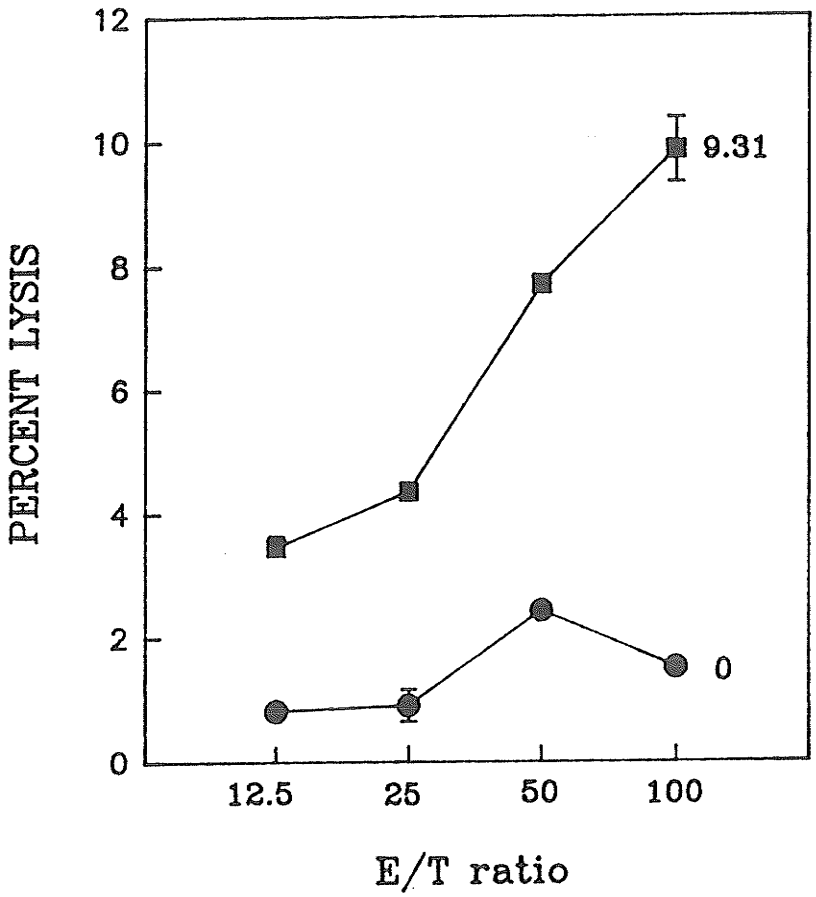

28
26
24
22
20
18
16
Body Weight (g)
I
II
III
Treatment Groups

Figure 6.2 A representative figure of 3 separate experiments showing Day 8 NK-like activity against BW1100 tumor targets in spleen cell suspensions recovered from a minimum of 3 BDF_1 recipients of untreated grafts (■) and grafts purged of $NK1.1^+$ cells with MoAb and complement (●). Error bars indicate the S.E. of the mean of triplicate samples. In those instances where no error bars are visible, the S.E. was smaller than the symbol size.


12
10
8
6
4
2
0
PERCENT LYSIS
9.31
0
12.5
25
50
100
E/T ratio

incorporation (no stimulators; Table 6.1). In comparison, responder cells treated with anti-NK1.1 and complement demonstrated a 12 - 13 fold increase. The reason for the facilitated proliferative response after treatment with anti-NK1.1 and complement is not known. It is possible that depletion with anti-NK1.1 may have removed $NK1.1^+$ cells that to some extent suppress MLR responsiveness.

6.4.4 IL-2 and IFN production in spleen cell cultures: a comparison between recipients of NK1.1-depleted and untreated grafts.

IL-2 and IFN were measured in spleen cell cultures prepared from recipients of untreated grafts and grafts depleted of NK1.1-positive cells to determine whether the lack of NK-like activity and the increased survival associated with anti-NK1.1 antibody treatment was related to a decrease in cytokine production. Results in table 6.2 showed that there is significant difference between recipients of untreated grafts and recipients of NK1.1-depleted grafts in the levels of IL-2 by spleen cell cultures established 2 days after induction. Similarly, there was no difference in the amount of IFN produced by spleen cell cultures on day 8.

6.4.5 CD3 expression on NK-like cytotoxic cells generated during GVH reactions.

The effect of depleting $CD3^+$ cells from the nylon

Table 6.1 The effect of anti-NK1.1 and complement on MLR-induced proliferation of poly I:C-stimulated B6 donor spleen cells.

Responder	Stimulator	NT	C	NK1.1 + C
B6	BDF_1		(DPM x 10^{-3})	
+	-	2.50 +/-0.36	3.29 +/-0.75	1.96 +/-0.26
+	+	14.20 +/-1.06	17.31 +/-0.98	25.05 +/-1.57

Values are the mean DPM +/- S.E. of triplicate cultures from a single experiment. MLR response by non-poly I:C-stimulated B6 responder spleen cells alone and in the presence of stimulator cells was 1.70 +/- 0.32 and 14.96 +/- 1.18 (8.8 fold increase), respectively. NT, no treatment; C, complement.

wool non-adherent spleen cell fraction by indirect-complement-mediated lysis on tumor cell cytotoxicity is shown in figure 6.3. Treatment with anti-CD3 and complement failed to deplete cytotoxic activity against any of the tumor targets. However, this treatment produced P815 killing that was significantly increased above that seen in complement control groups. In contrast, incubation of the effectors with anti-CD3 followed by rabbit anti-hamster IgG antiserum and complement caused a significant decrease in cytotoxic activity against both P815 and BW1100. This treatment had no effect on the level of YAC-1 lysis. Neither rabbit anti-hamster antiserum and complement nor complement alone reduced the level of cytotoxicity against any of the tumor targets. Similarly, incubation of effector cells with irrelevant hamster antibodies (normal hamster serum; Bio/Can Scientific, Mississauga, ON) had no effect on the level of cytotoxic activity (data not shown).

6.4.6 Expression of TCRγ/δ on cells mediating NK-like generated during GVH reactions.

To determine whether NK-like cells express TCRγ/δ, cytotoxic effector cells were incubated with anti-TCRγ/δ and depleted by indirect complement-mediated lysis. Treatment of the effector cells with anti-TCRγ/δ followed by rabbit anti-hamster antiserum and complement caused a 40 to 70 % reduction in the cytotoxic activity against BW1100 at an E:T ratio of 100:1 in three separate experiments (Figure 6.4).

Table 6.2 Comparison of IL-2 and IFN production in spleen cell cultures prepared from recipients of either untreated or anti-NK1.1-depleted grafts

	NT	NK1.1 + C
	(U/ml +/- S.E.)	
IL-2	37.84 +/- 8.54	35.12 +/- 7.10
IFN	16.67 +/- 3.33	20.00 +/- 0.00

Spleen cell cultures were prepared on day 2 for IL-2 (n=5) and day 8 for IFN (n=3). Student's t-test: IL-2 t=0.2449, 8 d.f.; IFN t=1, 4 d.f.. C, complement; NT, no treatment.

Figure 6.3 Analysis of CD3 expression on splenic cytotoxic effector cell populations isolated from graft recipients with acute GVH disease on day 8 of the reaction.

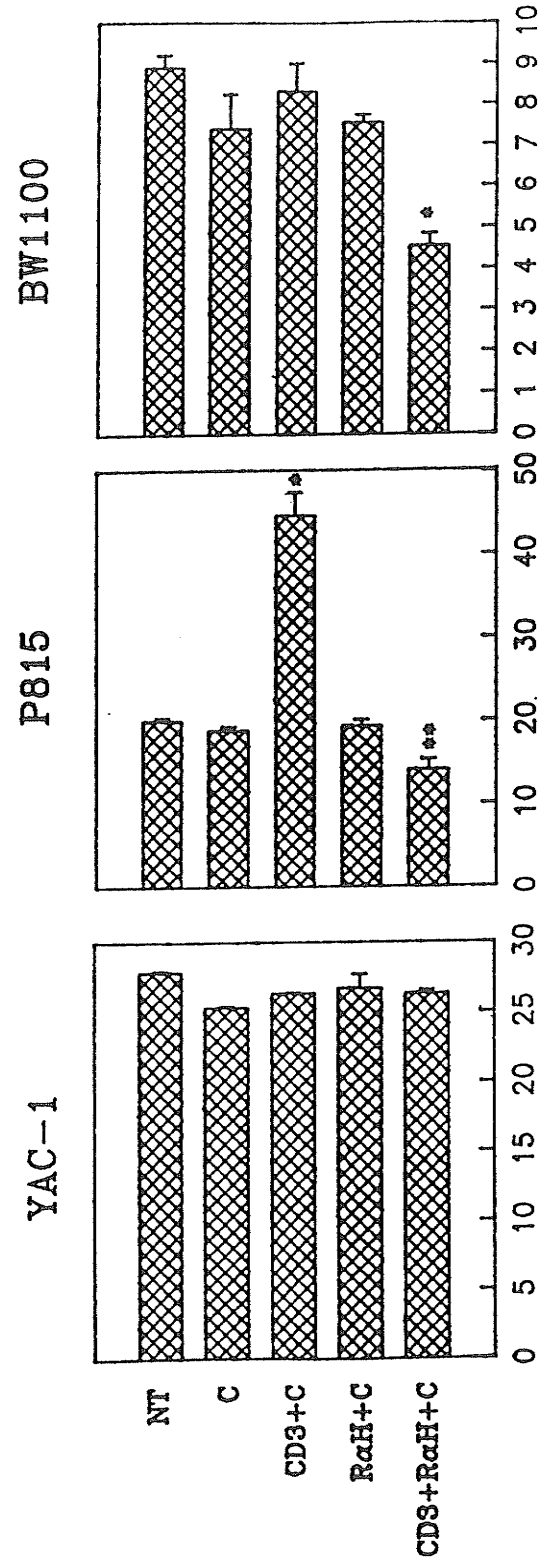
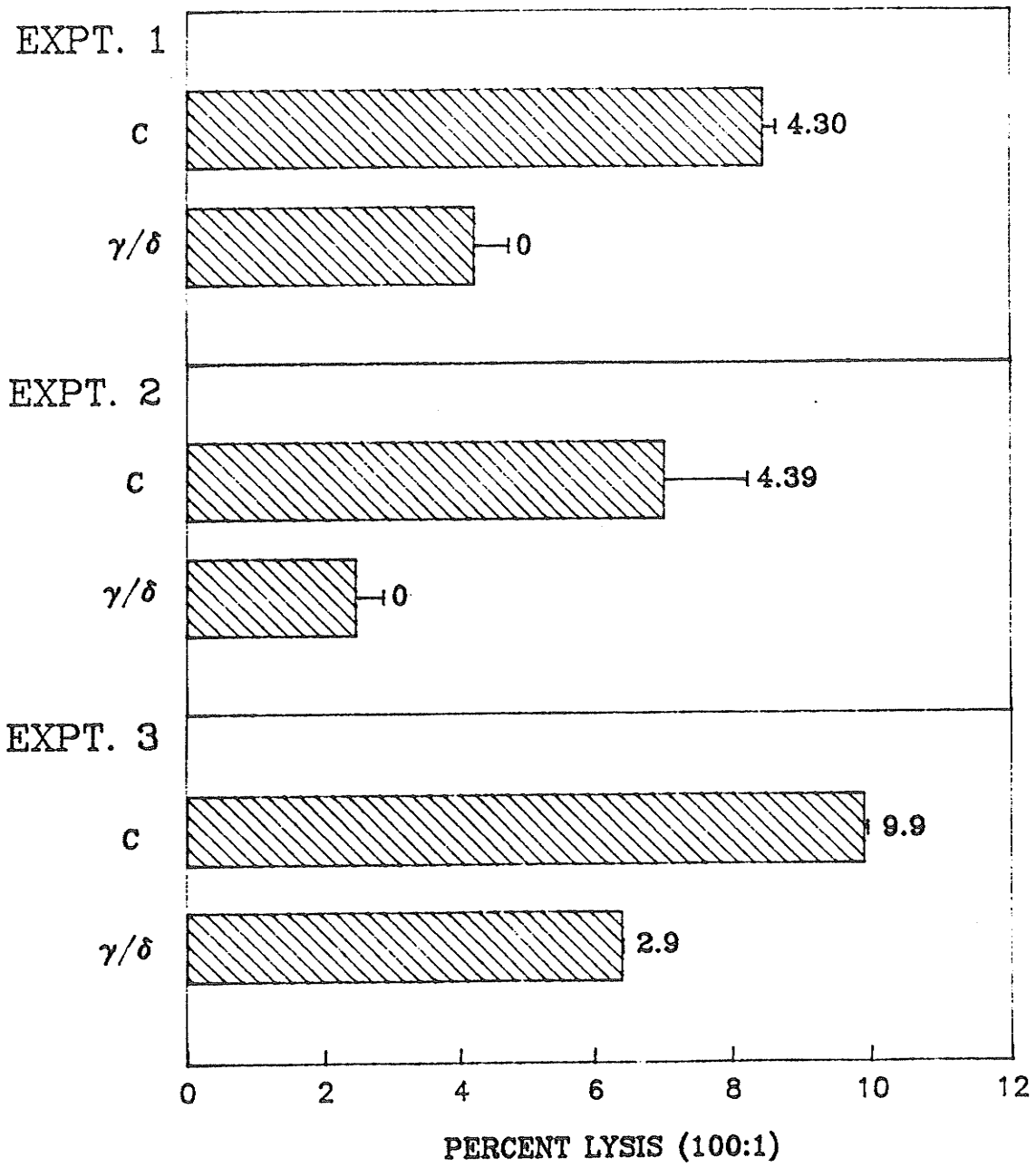

YAC-1
P815
BW1100
NT
C
CD3+C
RaH+C
CD3+RaH+C
0 5 10 15 20 25 30
0 10 20 30 40
0 1 2 3 4 5 6 7 8 9 10
PERCENT LYSIS (100:1)

Figure 6.4 Demonstration of TCRγ/δ expression on splenic NK-like cells isolated from mice with acute GVH disease on day 8 of the GVH reaction. NK-like activity was measured against BW1100 tumor targets. Cell suspensions were prepared from a minimum of 3 mice. In each experiment treatment with anti-TCRγ/δ + rab anti-hamster + complement (C) was shown by Student's t-test to cause a significant depletion of NK-like activity as compared to the C control (Expt.1 $p<0.005$, 4 d.f.; Expt.2 $p<0.01$, 4 d.f.; Expt.3 $p<0.005$, 4 d.f.).


EXPT. 1
C
γ/δ
4.30
0
EXPT. 2
C
γ/δ
4.39
0
EXPT. 3
C
γ/δ
9.9
2.9
0
2
4
6
8
10
12
PERCENT LYSIS (100:1)

This depletion in cytotoxicity represented a decrease in LU activity of 70 to 100%. Anti-TCRγ/δ and complement, and rabbit anti-hamster antiserum and complement did not significantly reduce BW1100 killing when compared to the complement control (data not shown).

6.5 Discussion

Previous studies have shown that activation of NK-like cells in the donor by poly I:C stimulation and their depletion from the graft with anti-NK1.1 plus complement, prior to induction, prevents mortality from acute GVH disease. Recipients of grafts depleted of NK-like cells also failed to develop the wasting syndrome associated with acute GVH disease. Since both NK cell activity and alloreactive CTL activity were observed in these recipients, it is evident that the mechanism that produces this wasting syndrome is not directly and exclusively mediated by NK cells and/or CTL.

In this study the level of splenic NK-like cell cytolytic activity was measured in recipients of NK1.1-depleted grafts and compared with the level observed in recipients of undepleted grafts. The lack of measurable NK-like cell cytotoxicity in mice in which acute GVH disease had been prevented suggests that NK-like cells, in contrast to NK cells and alloreactive T cells, may be involved in the development of the pathological features of acute GVH reactions. Depletion of NK1.1$^+$ cells from the graft did not diminish

alloreactivity of the graft or inhibit the production of IFN or IL-2 in the recipient. These findings clearly indicate that depletion with NK1.1 does not remove cells responsible for the generation of these important cytokines, which are critical elements in the allogeneic response of the reaction. Rather depletion appears to interfere with the effector mechanism in acute GVH disease, possibly by removing cells that cause tissue injury. It is possible that NK-like cells may be the relevant effector cell.

Most TCRγ/δ^+ T cells possess an LGL morphology and lack cell surface expression of the T cell markers CD4 and CD8. Also, freshly isolated γ/δ T cells are not cytolytic, but when stimulated with either IL-2 or IFN induce cytotoxic activity against both NK-sensitive and NK-resistant tumor targets (277,282). Because of the morphologic, phenotypic and functional similarities between NK-like cells and lymphokine-stimulated γ/δ T cells, experiments were performed to determine whether NK-like cells expressed CD3 and TCRγ/δ. Results showed that cells which mediate the killing of P815 and BW1100 tumor targets express CD3. The absence of CD3 expression on the cells restricted to killing YAC-1 target cells is consistent with the idea that they are conventional NK cells. The presence of CD3 on the cells responsible for P815 killing in this strain combination is not surprising since these cells possess a cell surface phenotype characteristic of CTL; ie $CD8^+$, $Thy\text{-}1^+$, $CD4^-$, $NK1.1^-$. Also,

since P815 is H-2^d, and, in the strain combination employed the reaction was directed at the H-2^d haplotype of the host ($BDF_1^{b/d}$), this would not be unexpected. Anti-CD3 without the second antibody facilitated killing of P815, but had no affect on BW1100, lysis. It is as yet unclear why NK-like cells did not exhibit facilitated killing of BW1100 following treatment with anti-CD3 as was observed with CTL activity against P815. Anti-CD3 has been shown to augment tumor target lysis by FCR-dependent and FCR-independent mechanisms (317,341,342). Spleen cells cultured with anti-CD3 for 24 - 48hr develop the ability to kill NK-resistant targets that is FCR-independent (342). Because the incubation of effectors with anti-CD3 in the present study was of such short duration (2hr), it is likely that BW1100 tumor cells do not express FCR and therefore killing of BW1100 would not be facilitated in FCR-dependent redirected lysis. Another possibility is that BW1100 does express FCR but binding is restricted to certain IgG isotypes (341).

Having demonstrated CD3 on the surface of NK-like cells, similar experiments were performed to determine whether these cells expressed TCRγ/δ. The results showed that much of the splenic NK-like cytotoxic activity is depletable with anti-TCRγ/δ^+ T cells. The failure to remove all NK-like cell activity with anti-TCRγ/δ and complement may indicate that some of the cells mediating NK-like activity do not express TCRγ/δ. T cells lacking CD4 and CD8, but expressing CD3 and

TCRα/β have been shown by others to mediate a pattern of killing similar to NK-like cells and in one study have been associated with clinical features of GVH disease (107-109). Therefore, it is possible that TCRα/β^+ cells with NK-like cell activity may also be contributing to the overall level of BW1100 lysis.

In summary, the data suggests that depletion of NK1.1$^+$ cells from grafts taken from poly I:C-stimulated donors prevents mortality from acute GVH disease by removing an effector cell population and not by blocking the allogeneic response during the lymphoproliferative phase of the reaction. It is suggested that TCRγ/δ^+ NK-like cells are the relevant effector cells being removed by this treatment.

6.6 Appendix

The material presented in this chapter has not been submitted for publication as yet. The cytotoxicty assays and the cytokine studies were done by the candidate. The MLR experiments were done by Mrs. Veronica Sanders with some technical assistance from the candidate.

CHAPTER 7

NK-LIKE CELLS IN THE PATHOGENESIS OF ACUTE GVH DISEASE: A HYPOTHETICAL MODEL

Allogeneic BMT has been used to treat a variety of neoplastic and non-neoplastic diseases. However, the high incidence of GVH disease following allogeneic BMT has greatly minimized the effectiveness of BMT. Attempts to identify the effector cell(s) responsible for GVH-disease have been equivocal. Studies performed in this laboratory have shown that two similar, yet distinct, non-MHC-restricted cytotoxic cell populations are activated during acute GVH reactions (332). One population resembles conventional NK cells both in cell-surface phenotype and in the range of target cells it can lyse. Phenotypically, these cells are $CD4^-$, $CD8^-$, Thy-$1^{+/-}$ and $ASGM_1^+$. These cells lyse NK-sensitive, but not NK-resistant tumor targets. The other cell population is entirely Thy-1^+. CTI experiments have demonstrated that these Thy-1^+ cells are able to lyse both NK-sensitive and NK-resistant tumor targets (335). Because of these differences the second cell population has been termed NK-like.

Experiments described by this candidate were designed to:

(1) further identify cell-surface markers specific to NK-like cells

(2) address the question of the host/donor origin of NK and NK-like cells in the spleens of F_1-hybrid mice with acute GVH reactions

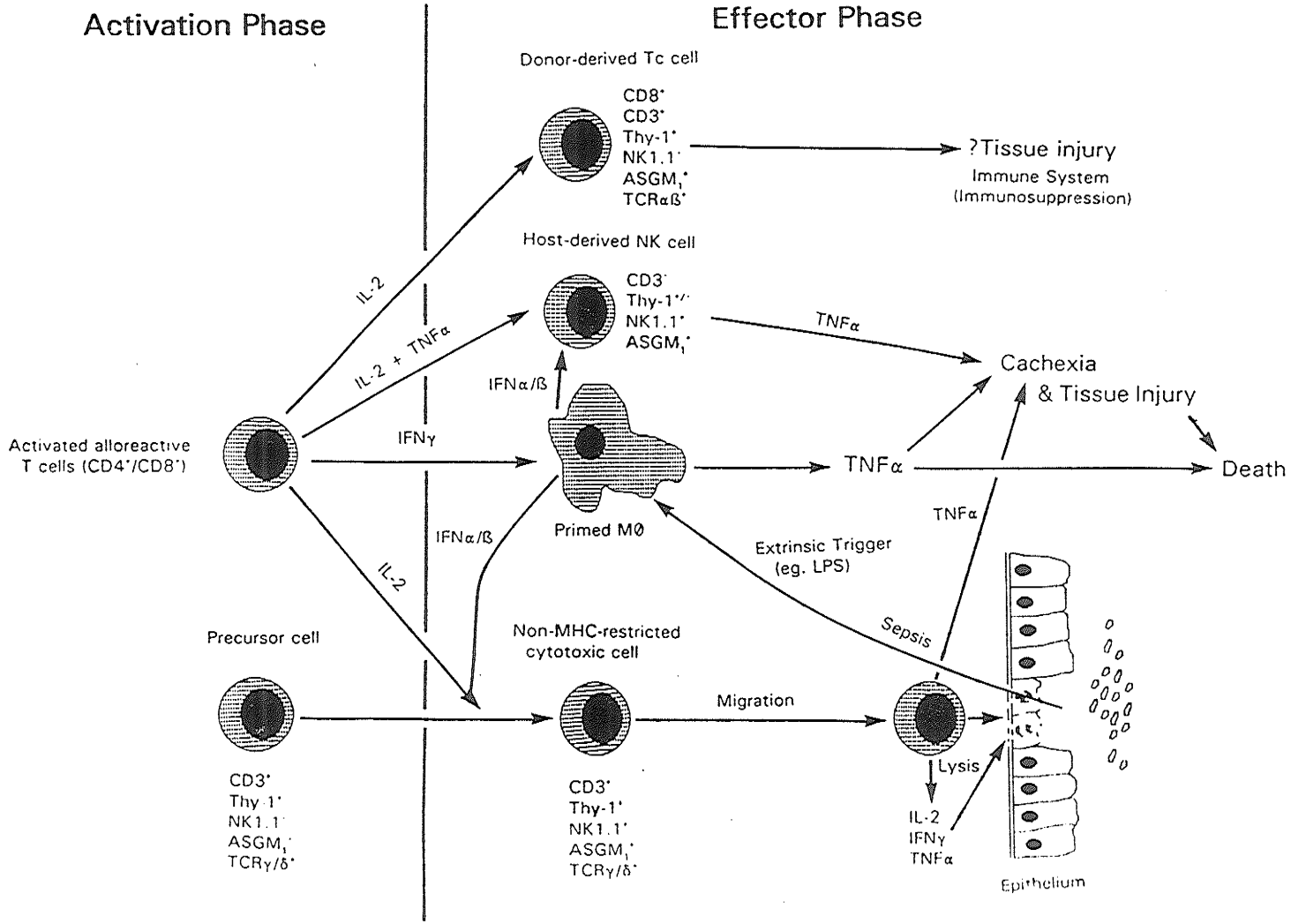
(3) study the relationship between the production of IL-2 and IFN, and the activation of NK and NK-like cell cytotoxicity

(4) determine the effect of purging NK and NK-like cells from the graft on the development of acute GVH disease in F_1-hybrid mice.

Results of these studies showed that augmented splenic NK cytotoxic cell activity was mediated predominantly by host cells; however, most of the splenic NK-like cell cytolytic activity was donor-derived. A comparison of cytokine levels in spleen cell cultures prepared from mice with chronic GVH reactions with those produced in mice with acute GVH reactions suggested that IL-2 and IFNα/β, but not IFNγ, are involved in the augmentation of host-derived NK cell activity. The data also suggested that IFNα/β alone or with IL-2 is required for the activation of NK-like cell cytotoxicity. *Ex vivo* treatment of the graft with anti-NK1.1 and complement was shown to alter the development of acute GVH disease, if and only if the donors had first been treated with poly I:C. Although recipients of these grafts still developed some features of acute GVH reactions, there was significantly reduced mortality in this group. The reduced mortality could not be attributed to a diminished alloreactive capacity of the grafted cells since IL-2 and IFN production in spleen cell cultures were unchanged as were *in vitro* MLR proliferative responses. Survival appeared to be related with the absence of cachexia and the lack of $CD3^+$, TCRγ/δ^+ cells mediating NK-like cell activity.

On the basis of these findings a hypothetical model

Figure 7.1 A schematic representation of potential effector cells involved in the pathogenesis of acute GVH disease.


Activation Phase
Effector Phase
Donor-derived Tc cell
CD8+
CD3+
Thy-1+
NK1.1-
ASGM1+
TCRαß+
?Tissue injury
Immune System
(Immunosuppression)
Host-derived NK cell
CD3-
Thy-1+/-
NK1.1+
ASGM1+
TNFα
Cachexia
& Tissue Injury
IL-2
IL-2 + TNFα
IFNα/ß
IFNγ
Activated alloreactive
T cells (CD4+/CD8+)
TNFα
Death
Primed MØ
IFNα/ß
IL-2
Extrinsic Trigger
(eg. LPS)
TNFα
Sepsis
Precursor cell
Non-MHC-restricted
cytotoxic cell
Migration
CD3+
Thy-1+
NK1.1-
ASGM1+
TCRγ/δ+
CD3+
Thy-1+
NK1.1+
ASGM1+
TCRγ/δ+
Lysis
IL-2
IFNγ
TNFα
Epithelium

describing the effector cell role of non-MHC-restricted cytotoxic cells in acute GVH disease has been developed and is shown in Figure 7.1. The model is believed to be a two step process involving the induction and activation of donor-derived alloreactive T cells by host alloantigens. The cytokines produced during this phase of the reaction then initiate effector mechanisms by stimulating the activation and differentiation of a variety of cell populations which in turn mediate the pathological changes and mortality associated with acute GVH disease.

7.1 Induction of Acute GVH reactions

In this model cytokines released by alloactivated T cells provide a regulatory signal to several effector cell populations. Among these effector cells, macrophages occupy a central position. The model suggests that priming of these cells by IFNγ triggers the release of cytokines that in turn cause the activation of other cell populations and mediate several effector functions. It is well recognized that priming by IFNγ is essential for LPS-induced TNFα secretion (200) and it has been shown that peritoneal macrophages from mice with acute GVH reaction secrete large amounts of TNFα when stimulated with LPS (202). Since macrophages exposed to IFNγ show an increased expression of class I and class II MHC molecules (343) these cells may also serve as important allogeneic targets. Immune suppression may be another

consequence of macrophage priming since PGE secretion by activated macrophages suppresses the proliferative responses to T and B cell mitogens in F_1-hybrid mice with GVH reactions (41). This immunosuppression appears to be reversible by treatment with anti-IFNγ (189). Finally, IFNγ priming is known to increase macrophage cytolytic activity (199).

The model also proposes that the secretion of IFNα/ß from activated macrophages is involved in the activation of the TCRγ/δ^+ cells that mediate NK-like cell activity. This idea is supported by the findings of others who have shown that mice stimulated with poly I:C generate Thy-1^+ non-MHC-restricted cytotoxic cells that are able to kill NK-sensitive and NK-resistant tumor targets (240,334). It has also been observed that incubation of PBL with rIFNß induces non-MHC-restricted killing in cells that are $CD3^+$, $CD4^-$, $CD8^-$, TCRα/$ß^-$ (260). While these studies support the idea that IFNα/ß mediates the activation of NK-like cells, the model does not exclude the possibility that other cytokines are also involved. For example, it is known that IL-2 and IFNα/ß synergize to increase NK cell killing (157,263,264). TNFα may also contribute to the activation of NK-like cell activity. It is now established that, although TNFα by itself is unable to induce non-MHC-restricted killing, it does potentiate IL-2-induced non-MHC-restricted killing (268).

7.2 Effector Mechanisms of Acute GVH Reactions

The model indicates that non-MHC-restricted cytotoxic T cells expressing the CD3/TCRγ/δ complex (the NK-like cells described by the candidate) mediate tissue injury in acute GVH reactions. These effector cells are thought to be recruited from a $CD3^+$, $NK1.1^-$ precursor cell pool and when stimulated with IFNα/β are induced to express NK1.1. This idea is supported by the work of other investigators who have shown that $CD3^+$, $CD4^-$, $CD8^-$ T cells do not express NK1.1 or cytotoxic activity in the resting state but do so following activation (219). While the candidate has determined that NK-like cells express TCRγ/δ, it should be noted that non-MHC-restricted killing can also be mediated by $CD3^+$, $CD4^-$, $CD8^-$, TCRα/β^+ cells (344).

The model proposes that, following activation, NK-like TCRγ/δ cells migrate from the lymph nodes and spleen to different organs where they cause epithelial cell injury. The actual mechanism by which this occurs is unknown. Two possibilities are proposed. Infiltrating NK-like cells may kill host epithelium by secreting cytokines. This idea is supported in part by studies showing that treatment of animals with anti-TNFα serum dramatically reduces the histopathologic effects and mortality from acute GVH disease (196). Because TNFα was only detected in tissue and not in serum of mice with acute GVH reactions, the authors concluded that locally infiltrating cells released TNFα and thereby mediated tissue

injury. There is some evidence that lectin stimulation of cell lines having a cell surface phenotype similar to NK-like cells induces the secretion of TNFα, as well as, IFNγ and TNFß (108).

Another way in which NK-like cells may lyse epithelial cells might involve receptor-ligand interaction. MoAb to FAM, a vimentin-like structure distinct from the CD3-associated TCR complex and present on both NK and IL-2 activated T cells, is known to inhibit tumor target cell lysis (298). It has also been observed that TCRγ/δ cells recognize allogeneic targets and kill target cells expressing HSP in association with non-conventional class I antigens (275,291,292). It is possible then IFN secretion by infiltrating cells may induce non-conventional class I antigen expression on epithelial cells. Expression of HSP in conjunction with the non-conventional class I antigens could thus make epithelial cells vulnerable to lysis by TCRγ/δ cells. It is of some interest that TNFα can induce HSP (74). Thus infiltrating cells such as NK-like cells or activated NK cells, may also facilitate the expression of HSP through the release of TNFα.

Regardless of the mechanism responsible for epithelial cell injury, it alone, albeit essential, does not appear to be sufficient to cause the mortality associated with GVH disease. Gnotobiotic animals with acute GVH reactions do not exhibit the cachexia or mortality observed with graft recipients kept in a normal enviroment (345-348). Although these animals

survive, they still show evidence of tissue injury and other features of GVH disease (347,348). BMT patients kept in a germ-free environment exhibit longer survival times (349). The model attempts to reconcile these paradoxical observations by suggesting that once the integrity of the epithelium has been compromised, bacteria are free to enter the circulation. The presence of endotoxin (LPS) in the circulation would provide the trigger for the release of TNFα from IFN-primed macrophages. It is this factor which mediates the cachexia and contributes to the mortality related to acute GVH disease.

How can the observation that the purging of NK1.1$^+$ cells from the graft prevents the mortality from acute GVH disease in F_1-hybrid mice be reached with this model. The most compelling explanation is that removal of TCRγ/δ^+, NK-like cells from the graft, while not abrogating induction and lymphoproliferation, prevents the damage to the epithelium which is necessary for the subsequent development of sepsis. In the absence of an extrinsic trigger, macrophages, although primed, would not release TNFα, thus preventing the development of cachexia and mortality.

The model predicts that purging of NK1.1$^+$ cells should have no effect on the induction of the reaction, thus other cell populations, such as donor-derived host-specific CTL and host-derived NK cells, are still activated. It appears that these cell populations are not directly involved in the lethal effects of acute GVH disease in an F_1-hybrid model.

Cytokine studies suggest that IL-2 synergizes with IFNα/ß to augment NK activity; however, this does not exclude the potential contribution of other cytokines. For example, TNFα release by alloactivated T cells may also be involved in augmenting NK cell activity (268,269). Activated NK cells have been shown to produce TNFα (198) and may also contribute to the development of cachexia. Also, NK cell production of IFNγ in response to IL-2 (350) may add to further up-regulation of class I and class II antigen expression on antigen-presenting cells.

In conclusion, the data presented by the candidate support the idea that TCRγ/δ^+ T cells expressing cytotoxic cell activity are instrumental in a pathological process that culminates in the development of acute, lethal GVH disease. If these cells are removed from the graft before transplant, cachexia and mortality can be prevented.

References

1. Murphy, J.B. 1916. The effect of adult chicken organ grafts on the chick embryo. *J. Exp. Med. 24:1.*
2. Dempster, W.J. 1952. Kidney homotransplantation. *Brit. J. Surg. 40:447.*
3. Simonsen, M. 1953. Biological incompatibility in kidney transplantation in dogs. *Acta Path. Microbiol. Scand. 32:36.*
4. Simonsen, M. 1957. The impact on the developing embryo and newborn animal of adult homologous cells. *Acta Path. Microbiol. Scand. 40:480.*
5. Billingham, R.E. 1958. Studies on the reaction of injected lymphoid tissue cells against the host. *Ann. N.Y. Acad. Sci. 73:782.*
6. Billingham, R.E. 1966. The biology of graft-versus-host reactions. *The Harvey Lect. 62:21.*
7. Billingham, R.E., Defendi, V., Silvers, W.K., and Stienmuller, D. 1962. Quantitative studies on the induction of tolerance of skin homografts and on runt disease in neonate rats. *J. Nat. Cancer Instit. 28:365.*
8. Gleichmann, E., Pals, S.T., Rolink, A.G., Radaskiewicz, T., and Gleichmann, H. 1984. Graft-versus-host reactions: clues to the etiopathology of a spectrum of immunological diseases. *Immunol. Today 5:324.*

9. Lapp, W.S., Ghayur, T., Mendes, M., Seddik, M., and Seemayer, T. 1985. The functional and histological basis for graft-versus-host-induced immunosuppression. *Immunol. Rev. 88:107.*

10. Howard, J.G., and Woodruff, M.F.A. 1961. Effect of the graft-versus-host reaction on the immunological responsiveness of the mouse. *Proc. Roy. Soc. 154:532.*

11. Möller, G. 1971. Suppressive effect of graft versus host reactions on the immune response to heterologous red cells. *Immunol. 20:597.*

12. Seddik, M., Seemayer, T., and Lapp, W.S. 1984. The graft-versus-host reaction and immune function: I. T helper cell immunodeficiency associated with graft-versus-host-induced thymic epithelial cell damage. *Transplant. 37:281.*

13. Via, C.S. and Shearer, G.M. 1988. Murine graft-versus-host disease as a model for the development of autoimmunity. Relevance of cytotoxic T lymphocytes. *Ann. N.Y. Acad. Sci. 532:44.*

14. Moser, M., Mizuochi, T., Sharrow, S.O., Singer, A., and Shearer, G.M. 1987. Graft-vs.-host reaction limited to class II MHC differences results in a selective deficiency in $L3T4^+$ but not in $Lyt2^+$ T helper cells. *J. Immunol. 138:1355.*

15. Rolink, A.G. and Gleichmann, E. 1983. Allosuppressor- and allohelper-T cells in acute and chronic graft-versus-host (GVH) disease: III. Different Lyt subsets of donor T cells induce different pathological syndromes. *J. Exp. Med. 158:546.*

16. Via, C.S., Sharrow, S.O., and Shearer, G.M. 1987. Role of cytotoxic T lymphocytes in the prevention of a lupus-like disease occurring in a murine model of graft-versus-host disease. *J. Immunol. 139:1840.*

17. Kimura, M., and Gleichmann, E. 1987. Depressed antibody responses to exogenous antigens in mice with lupus-like graft-versus-host disease. *Clin. Immunol. Immunopath. 43:97.*

18. Hakim, F.T. and Shearer, G.M. 1990. Immunologic and hematopoietic deficiencies of graft-vs.-host disease. in, Burakoff, S.J., Deeg, H.J., Ferrara, J., and Atkinson., K. eds., Graft-vs.-Host Disease: Immunology, Pathophysiology and Treatment. New York, Marcel Dekker, p133.

19. Grushka, M. and Lapp, W.S. 1974. Effect of lymphoid tissue transplants on the intensity of existing graft-versus-host reaction. *Transplant. 17:157.*

20. Seddik, M., Seemayer, T., and Lapp, W.S. 1986. The graft-versus-host reaction and immune function: IV. B cell functional defect associated with a depletion of splenic colony-forming units in marrow of graft-versus-host-reactive mice. *Transplant. 41:242.*

21. Lapp, W.S., Wechsler, A., and Kongshavn, P. 1974. Immune restoration of mice immunosuppressed by a graft-versus-host reaction. *Cell. Immunol. 11:419.*

22. Cudkowicz, G. and Bennett, M. 1971. Peculiar immunobiology of bone marrow allografts: II. Rejection of parental grafts by resistant F_1 hybrid mice. *J. Exp. Med. 134:1513.*

23. Dalianis, T. and Ahrlund-Richter, L. 1992. One explanation for F_1 antiparent responses. *Immunol. Today 13:248.*

24. Murphy, W.J., Kumar, V., and Bennett, M. 1990. Natural killer cells activated with interleukin 2 *in vitro* can be adoptively transferred and mediate hematopoietic histocompatibility-1 antigen specific bone marrow rejection *in vivo. Eur. J. Immunol. 29:1729.*

25. Sentman, C.L., Hackett, J.$^{Jr.}$, Kumar, V., and Bennett, M. 1989. Identification of a subset of murine natural killer cells that mediates rejection of *Hh-1*^{d} but not *Hh-1*^{b} bone marrow grafts. *J. Exp. Med. 170:191.*

26. Yankelevich, B., Knobloch, C., Nowicki, M. and Dennert, G. 1989. A novel cell type responsible for marrow graft rejection in mice: T cells with NK phenotype cause acute rejection of marrow grafts. *J. Immunol. 142:3423.*

27. Lorenz, E., Uphoff, D.E., Reid, T.R. and Shelton, E. 1951. Modification of irradiation injury in mice and guinea pigs by bone marrow infections. *J. Natl. Cancer. Inst. 12:197.*

28. Santos, G.W. 1983. History of bone marrow transplantation. *Clin. Haematopath. 12:611.*

29. Woodruff, M.F.A. and L.O. Simpson. 1955. Induction of tolerance to skin homografts in rats by injection of cells from the prospective donor soon after birth. *Br. J. Exp. Path. 36:494.*

30. Billingham, R.E. 1958. Studies on the reaction of injected homologous lymphoid tissue cells against the host. *Ann. N.Y. Acad. Sci. 73:782.*

31. Howard, J.G., Michie, D., and Simonsen, M. 1961. Splenomegaly as a host response in graft-versus-host disease. *Br. J. Exp. Path. 42:478.*

32. Fox, M. 1962. Cytological estimation of proliferating donor cells driving graft-versus-host disease in F_1 hybrid mice injected with parental spleen cells. *Immunol. 5:489.*

33. Elkins, W.L. 1970. Specific and non-specific lymphoid cell proliferation in the pathogenesis of graft-versus-host reactions. *Transplant. 9:273.*

34. Romano, T.J., Ponzio, N.M., Thorbecke, G.J. 1976. Graft-vs-host reactions in F_1 mice induced by parental lymphoid cells: nature of recruited F_1 cells. *J. Immunol. 116:1618.*

35. Piguet, P.F., Dewey, H.K., and Vassalli, P. 1975. Study of the cells proliferating in parent versus F_1 hybrid mixed lymphocyte culture. *J. Exp. Med. 141:775.*

36. Elie, R. and Lapp, W.S. 1977. Graft versus host-induced immunosuppression: mechanism of depressed T-cell helper function *in vitro. Cell. Immunol. 34:38.*

37. Rolstad, B. and Fossum, S. 1977. The host component of the popliteal lymph node graft-versus-host (GVH) reaction: identification if cells in the node and kinetics of cell proliferation. *Tissue Antigens 9:96.*

38. Ford, W.L., Rolstad, B., Fossum, S., Hunt, S.V., Smith, M.E., and Sparshott, S.M. 1981. The stimulus to host cell proliferation in graft-versus-host reactions. *Scand. J. Immunol. 14:705.*

39. Lapp, W.S. and Möller, G. 1969. Prolonged survival of *H-2* incompatible skin allografts on F_1 animals treated with parental lymphoid cells. *Immunol. 17:339.*

40. Parthenais, E. and Lapp, W.S. 1978. Evidence for two types of non-specific suppressor cells activated by the graft-versus-host reaction in mice. *Scand. J. Immunol. 7:215.*

41. Lapp, W.S., Mendes, M., Kirchner, H., and Gernsa, D. 1980. Prostaglandin synthesis by lymphoid tissue of mice experiencing a graft-versus-host reaction: relationship to immunosuppression. *Cell. Immunol. 50:271.*

42. Maier, T. and Claman, H.N. 1990. Experimental GVHD across minor histocompatibility barriers. in, Burakoff, S.J., Deeg, H.J., Ferrara, J. and Atkinson, K. eds., Graft-vs.-Host Disease: Immunology, Pathophysiology and Treatment. New York, Marcel Dekker, p75.

43. Maier, T., Holda, J.H. and H.N. Claman. 1989. Murine natural suppressor cells in the newborn, in bone marrow, and after cyclophosphamide: genetic variations and dependence on IFN-γ. *J. Immunol. 143:491.*

44. Smith, S.R., Terminelli, C., Kenworthy-Bott, L., and Phillips, D.L. 1991. A study of cytokine production in acute graft-vs-host disease. *Cell. Immunol. 134:336.*

45. Knobloch, C. and Dennert, G. 1988. Asialo-GM_1-positive killer cells are generated in F_1 mice injected with parental spleen cells. *J. Immunol. 140:744.*

46. Mendes, M., Rode, H., Peres, A., Kongshavn, P.A.L., and Lapp, W.S. 1985. Interleukin-1 and interleukin-2 defects associated with murine graft-versus-host-induced immunodeficiency. *Transpl. 39:418.*

47. Xenocostas, A., Lapp, W.S., and Osmond, D.G. 1987. Suppression of B lymphocyte genesis in the bone marrow by systemic graft-versus-host reaction. *Transplantation 43:549.*

48. Xenocostas, A., Osmond, D.G., and Lapp, W.S. 1991. The effect of the graft-versus-host reaction on B lymphocyte production in bone marrow of mice: depressed genesis of early progenitors prior to μ heavy chain expression. *Transplantation 51:1089.*

49. Tracey, K.J. and Cerami, A. 1990. Metabolic responses to cachectin/TNF: a brief review. *Ann. N.Y. Acad. Sci. 587:325.*

50. Balkwill, F., Burke, F., Talbot, D., Tavernier, J., Osborne, R., Naylor, S., Durbin, H., and Fiers, W. 1987. Evidence for tumour necrosis factor/cachectin production in cancer. *Lancet 2:1229.*

51. Lähdevirta, J., Maury, C.P.J., Teppo, A.M., and Repo, H. 1988. Elevated levels of circulating cachectin/Tumor necrosis factor in patients with acquired immunodeficiency syndrome. *Am. J. Med. 85:289.*

52. Scuderi, P., Lam, K.S., Ryan, K., Petersen, E., Sterling, K.E., Finley, P.R., Ray, C.G., Slymien, D.J., and Salmom, S.E. 1986. Raised serum levels of Tumour necrosis factor in parasitic infections. *Lancet 2:1364.*

53. Eckel, R. 1989. Lipoprotein lipase: a multifunctional enzyme relevant to common metabolic diseases. *N. Engl. J. Med. 320:1060.*

54. Kawakami, M., et al. 1990. Specificity in metabolic effects of cachectin/TNF and other related cytokines. *Ann. N.Y. Acad. Sci. 587:339.*

55. Kawakami, M. and Cerami, A. 1981. Studies of endotoxin-induced decrease in lipoprotein lipase activity. *J. Exp. Med. 154:631.*

56. Beutler, B., Mahoney, J., Le Trang, N., Pekula, P., and Cerami, A. 1985. Purification of cachectin, a lipoprotein lipase-suppressing hormone secreted by endotoxin-induced RAW 264.7 cells. *J. Exp. Med. 161:984.*

57. Lee, M.D., Zentella, A., Pekula, P.H., and Cerami, A. 1987. Effect of endotoxin-induced monokines on glucose metabolism in the muscle cell line L6. *Proc. Natl. Acad. Sci. USA 84:2590.*

58. Tracey, K.J., Wei, H., Manogue, K.R., Fong, Y., Hesse, D.G., Nguyen, H.T., Kuo, G.C., Beutler, B., Cotran, R.S., Cerami, A., and Lowry, S.F. 1988. Cachectin/tumor necrosis factor induces cachexia, anemia, and inflammation. *J. Exp. Med. 167:1211.*

59. Dinarello, C.A. 1988. Biology of interleukin 1. *FASEB J. 2:108.*

60. Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown-Shimer, S., and Cerami, A. 1986. Identification of a common nucleotide sequence in the 3'-untranslated region mRNA molecules specifying inflammatory mediators. *Proc. Natl. Acad. Sci. USA 83:1670.*

61. Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N., and Williamson, B. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA 72:3666.*

62. Coley, W.B. 1896. The therapeutic value of the mixed toxins of the streptococcus of erysipelas and bacillus prodigious in the treatment of inoperable malignant tumors with a report of one hundred sixty cases. *Am. J. Med. Sci. 112:251.*

63. Spriggs, D.R., Deutsch, S., and Kufe, D.W. 1992. Genomic structure, induction, and production of TNF-α. *Immunol. Ser. 56:1.*

64. Kriegler, M., Perez, C., Defay, K., Albert, I., and Lu, S.D. 1988. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell 53:45.*

65. Luettig, B., Decker, T., and Lohmann-Mathes, M.L. 1989. Evidence for the existence of two forms of membrane tumor necrosis factor: an integral protein and a molecule attached to its receptor. *J. Immunol. 143:4034.*

66. Liu, C., Detmers, P.A., Jiang, S., and Young, J.D. 1989. Identification and characterization of a membrane bound cytotoxin of murine cytotoxic lymphocytes that is related to tumor necrosis factor/cachectin. *Proc. Natl. Acad. Sci. 86:3286.*

67. Birkland, T., Sypek, J.P., and Wyler, D.J. 1992. Soluble TNF and membrane TNF expressed on $CD4^+$ T lymphocytes differ in their ability to activate macrophage antileishmanial defense. *J. Leuk. Biol. 57:296.*

68. Kunkel, S.L., Remick, D.G., Strieter, R.M., and Larrick, J.W. 1989. Mechanisms that regulate the production and effects of tumor necrosis factor-α. *Crit. Rev. Immunol. 9:93.*

69. Loetscher, H., Pan, Y.E., Lohm, H., Gentz, R., Brockhaus, M., Tabuchi, H., and Lesslauer, W. 1990. Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell 61:351.*

70. Tsujimoto, M. 1992. Regulation of TNF receptors. *Immunol. Ser. 56:149.*

71. Goodwin, R.G., Anderson, D., Jerzy, R., Davis, T., Brannan, C.I., Copeland, N.G., Jenkins, N.A., and Smith, C.A. 1991. Molecular cloning and expression of the type 1 and type 2 murine receptors for tumor necrosis factor. *Mol. Cell. Biol. 11:3020.*

72. Tartaglia, L.A., Weber, R.F., Figari, I.S., Reynolds, C., Palladino, M.A., and Goeddel, D.V. 1991. The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc. Natl. Acad. Sci. USA 88:9292.*

73. Tartaglia, L.A., and Goeddel, D.V. 1992. Two TNF receptors. *Immunol. Today 13:151.*

74. Larrick, J.W., and Wright, S.C. 1990. Cytotoxic mechanism of tumor necrosis factor-α. *FASEB 4:3215.*

75. Tracey, K.J., Beutler, B., Lowry, S.F., Merryweather, J., Wolpe, S., Milsark, I.W., Hariri, R.J., Fahey III, T.J., Zentella, A., Albert, J.D., Shires, G.T. and Cerami, A. 1986. Shock and tissue injury induced by recombinant human cachectin. *Science 234:470.*

76. Rappaport, H., Khalil, A., Halle-Pannenko, O., Pritchard, L., Dantchev, D., and Mathé, G. 1979. Histopathologic sequence of events in adult mice undergoing lethal graft-versus-host reaction developed across H-2 and/or non-H-2 histocompatibility barriers. *Am. J. Path. 96:121.*

77. Seemayer, T., Lapp, W.S., and Bolande, R.P. 1977. Thymic involution in murine graft-versus-host reaction. *Am. J. Path. 88:119.*

78. Seemayer, T.A., Lapp, W.S., and Bolande, R.P. 1978. Thymic epithelial injury in graft-versus-host reactions following adrenalectomy. *Am. J. Path. 93:325.*

79. Ghayur, T., Seemayer, T.A., Xenocostas, A., and Lapp, W.S. 1988. Complete sequential regeneration of graft-vs.-host-induced severely dysplastic thymuses: implications for the pathogenesis of chronic graft-vs.-host disease. *Am. J. Path. 133:39.*

80. Woodruff, J.M., Eltringham, J.R., and Casey, H.W. 1969. Early secondary disease in the Rhesus monkey: I. a comparative histopathologic study. *Lab. Invest. 20:499.*

81. Woodruff, J.M., Hansen, J.A., Good, R.A., Santos, G.W., and Slavin, R.E. 1976. The pathology of graft-versus-host reaction (GVHR) in adults receiving bone marrow transplants. *Transplant. Proc. 8:675.*

82. Woodruff, J.M., Butcher, W.I., and Hellerstein, L.J. 1972. Early secondary disease in the Rhesus monkey: II. electron microscopy of changes in mucus membranes and external epithelia as demonstrated in the tongue and lip. *Lab. Invest. 27:85.*

83. Cantor, H. 1972. The effects of anti-theta antiserum upon graft-versus-host activity of spleen and lymph node cells. *Cell. Immunol. 3:461.*

84. Martin, P.J. and Kernan, N.A. 1990. T-cell depletion for the prevention of graft-vs.-host disease. in, Burakoff, S.J., Deeg, H.J., Ferrara, J., and Atkinson, K. eds., Graft-vs.-Host Disease: Immunology, Pathophysiology and Treatment. New York, Marcel Dekker, p371.

85. Weider, P.L., Sullivan, K.M., Flournoy, N., Storb, R., and Thomas, E.D. 1981. Antileukemic effect of chronic graft-versus-host disease. *N. Engl. J. Med. 304:1529.*

86. Drobyski, W.R., Piaskowski, V., Ash, R.C., Casper, J.T., and Truitt, R.L. 1990. Preservation of lymphokine-activated killer activity following T cell depletion of human bone marrow. *Transpl. 50:625.*

87. Keever, C.A., Small, T.N., Flonenberg, N., Heller, G., Pekle, K., Black, P., Pecora, A., Gillio, A., Kernan, N.A., and O'Reilly, R.J. 1989. Immune reconstitution following bone marrow transplantation: comparison of recipient of T-cell depleted marrow with recipients of conventional marrow grafts. *Blood 73:1340.*

88. Gale, R.P and Reisner, Y. 1986. Graft rejection and graft-versus-host disease: mirror images. *Lancet 1(2):1468.*

89. Vallera, D.A., Soderling, C.C.B., Carlson, G.J. and Kersey, J.H. 1982. Bone marrow transplant across major histocompatibility barriers in mice: II. T cell requirement for engraftment in total lymphoid irradiation-conditioned recipients. *Transpl. 33:243.*

90. Kernan, N.A. 1990. Graft failure following transplantation of T-cell-depleted marrow. in, Burakoff, S.J., Deeg, H.J., Ferrara, J., and Atkinson, K. eds., Graft-vs.-Host Disease: Immunology, Pathophysiology and Treatment. New York, Marcel Dekker, p557.

91. Mason, D.W. 1981. Subsets of T cells in the rat mediating lethal graft-versus-host disease. *Transpl. 32:222.*

92. Korngold, R. and Sprent, J. 1987. T cell subsets and graft-versus-host disease. *Transpl. 44:335.*

93. Sprent, J., Schaeffer, M., Lo, D., and Korngold, R. 1986. Properties of purified T cell subsets: II. *in vivo* responses to class I vs. class II differences. *J. Exp. Med. 163:998.*

94. Korngold, R. and Sprent, J. 1985. Surface markers of T cells causing lethal graft-versus-host disease to class I and class II H-2 differences. *J. Immunol. 135:3004.*

95. Ferrara, J. and Buarakoff, S.J. 1990. The pathophysiology of acute graft-vs.host disease in a murine bone marrow transplant model. in, Burakoff, S.J., Deeg, H.J., Ferrara, J., and Atkinson, K. eds., Graft-vs.-Host Disease: Immunology, Pathophysiology and Treatment. New York, Marcel Dekker, p9.

96. Ferrara, J.L.M., and Deeg, H.J. 1991. Mechanisms of disease: graft-versus-host disease. *N. Engl. J. Med. 324:667.*

97. Kelso, A., Troutt, A.B., Maraskovsky, E., Gough, N.M., Morris, L., Peck, M.H., and Thomson, J.A. 1991. Heterogeneity in lymphokine profiles of $CD4^+$ and $CD8^+$ T cells and clones activated *in vivo* and *in vitro*. *Immunol. Rev. 123:85.*

98. Elkins, W.L. 1976. Correlation of graft-versus-host mortality and positive CML assay in the mouse. *Transpl. Proc. 8:343.*

99. Elkins, W.L. 1978. Effector mechanism and graft-versus-host disease. *Transpl. Proc. 10:15.*

100. Loveland, B.E. and McKenzie, I.F.C. 1982. Which T cells cause graft rejection. *Transpl. 33:217.*

101. Cerottini, J.-C., Nordin, A.A., and Brunner, K.T. 1971. Cellular and humoral response to transplantation antigens: I. Development of alloantibody-forming cells and cytotoxic lymphocytes in the graft-versus-host reaction. *J. Exp. Med. 134:553.*

102. Piguet, P.F. 1985. GVHR elicited by products of class I or class II loci of the MHC: analysis of the response of mouse T lymphocytes to products of class I and class II loci of the MHC in correlation with GVHR-induced mortality, medullary aplasia, and enteropathy. *J. Immunol. 135:1637.*

103. Borland, A., Mowat, A.Mc.I., and Parrott, D.V.M. 1983. Augmentation of intestinal and peripheral natural killer cell activity during the graft-versus-host reaction in mice. *Transpl. 36:513.*

104. Jadus, M.R. and Peck, A.B. 1983. Lethal murine graft-versus-host disease in the absence of detectable cytotoxic T lymphocytes. *Transpl. 36:281.*

105. Mowat, A.Mc.I. and Felstein, M.V. 1990. Intestinal graft-vs.-host reaction. in, Burakoff, S.J., Deeg, H.J., Ferrara, J. and Atkinson, K. eds., Graft-vs.-Host Disease: Immunology, Pathophysiology and Treatment. New York, Marcel Dekker, p205.

106. van Els, C.A.C.M., Bakker, A., Zwinderman, A.H., Zwaan, F.E., and Goulmy, E. 1990. Effector mechanism in graft-versus-host disease in response to minor histocompatibility antigens: I. absence of correlation with cytotoxic effector cells. *Transpl. 50:62.*

107. Wirt, D.P., Brooks, E.G., Vaidya, S., Klimpel, G.R., Waldmann, T.A., and Goldblum, R.M. 1989. Novel T-lymphocyte population in combined immunodeficiency with features of graft-versus-host disease. *N. Engl. J. Med. 321:370.*

108. Brooks, E.G., Wirt, D.P., Goldblum, R.M., Vaidya, S., Asuncion, M.T., Patterson, J.C., Ware, C.F., and Klimpel, G.R. 1990. Double-negative ($CD4^-CD8^-$) T cells with $\alpha/ß$ T cell receptor: non-MHC-restricted cytolytic activity and lymphokine production. *J. Immunol. 144:4507.*

109. Brooks, E.G., Wirt, D.P., Klimpel, G.R., Vaidya, S., and Goldblum, R.M. 1989. Combined immunodeficiency with graft versus host-like autoimmunity and double negative lymphocytes: response to cyclosporine A. *Clin. Res. 37:56A.*

110. Singh, J.N., Sabbadini, E., and Sehon, A.H. 1971. Nonspecific cytotoxicity in graft-versus-host reactions. *Transpl. 11:475.*

111. Singh, J.N., Sabbadini, E., and Sehon, A.H. 1972. Cytotoxicity in graft-versus-host reactions: I. role of donor and host spleen cells. *J. Exp. Med. 136:39.*

112. Roy, C., Ghayur, T., Kongshavn, and Lapp, W.S. 1982. Natural killer activity by spleen, lymph node and thymus cells during the graft-versus-host reaction. *Transpl. 34:144.*

113. Ghayur, T., Seemayer, T.A., and Lapp, W.S. 1987. Kinetics of natural killer cell cytotoxicity during the graft-versus-host reaction. *Transpl. 44:254.*

114. Mowat, A.Mc.I., Borland, A., and Parrott, D.V.M. 1985. Augmentation of natural killer cell activity by anti-host delayed-type hypersensitivity during the graft-versus-host reaction in mice. *Scand. J. Immunol. 22:389.*

115. Mowat, A.Mc.I. and Felstein, M.V. 1987. Experimental studies of immunologically mediated enteropathy. *Immunol. 61:179.*

116. Pattengale, P.K., Ramstedt, U., Gidlund, M., Orn, A., Axberg, I., and Wigzell, H. 1983. Natural killer activity in (C57BL/6xDBA/2)F_1 hybrids undergoing acute and chronic graft-vs.-host reaction. *Eur. J. Immunol. 13:912.*

117. Ghayur, T., Seemayer, T.A., Kongshavn, P.A.L., Gartner, J.G., and Lapp, W.S. 1987. Graft-versus-host reactions in the beige mouse: an investigation of the role of host and donor natural killer cells in the pathogenesis of graft-versus-host disease. *Transpl. 44:261.*

118. Halle-Pannenko, O. and Bruley-Rosset, M. 1985. Decreased graft-versus-host reaction and cytolytic potential of Beige mice. *Transpl. 39:85.*

119. Roder, J. and Duwe, A. 1979. The *beige* mutation in the mouse selectively impairs natural killer cell function. *Nature 278:451.*

120. Windhorst, D.B. and Padgett, G. 1973. The Chediak-Higashi syndrome and the homologous trait in animals. *J. Invest. Derm. 60:529.*

121. Saxena, R.K., Saxena, Q.B. and Adler, W.H. 1982. Defective T cell response in beige mutant mice. *Nature 295:240.*

122. Charley, M.R., Mikhael, A., Hoot, G., Hackett, J., and Bennett, M. 1985. Studies addressing the mechanism of anti-Asialo GM_1 prevention of graft-versus-host disease due to minor histocompatibility antigenic differences. *J. Invest. Derm. 85:121s.*

123. Charley, M.R., Mikhael, A., Bennett, M., Gilliam, J.N., and Sontheimer, R.D. 1983. Prevention of lethal, minor-determinate graft-versus-host disease in mice by the *in vivo* administration of anti-asialo GM_1. *J. Immunol. 131:2101.*

124. Varkila, K. and Hurme, M. 1985. Natural killer (NK) cells and graft-versus-host disease (GVHD): no correlation between NK cell levels and GVHD in the murine $P \rightarrow F_1$ model. *Immunol. 54:121.*

125. Varkila, K. 1987. Depletion of asialo-$GM1^+$ cells from the F_1 recipient mice prior to irradiation and transfusion of parental spleen cells prevents mortality to acute graft-versus-host disease and induction of anti-host specific cytotoxic T cells. *Clin. Exp. Immunol. 69:652.*

126. Varkila, K and Hurme, M. 1985. Acute graft versus host disease mortality in murine P vs F_1 model can be prevented by pretreatment of the recipient mice with anti asialo GM_1 antibody. *J. Leuk. Biol. 38:124.*

127. Ghayur, T., Seemayer, T.A., and Lapp, W.S. 1988. Prevention of murine graft-versus-host disease by inducing and eliminating $ASGM1^+$ cells of donor origin. *Transpl. 45:586.*

128. Ghayur, T., Xenacostas, A., Seemayer, T.A., and Lapp, W.S. 1991. Induction, specificity, and elimination of Asialo-$GM1^+$ graft-versus-host effector cells of donor origin. *Scand. J. Immunol. 34:497.*

129. Ting, C.-C., Bluestone, J.A., Hargrove, M.E., and Loh, N.-N. 1986. Expression and function of asialo GM_1 in alloreactive cytotoxic T lymphocytes. *J. Immunol. 137:2100.*

130. Stitz, L., Baenziger, J., Pircher, H., Hengartner, H., and Zinkernagel, R.M. 1986. Effect of rabbit anti-asialo GM_1 treatment *in vivo* or with anti-asialo GM_1 plus complement *in vitro* on cytotoxic T cell activities. *J. Immunol. 136:4674.*

131. Stuttles, J., Schwarting, G.A., and Stout, R.D. 1986. Flow cytometric analysis reveals the presence of asialo G_{m1} on the surface membrane of alloimmune cytotoxic T lymphocytes. *J. Immunol. 136:1586.*

132. Knobloch, C. and Dennert, G. 1988. Asialo-GM_1-positive T killer cells are generated in F_1 mice injected parental spleen cells. *J. Immunol. 140:744.*

133. Stout, R.D., Schwarting, G.A., and Stuttles, J. 1987. Evidence that expression of asialo-G_{m1} may be associated with cell activation: correlation asialo-G_{m1} expression with increased total cellular RNA and protein content in normal thymocytes and spleen cell populations. *J. Immunol. 139:2123.*

134. Beck, B.N., Gillis, S., and Henney, C.S. 1982. Display of the neutral glycolipid ganglio-N-tetraosylceramide (Asialo G_{m1}) on cells of the natural killer and T lineages. *Transpl. 33:118.*

135. Blazar, B., Soderling, C.C.B., Koo, G.C., and Vallera, D.A. 1988. Absence of a facilitatory role for NK1.1-positive donor cells in engraftment across a major histocompatibility barrier in mice. *Transpl. 45:876.*

136. Guillén, F.J., Ferrara, J., Hancock, W.W., Messadi, D., Fonferko, E., Burakoff, S., and Murphy, G.F. 1986. Acute cutaneous graft-versus-host disease to minor histocompatibility antigens in a murine model: evidence that large granular lymphocytes are effector cells in the immune response. *Lab. Invest. 55:35.*

137. Ferrara, J, Mauch, P., Murphy, G., and Burakoff, S. 1985. Bone marrow transplantation: genetic and cellular basis of resistance to engraftment and acute graft-versus-host disease. *Sur. Immunol. Res. 4:253.*

138. Lopez, C., Kirkpatrick, D., Sorell, M., O'Reilly, R.J. & Ching C. 1979. Association between pre-transplant natural killer cell activity and graft-versus-host disease after stem cell transplantation. *Lancet 2:1103.*

139. Livnat, S., Seigneuret, M., Storb, R., and Prentice, R.L. 1980. Analysis of cytotoxic effector cell function in patients with leukemia or aplastic anemia before and after marrow transplantation. *J. Immunol. 124:481.*

140. Gratama, J.W., Lipovich-Oosterveer, M.A., Ronteltop, C., Sinnige, L.G.F., Jansen, J., Van Der Griend, R.J., and Bolhuis, R.L.H. 1985. Natural immunity and graft-versus-host disease. *Transpl. 40:256.*

141. Dokhelar, M.C., Wiels, J., Lipinski, M., Tetaud, C., Devergie, A., Gluckman, E., and Tursz, T. 1981. Natural killer cell activity in human bone marrow recipients: early appearance of peripheral natural killer activity in graft-versus-host disease. *Transpl. 31:61.*

142. Hercend, T., Takvorian, T., Nowill, A., Tantravahi, R., Moingeon, P., Anderson, K.C., Murray, C., Bohuon, C., Ythier, A., and Ritz, J. 1986. Characterization of natural killer cells with antileukemia activity following allogeneic bone marrow transplantation. *Blood 67:722.*

143. Rooney, C.M., Wimperis, J.Z., Brenner, M.K., Patterson, J., Hoffbrand, A.V., and Prentice, H.G. 1986. Natural killer cell activity following T-cell depleted allogeneic bone marrow transplantation. *Brit. J. Haemat. 62:413.*

144. Gillis, S. and Smith K.A. 1977. Long term culture of tumour-specific cytotoxic T cells. *Nature 268:154.*

145. Gillis, S., Ferm, M.M., Ou, W. and Smith, K.A. 1978. T cell growth factor: Parameters of production and a qualitative microassay. *J. Immunol. 120:2027.*

146. Malek, T.R., Schmidt, J.A., and Shevach, E.M. 1985. The murine IL 2 receptor: III. cellular requirements for the induction of IL 2 receptor expression on T cell populations. *J. Immunol. 134:2405.*

147. Smith, K.A. 1988. Interleukin-2: inception, impact, and implications. *Science 240:1169.*

148. Miyajima, A., Kitamura, T., Harada, N., Yokota, T., and Arai, K-I. 1992. Cytokine receptors and signal transduction. *Ann. Rev. Immunol. 10:295.*

149. Nakanishi, K., Hirose, S., Yoshimoto, T., Ishizoshi, H., Hiroishi, K., Tanaka, T., Kono, T., Miyasaka, M., Tanaguchi, T., and Higashino, K. 1992. Role and regulation of interleukin (IL-)-2 receptor alpha and beta chains in IL-2-driven B cell growth. *Proc. Natl. Acad. Sci. 89:3551.*

150. Voss, S.D., Sondel, P.M., and Robb, R.J. 1992. Characterization of IL-2 receptors (IL-2R) expressed on human natural killer cells activated *in vivo* by IL-2: association of the p64 IL-2Rγ chain with IL-2Rß chain in functional intermediate-affinity IL-2R. *J. Exp. M. 176:531.*

151. Takashita, T., Asao, H., Suzuki, J., and Sugamura, K. 1990. An associated molecule, p64, with high-affinity interleukin 2 receptor. *Inter. Immunol. 2:477.*

152. Hatakeyama, M., Kono, T., Kobayashi, N., Kawahara, A., Levin, S.D., Perlmutter, R.M., and Tanaguchi, T. 1991. Interaction of the IL-2 receptor with the src-family kinase $p56^{lck}$: identification of novel intermolecular association. *Science 252:1523.*

153. Torigoe, T., Saragovi, H.U., and Reed, J.C. 1992. Interleukin 2 regulates the activity of the lyn protein tyrosine kinase in a B cell line. *Proc. Natl. Acad. Sci. 89:2674.*

154. Ralph, P., Jeong, G., Welte, C., Mertelsmann, R., Rabin, H., Henderson, L.E., Souza, L.M., Boone, T.C. and Robb, R.J. 1984. Stimulation of immunoglobulin secretion in human B lymphocytes as a direct effect of high concentration of IL 2. *J. Immunol. 133:2442.*

155. Suzuki, R., Handa, K., Itoh, K., and Kumagi, K. 1983. Natural killer (NK) cells as a responder to interleukin 2 (IL 2): I. proliferative response and establishment of clones. *J. Immunol. 130:981.*

156. Biron, C.A., Young, H.A., and Kasaian, M.T. 1990. Interleukin 2-induced proliferation of murine natural killer cells in vivo. *J. Exp. Med. 171:173.*

157. Henney, C.S., Kuribayashi, K., Kern, D.E, and Gillis, S. 1981. Interleukin-2 augments NK cell activity. *Nature 291:335.*

158. Malkovsky, M., Loveland, B., North, M., Asherson, G.L., Gao, L., Ward, P., and Fiers, W. 1987. Recombinant interleukin-2 directly augments the cytotoxicity of human monocytes. *Nature 325:262.*

159. Eberlein, T.J., Rosenstein, M.M., and Rosenberg, S.A. 1982. Regression of a disseminated syngeneic solid tumor by a systemic transfer of lymphoid cells expanded in IL-2. *J. Exp. Med. 156:385.*

160. Mulé, J.J., Shu, S., and Rosenberg, S.A. 1985. The anti-tumor efficacy of lymphokine-activated killer cells and recombinant interleukin 2 in vivo. *J. Immunol. 135:646.*

161. Puri, R.K. and Leland, P. 1991. *In vivo* treatment with interferon causes augmentation of IL-2 induced lymphokine-activated killer cells in the organs of mice. *Clin. Exp. Immunol. 85:317.*

162. Niven, J. and Lapp, W.S. 1986. Interleukin-2 production in early murine graft versus host disease. *J. Leuk. Biol. 36:440.*

163. Pritchard, L.L. and Halle-Pannenko, O. 1985. Abrogation of lethal graft-versus-host reaction to non-H-2 antigens correlates with suppression of proliferative but not cytotoxic effector cell activity. *Transpl. Proc. 17:439.*

164. Kornbluth, M., You-ten, E., Desbarats, J., Gamache, S., Xenocostas, A., and Lapp, W. 1991. T cell subsets in the thymus of graft-versus-host immunosuppressed mice: sensitivity of the $L3T4^{+}Lyt\text{-}2^{-}$ subset to cortisone. *Transplantation 51:262.*

165. Tsoi, M.-S., Mori, T., Brkic, S., Dobbs, S., Gillis, S., Santos, E., Thomas, E.D., and Storb, R. 1984. Ineffective cellular interaction and interleukin 2 deficiency causing T cell defects in human allogeneic marrow recipients early after grafting and in those with chronic graft-versus-host disease. *Transpl. Proc. 16:1470.*

166. Malkovsky, M., Brenner, M.K., Hunt, R., Rastan, S., Doré, C., Brown, S., North, M.E., Asherson, G.L., Prentice, H.G., and Medawar, P.B. 1986. T-cell depletion of allogeneic bone marrow prevents acceleration of graft-versus-host disease induced by exogenous interleukin 2. *Cell. Immunol. 103:476.*

167. Sykes, M., Romick, M.L., Hoyles, K.A., and Sachs, D.H. 1990. In vivo administration of interleukin 2 plus T cell-depleted syngeneic marrow prevents graft-versus-host disease mortality and permits alloengraftment. *J. Exp. Med. 171:645.*

168. Sykes, M., Hoyles, K.A., Romick, M.L., and Sachs, D.H. 1990. *In vitro* and *in vivo* analysis of bone marrow-derived $CD3^+$, $CD4^-$, $CD8^-$, $NK1.1^+$ cell lines. *Cell. Immunol. 129:478.*

169. Sondel, P.M., Hank, J.A., Kohler, P.C., Chen, B.P., Minkoff, D.Z., and Molenda, J.A. 1986. Destruction of autologous human lymphocytes by interleukin 2-activated cytotoxic cells. *J. Immunol. 137:502.*

170. Lotze, M.T., Matory, Y.L., Ettinghausen, S.E., Rayner, A.A., Sharrow, S.O., Seipp, C.A.Y., Custer, M.C., and Rosenberg, S.A. 1985. In vivo administration of purified human interleukin 2: II. half life, immunologic effects, and expansion of peripheral lymphoid cells in vivo with recombinant IL 2. *J. Immunol. 135:2865.*

171. Piguet, P.F., Grau, G., Irle, C., and Vassalli, P. 1986. Administration of recombinant interleukin 2 to mice enhances production of hemopoietic and natural killer cells. *Eur. J. Immunol. 16:1257.*

172. Volk, H.-D., Brocke, S., Osawa, H., and Diamantstein, T. 1986. Effects of in-vivo administration of a monoclonal antibody specific for the interleukin-2 receptor on the acute graft-versus-host reaction in mice. *Clin. Exp. Immunol. 66:126.*

173. Kirchner, H. 1984. Interferons, a group of multiple lymphokines. *Springer Semin. Immunopath. 7:347.*

174. Isaacs, A. and Lindenmann, J. 1957. Virus interference. I. The interferon. *Proc. Roy. Soc. 147:258.*

175. Pestka, S., and Langer, J.A. 1987. Interferons and their actions. *Ann. Rev. Biochem. 56:727.*

176. Peters, M. 1990. Immunological aspects of antiviral therapy. *Springer Semin. Immunopath. 12:47.*

177. Yamamoto, Y. and Kawade, Y. 1980. Antigenicity of mouse interferons: distinct antigenicity of the two L cell interferon species. *Virol. 103:80.*

178. Branca, A.A., and Baglioni, C. 1981. Evidence that types I and II interferons have different receptors. *Nature 294:768.*

179. Uzé, G., Lutfalla, G., Bamdu, M.-T., Proudhon, D., and Mogensen, K.E. 1992. Behaviour of a cloned murine interferon α/ß receptor expressed in homospecific or heterospecific background. *Proc. Natl. Acad. Sci. 89:4774.*

180. Hemmi, S., Peghini, P., Metzler, M., Merlin, G., Dembic, Z., and Aguet, M. 1989. Cloning of murine interferon γ receptor cDNA: expression in human cells mediates high affinity binding but is not sufficient to confer sensitivity to murine interferon γ. *Proc. Natl. Acad. Sci. 86:9901.*

181. Paucker, K., Cantell, K., and Henle, W. 1962. Quantitative studies on viral interference in suspended L cells: III. Effect of interfering viruses and interferon on the growth rate of cells. *Virol. 17:324.*

182. Lapierre, L.A., Fiers, W., and Pober, J.S. 1988. Three distinct classes of regulatory cytokines control endothelial cell MHC antigen expression: interactions with immune γ interferon differentiate the effects of tumor necrosis factor and lymphotoxin from those of leukocyte α and fibroblast ß interferons. *J. Exp. Med. 167:794.*

183. Snapper, C.M. and Paul, W.E. 1987. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science 236:944.*

184. Coffman, R.L., Seymour, B.W.P., Lebman, D.A., Hiraki, D.D., Christiansen, J.A., Shrader, B., Cherwinski, H.M., Savelkoul, H.F.J., Finkelman, F.D., Bond, M.W., and Mossman, T.R. 1988. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol. Rev. 102:5.*

185. Reyes, V.E. and Klimpel, G.R. 1987. Interferon α/ß synthesis during acute graft-versus-host disease. *Transpl. 43:412.*

186. Cleveland, M.G., Lane, R.G., and Klimpel, G.R. 1987. Enhanced interferon-α/ß(IFN-α/ß) and defective IFN-γ production in chronic graft versus host disease: a potential mechanism for immunosuppression. *Cell. Immunol. 110:120.*

187. Cleveland, M.G., Lane, R.G., and Klimpel, G.R. 1988. Spontaneous IFN-ß production: a common feature of natural suppressor systems. *J. Immunol. 141:2043.*

188. Cleveland, M.G., Annable, C.R., and Klimpel, G.R. 1988. In vivo and in vitro production of IFN-ß and IFN-γ during graft vs host disease. *J. Immunol. 141:3349.*

189. Klimpel, G.R., Annable, C.R., Cleveland, M.G., Jerrells, T.R., and Patterson, J.C. 1990. Immunosuppression and lymphoid hypoplasia associated with chronic graft versus host disease is dependent upon IFN-γ production. *J. Immunol. 144:84.*

190. Peres, A., Seemayer, T.A., and Lapp, W.S. 1986. The effects of polyinosinic:polycytidylic acid (pI:C) on the GVH reaction: immunopathological observations. *Clin. Immunol. Immunopath. 39:102.*

191. Peres, A., Amlani, S., Kornbluth, M., Seemayer, T.A., and Lapp, W.S. 1989. The effects of polyinosinic:polycytidylic acid on the graft-versus-host reaction. *Transpl. 48:80.*

192. Mizuno, S.-I., Morishima, Y., Kodera, Y., Ohno, R., Yokomaku, S., Sao, H., Yoshikawa, S. 1986. Gamma-interferon production capacity and T lymphocyte subpopulation after allogeneic bone marrow transplantation. *Transpl. 41:311.*

193. Niederwieser, D., Herold, M., Woloszcuk, W., Aulitzky, W., Meister, B., Tilg, H., Gastl, G., Bowden, R., and Huber, C. 1990. Endogenous IFN-gamma during human bone marrow transplantation: analysis of serum levels of interferon and interferon-dependent secondary messages. *Transpl. 50:620.*

194. Klingemann, H.-G., Grigg, A.P., Wilkie-Boyd, K., Barnett, M.J., Eaves, A.C., Reece, D.E., Shepherd, J.D., Phillips, G.L. 1991. Treatment with recombinant interferon (α-2ß) early after bone marrow transplantation in patients at high risk for relapse. *Blood 78:3306.*

195. Fontaine, P. and Perrault, C. 1990. Diagnosis of graft-versus-host disease in mice transplanted across minor histocompatibility barriers. *Transpl. 49:1177.*

196. Piguet, P.-F., Grau, G.E., Allet, B., and Vassalli, P. 1987. Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs.-host disease. *J. Exp. Med. 166:1280.*

197. Piguet, P.F., Grau, G.E., Collart, M.A., Vassalli, P., and Kapanci, Y. 1989. Pneumopathies of graft-versus-host reaction: alveolitis associated with an increased level of tumor necrosis factor mRNA and chronic interstitial pneumonitis. *Lab. Invest. 61:37.*

198. Beutler, B. 1988. Tumor necrosis, cachexia, shock, and inflammation: a common mediator. *Ann. Rev. Biochem. 57:505.*

199. Pace, J.L., Russell, S.W., Torres, B.A., Johnson, H.M. and Gray, P.W. 1983. Recombinant mouse γ interferon induces the priming step in macrophage activation for tumor cell killing. *J. Immunol. 130:2011.*

200. Gifford, G.E. and Lohmann-Matthes, M.-L. 1987. Gamma interferon priming of mouse and human macrophages for induction of tumor necrosis factor production by bacterial lipopolysaccharide. *J. Natl. Cancer Inst. 78:121.*

201. Collart, M.A., Belin, D., Vassalli, J.D., de Kossodo, S., and Vassalli, P. 1986. Gamma interferon enhances macrophage transcription of tumor necrosis factor/cachectin, interleukin-1, and urokinase genes, which are controlled by short-lived repressors. *J. Exp. Med. 164:2113.*

202. Nestel, F.P., Price, K.S., Seemayer, T.A., and Lapp, W.S. 1992. Macrophage priming and lipopolysaccharide release of tumor necrosis factor α during graft-versus-host disease. *J. Exp. Med. 175:405.*

203. Symington, F.W., Pepe, M.S., Chen, A.B., and Deliganis, A. 1990. Serum tumor necrosis factor alpha associated with acute graft-versus-host disease in humans. *Transpl. 50:518.*

204. Holler, E., Kolb, H.J., Möller, A., Kempeni, S., Liesenfeld, S., Pechumer, H., Lehmacher, W., Ruckdeschel, G., Gleixner, B., Riedner, C., Ledderose, G., Brehm, G., Mittermüller, J., and Wilmanns, W. 1990. Increased serum levels of tumor necrosis factor α precede major complications of bone marrow transplantation. *Blood 75:1011.*

205. Sims, J.E., March, C.J., Cosman, D., Widmer, M.B., MacDonald, H.R., McMahan, C.J., Grubin, C.E., Wignall, J.M., Jackson, J.L., Call, S.M., Friend, D., Alpert, A.R., Gillis, S., Urdal, D.L., and Dower, S.K. 1988. cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science 241:585.*

206. Boraschi, D., Rambaldi, A., Sica, A., Ghiara, P., Colotta, F., Wang, J.M., de Rossi, M., Zoia, C., Renuzzi, G., Bussolino, F., et al. 1991. Endothelial cells express the interleukin-1 receptor type I. *Blood 78:1262.*

207. Dower, S.K., Sims, J.E., Stanton, T.H., Slack, J., McMahan, C.J., Valentine, M.A., and Bomsztyk, K. 1990. Molecular heterogeneity of IL-1 receptors. *Ann. N.Y. Acad. Sci. 594:231.*

208. McCarthy Jr, P.L., Abhyankar, S., Neben, S., Newman, G., Sieff, C., Thompson, R.C., Burakoff, S.J., and Ferrara, J.L.M. 1991. Inhibition of interleukin-1 by an interleukin-1 receptor antagonist prevents graft-versus-host disease. *Blood 78:1915.*

209. Zinkernagel, R.M. and Doherty, P.C. 1974. Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic chorio-meningitis within a syngeneic or semiallogeneic system. *Nature 248:701.*

210. Hercend, T. and Schmidt, R.E. 1988. Characteristics and uses of natural killer cells. *Immunol. Today 9:291.*

211. Ritz, J., Campen, T.J., Schmidt, R.E., Royer, H.D., Hercend, T., Hussey, R.E., and Reinherz, E.L. 1985. Analysis of T-cell receptor gene rearrangement and expression in human natural killer cells. *Science 228:1540.*

212. Loh, E.Y., Cwirla, S., Serafini, A.T., Phillips, J.H., and Lanier, L.L. 1988. Human T-cell-receptor δ chain: genomic organization, diversity, and expression in populations of cells. *Proc. Natl. Acad. Sci. USA 85:9714.*

213. Biron, C.A., Van Den Elsen, P., Tutt, M.M., Medveczky, P., Kumar, V., and Terhorst, C. 1987. Murine natural killer cells stimulated in vivo do not express the T cell receptoer α, ß, γ, T3δ, or T3ε genes. *J. Immunol. 137:1704.*

214. Anderson, Caligiuri, M., Ritz, J. and Schlossman, S.F. 1989. CD3-negative natural killer cells express zeta TCR as part of a novel molecular complex. *Nature 341:159.*

215. Lanier, L.L., Spits, H., and Phillips, J.H. 1992. The developmental relationship between NK cells and T cells. *Immunol. Today 13:392.*

216. Ravetch, J.V. and Kinet, J.-P. 1991. Fc Receptors. *Ann. Rev. Immunol. 9:457.*

217. Lanier, L.L, Testi, R., Bindl, J., and Phillips, J.H. 1989. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J. Exp. Med. 169:2233.*

218. Koyasu, S., D'Adamio, L., Arulanandam, A.R.N., Abraham, S., Clayton, L.K., and Reinherz, E.L. 1992. T cell receptor complexes containing FcεRIγ homodimers in lieu of CD3zeta and CD3eta components: a novel isoform expressed on large granular lymphocytes. *J. Exp. Med. 175:203.*

219. Ballas, Z.K. and Rasmussen, W. 1991. Lymphokine-activated killer (LAK) cells: VI. $NK1.1^{+}$, $CD3^{+}$, Lak effectors are derived from $CD4^{-}$, $CD8^{-}$, $NK1.1^{-}$ precursors. *Cell. Immunol. 134:296.*

220. Takasugi, M., Mickey, M.R., and Terasaki, P.I. 1973. reactivity of lymphocytes from normal persons on cultured tumor cells. *Cancer res. 33:2898.*

221. Rosenberg, E.B., Herberman, R.B., Levine, P.H., Halterman, R.H., McCoy, J.L., and Wunderlich, J.R. 1972. Lymphocyte cytotoxicity reactions to leukemia-associated antigens in identical twins. *Inter. J. Cancer 9:648.*

222. Oldham, R.K. and Herberman, R.B. 1973. Evaluation of cell-mediated cytotoxic reactivity against tumor associated antigens with ^{125}I-iododeoxyuridine labelled target cells. *J. Immunol. 111:1862.*

223. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol. 47:187.*

224. Herberman, R.B. and Holden, H.T. 1978. Natural cell-mediated immunity. *Adv. Cancer Res. 27:305.*

225. Cantor, H., Kasai, M., Shen, F.W., Leclerc, J.C., and Glimcher, L. 1979. Immunogenetic analysis of "natural killer" activity in the mouse. *Immunol. Rev. 44:1.*

226. Timonen, T. and Saksela, E. 1980. Isolation of human NK cells by density gradient centrifugation. *J. Immunol. Meth. 36:285.*

227. Timonen, T., Ortaldo, J.R. and Herberman, R.B. 1981. Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. *J. Exp. Med. 153:569.*

228. Kumagai, K., Itoh, K., Suzuki, R., Hinuma, S., and Saitoh, F. 1982. Studies of murine large granular lymphocytes: I. identification as effector cells in NK and K cytotoxicity. *J. Immunol. 129:388.*

229. Itoh, K., Suzuki, R., Umezu, Y., Hanaumi, K., and Kumagai, K. 1982. Studies of murine large granular lymphocytes: II. tissue, strain, and age distributions of LGL and LAL. *J. Immunol. 129:395.*

230. Koo, G.C., Jacobsen, J.B., Hammerling, G.J., and Hammerling, U. 1980. Antigenic profile of murine natural killer cells. *J. Immunol. 125:1003.*

231. Kiessling, R., Klein, E., Pross, H., and Wigzell, H. 1975. "Natural" killer cells in the mouse: II. cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur. J. Immunol. 5:117.*

232. Glimcher, L., Shen, F.W., and Cantor, H. 1977. Identification of a cell-surface antigen selectively expressed on the natural killer cell. *J. Exp. Med. 145:1.*

233. Beaumont, T.J., Roder, J.C., Elliot, B.E., Kerbel, R.S., Dennis, J.W., Kasai, M., and Okumura, K. 1982. A comparative analysis of cell surface markers on murine NK cells and CTL target-effector conjugates. *Scand. J. Immunol. 16:123.*

234. Young, W.W., Hakomori, S.-I., Durdik, J.M., and Henney, C.S. 1980. Identification of ganglio-*N*-tetraosylceramide as a new cell surface marker for murine natural killer (NK) cells. *J. Immunol. 124:199.*

235. Kasai, M., Iwamori, M., Nagai, Y., Okumura, K., and Tada, T. 1980. A glycolipid on the surface of mouse natural killer cells. *Eur. J. Immunol. 10:175.*

236. Merluzzi, V.J. 1985. Comparison of murine lymphokine-activated killer cells, natural killer cells, and cytotoxic T lymphocytes. *Cell. Immunol. 95:95.*

237. Burton, R.C., Koo, G.C., Smart, Y.C., Clark, D.A., and Winn, H.J. 1988. Antigens of murine natural killer cells. *Int. Rev. Cytology 111:185.*

238. Herberman, R.B., Nunn, M.E., and Holden H.T. 1978. Low density of Thy 1 antigen on the mouse effector cells mediating natural cytotoxicity against tumor cells. *J. Immunol. 121:304.*

239. Mattes, M.J., Sharrow, S.O., Herberman, R.B., and Holden, H.T. 1980. Identification and separation of Thy-1 positive mouse spleen cells active in natural cytotoxicity and antibody-dependent cell mediated cytotoxicity. *J. Immunol. 123:285.*

240. Shah, P.D., Keji, J., Gilbertson, S.M. & Rowley D.A. 1986. Thy-1$^+$ and Thy-1$^-$ Natural Killer Cells: only Thy-1$^-$ natural killer cells suppress dendritic cells. *J. Exp. Med. 163:1012.*

241. Smith, J.B. and Eaton G.J. 1976. Suppressor cells in spleens from "nude" mice: their effect on the mitogenic response of B lymphocytes. *J. Immunol. 117:319.*

242. Pollack, S.B., Tam, M.R., Nowinski, R.C. and Emmons, S.L. 1979. Presence of T cell-associated surface antigens on murine NK cells. *J. Immunol. 123:1818.*

243. Koo G.C., and Peppard J.R. 1984. Establishment of monoclonal anti-NK-1.1 antibody. *Hybridoma 3:301.*

244. Hackett, J., Tutt, M., Lipscomb, M., Bennett, M., Koo, G., and Kumar, V. 1986. Origin and differentiation of natural killer cells: functional and morphologic studies of purified NK-1.1$^+$. *J. Immunol. 136:3124.*

245. Koo, G.C., Dumont, F.J., Tutt, M., Hackett, J., and Kumar, V. 1986. The NK1.1$^-$ mouse: a model to study differentiation of murine NK cells. *J. Immunol. 137:3742.*

246. Seaman, W.E., Sleisenger, M., Eriksson, E., and Koo, G.C. 1987. Depletion of natural killer cells in mice by monoclonal antibody to NK1.1: reduction in host defense against malignancy without loss of cellular or humoral immunity. *J. Immunol. 138:4539.*

247. Burton, R.C., and Winn, H.J. 1981. Definitive evidence that natural killer (NK) cells inhibit experimental tumor metastasis *in vivo. J. Immunol. 126:1985.*

248. Hanna, N., and Burton, R.C. 1981. Studies on natural killer (NK) cells: I. NK cell specific antibodies in CE anti-CBA serum. *J. Immunol. 127:1754.*

249. Hackett, J., Bennett, M., and Kumar, V. 1985. Origin and differentiation of natural killer cells: I. characteristics of a transplantable NK cell precursor. *J. Immunol. 134:3731.*

250. Ryan, J.C., Turk, J., Niemi, E.C., Yokoyama, W.M., and Seaman, W.E. 1992. Molecular cloning of the NK1.1 antigen, a member of the NKR-P1 family of natural killer cell activation molecules. *J. Immunol. 149:1631.*

251. Giorda, R., Rudert, W.A., Vavassori, C., Chambers, W.H., Hiserodt, J.C., and Trucco, M. 1990. NKR-P1, a signal transduction molecule on natural killer cells. *Science 249:1298.*

252. Targan, S. and Dorey, F. 1980. Interferon activation of "pre-spontaneous killer" (Pre-SK) cells and alteration in kinetics of lysis of both "Pre-SK" and active SK cells. *124:2157.*

253. Timonen, T., Ortaldo, J.R., and Herberman, R.B. 1982. Analysis by a single cell cytotoxicity assay of natural killer (NK) cell frequencies among human large granular lymphocytes and of the effects of interferon on their activity. *J. Immunol. 128:2514.*

254. Biron, C.A., Sonnenfeld, G., and Welsh, R.M. 1984. Interferon induces natural killer cell blastogenesis in vivo. *J. Leuk. Biol. 35:31.*

255. Kasaian, M.T. and Biron, C.A. 1990. Cyclosporin A inhibition of interleukin 2 gene expression, but not natural killer cell proliferation, after interferon induction in vivo. *J. Exp. Med. 171:745.*

256. Trinchieri, G. and Santoli, D. 1990. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells: enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. *J. Exp. Med. 147:1314.*

257. Djeu, J.Y., Heinbaugh, J.A., Holden, H.T., and Herberman, R.B. 1979. Augmentation of mouse natural killer cell activity by interferon and interferon inducers. *122:175.*

258. Djeu, J.Y., Heinbaugh, J.A., Holden, H.T., and Herberman, R.B. 1979. Role of macrophages in the augmentation of mouse natural killer cell activity by poly I:C and interferon. *J. Immunol. 122:182.*

259. Lucero, M.A., Fridman, W.H., Provost, M.-A., Billardon, C., Pouillart, P., Dumont, J., and Falcoff, E. 1981. Effect of various interferons on the spontaneous cytotoxicity exerted by lymphocytes from normal and tumor-bearing mice. *Cancer Res. 41:294.*

260. Borst, J., van de Griend, R.J., van Oostveen, J.W., Ang, S.-L., Melief, C.J., Seidman, J.G., and Bolhuis, R.L.H. 1987. A T-cell receptor γ/CD3 complex found on cloned functional lymphocytes. *Nature 325:683.*

261. Lotzova, Savary, C.A., Gutterman, J.U., and Hersh, E.M. 1982. Modulation of natural killer cell-mediated cytotoxicity by partially purified and cloned interferon-α. *Cancer Res. 42:2480.*

262. Maluish, A.E., Ortaldo, J.R., Conlon, J.C., Sherwin, S.A., Leavitt, R., Strong, D.M., Weirnik, P., Oldham, R.K., and Herberman, R.B. 1983. Depression of Natural killer cytotoxicity after *in vivo* administration of recombinant leukocyte interferon. *J. Immunol. 131:503.*

263. Brunda, M.J., Tarnowski, D., and Davatelis, V. 1986. Interaction of recombinant interferons with recombinant IL-2: Differential effects on NK cell activity and IL-2-activated killer cells. *Int. J. Cancer 37:787.*

264. Svedersky, L.P., Shepard, H.M., Spencer, S.A., Shalaby, M.R., and Palladino, M.A. 1984. Augmentation of human natural cell-mediated cytotoxicity by recombinant human interleukin 2. *J. Immunol. 133:714.*

265. Lanier, L.L., Benike, C.J., Phillips, J.H., and Engleman, E.G. 1985. Recombinant interleukin 2 enhanced natural killer cell-mediated cytotoxicity in human lymphocyte subpopulations expressing the Leu 7 and Leu 11 antigens. *J. Immunol. 134:794.*

266. Trinchieri, G., Matsumoto-Kabayashi, M., Clark, S.C., Seehra, J., London, L., and Perussia, B. 1984. Response of resting human peripheral blood natural killer cells to interleukin 2. *J. Exp. Med. 160:1147.*

267. Phillips, J.H. and Lanier, L.L. 1986. Dissection of the lymphokine-activated killer phenomenon: relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. *J. Exp. Med. 164:814.*

268. Ostensen, M.E., Thiele, D.L. and Lipsky, P.E. 1987. Tumor necrosis factor-α enhances cytolytic activity of human natural killer cells. *J. Immunol. 138:4185.*

269. Chouaib, S., Bertoglio, J., Blay, J.-Y., Marchiol-Fournigault, C., and Fradelizi, D. 1988. Generation of lymphokine-activated killer cells: synergy between tumor necrosis factor and interleukin 2. *Proc. Natl. Acad. Sci. USA 85:6875.*

270. Peppoloni, S., Bossù, P., Boraschi, D., and Tagliabue, A. 1989. A short synthetic peptide fragment of human interleukin-1ß increases both human and murine natural killer activity. *Nat. Immun. Cell Growth Regul. 8:10.*

271. Kobayashi, M., Fitz, L., Ryan, M., Hewick, R.M., Clark, S.C., Chan, S., Loudon, R., Sherman, F., Perussia, B., and Trinchieri, G. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF) a cytokine with multiple biologic effect on human lymphocytes. *J. Exp. Med. 170:827.*

272. D'Andrea, A., Rengaragi, M., Valiante, N.M., Chehimi, J., Kubin, M., Aste, M., Chan, S.H., Kobayashi, M., Young, D., Nickbarg, E., Chizzonite, R., Wolf, S.F., and Trinchieri, G. 1992. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J. Exp. Med. 176:1387.*

273. Preussia, B., Chan, S.H., D'Andrea, A., Tsuji, K., Santoli, D., Posposil, M., Young, D., Wolf, S.F., and Trinchieri, G. 1992. Natural killer (NK) cell stimulatory factor or IL-12 has differential effects on the proliferation of TCRα/ß, TCRγ/δ T lymphocytes and NK cells. *J. Immunol. 149:3495.*

274. Matis, L.A., Cron, R.G., and Bluestone, J.A. 1987. Major histocompatibility complex-linked specificity of gamma/delta receptor-bearing T lymphocytes. *Nature 330:262.*

275. Ciccone, E., Viale, O., Pende, D., Malnati, M., Battista-Ferrara, G., Barocci, S., Moretta, A., and Moretta, L. 1988. Antigen recognition by human T cell receptor-gamma-positive lymphocytes: specific lysis of allogeneic cells after activation in mixed lymphocyte culture. *J. Exp. Med. 167:1517.*

276. Porcelli, S., Brenner, M.B., and Band, H. 1991. Biology of the human γδT-cell receptor. *Immunol. Rev. 120:137.*

277. Ferrini, S., Zarcone, D., Viale, M., Cerutti, G., Millo, R., Moretta, A., and Grossi, C.E. 1989. Morphologic and functional characterization of human peripheral blood T cells expressing the T cell receptor γ/δ. *Eur. J. Immunol. 19:1183.*

278. Mukherji, B., Guha, A., Loomis, R., and Ergin, M.T. 1987. Cell-mediated amplification and down regulation of cytotoxic immune response against autologous human cancer. *J. Immunol. 138:1987.*

279. Cron, R.Q., Gajewski, T.F., Sharrow, S.O., Fitch, F.W., Matis, L.A., and Bluestone, J.A. 1989. Phenotypic and functional analysis of murine $CD3^+$,$CD4^-$,$CD8^-$ TCR-γ/δ-expressing peripheral T cells. *J. Immunol. 142:3754.*

280. Ferrini, S., Bottino, C., Biassoni, R., Poggi, A., Sekaly, R.P., Moretta, L., and Moretta, A. 1987. Characterization of $CD3^+$, $CD4^-$, $CD8^-$ clones expressing the putative T cell receptor γ gene product: analysis of the activation pathways leading to interleukin 2 production and triggering of the lytic machinery. *J. Exp. Med. 166:277.*

281. Mingari, M.C., Ferrini, S., Pende, D., Bottino, C., Prigione, I., Moretta, A., and Moretta, L. 1987. Phenotypic and functional analysis of human $CD3^+$ and $CD3^-$ clones with "lymphokine-activated killer" (LAK) activity. Frequent occurrence of $CD3^+$ LAK clones which produce IL-2. *Int. J. Cancer 40:495.*

282. Moingeon, P., Ythier, A., Goubin, G., Faure, F., Nowill, A., Delmon, L., Rainaud, M., Forestier, F., Daffos, F., Bohuon, C., and Hercend, T. 1986. A unique T-cell receptor complex expressed on human fetal lymphocytes displaying natural-killer-like activity. *Nature 323:638.*

283. Brenner, M.B., Mclean, J., Scheft, H., Riberdy, J., Ang, S.-L., Seidman, J.G., Devlin, P., and Krangel, M.S. 1987. Two forms of the T-cell receptor γ protein found in the peripheral blood cytotoxic T lymphocytes. *Nature 325:689.*

284. Grossi, C.E., Coccone, E., Migone, N., Bottino, C., Zarcone, D., Mingari, M.C., Ferrini, S., Tambussi, G., Viale, O., Casorati, G., Millo, R., Moretta, L., and Moretta, A. 1989. Human T cells expressing the γ/δT-cell receptor (TcR-1): $C_\gamma 1$- and $C_\gamma 2$-encoded forms of the receptor correlate with distinctive morphology, cytoskeletal organization and growth characteristics. *Proc. Natl. Acad. Sci. USA 86:1619.*

285. Bluestone, J.A., Cron, R.Q., Barrett, T.A., Houlden, B., Sperling, A.I., Dent, A., Hedrick, S., Rellahan, B., and Matis, L.A. 1991. Repertoire development and ligand specificity of murine TCRγ/δ cells. *Immunol. Rev. 120:5.*

286. Groh, V., Porcelli, S., Fabbi, M., Lanier, L.L., Picker, L.J., Anderson, T., Warnke, R.A., Bhan, A.K., Strominger, J.L., and Brenner, M.B. 1989. Human lymphocytes bearing T cell receptor γ/δ are phenotypically diverse and evenly distributed throughout the lymphoid system. *J. Exp. Med. 169:1277.*

287. Van Kaer, L., Wu, M., Ichikawa, Y., Ito, K., Bonneville, M., Ostrand-Rosenberg, S., Murphy, D.B., and Tonegawa, S. 1991. Recognition of MHC TL gene products by γ/δ T cells. *Immunol. Rev. 120:89.*

288. Ferrick, D.A., Sydora, B., Wallace, V., Gemmeli-Hori, L., Kronenberg, M., and Mak, T.W. 1991. Self-reactive γ/δ T lymphocytes: implications for cell ontogeny and reactivity. *Immunol. Rev. 120:51.*

289. Bluestone, J.A., Cron, R.Q., Cotterman, M., Houlden, B.A., and Matis, L.A. 1988. Structure and specificity of T cell receptor γ/δ on major histocompatibility complex antigen-specific $CD3^+$, $CD4^-$, $CD8^-$ T lymphocytes. *J. Exp. Med. 168:1899.*

290. Ito, K., Van Kaer, L., Bonneville, M., Hsu, S., Murphy, D.B., and Tonegawa, S. 1990. Recognition of the product of a novel MHC TL region gene (27^b) by a mouse γ/δ T cell receptor. *Cell 62:549.*

291. Vidovic, D., Roglic, M., McCune, K., Guerder, S., MacKay, C., and Dembic, Z. 1989. Qa-1 restricted recognition of foreign antigen by a γ/δ T-cell hybridoma. *Nature 340:646.*

292. Janeway, C.A., Jones, B., and Hayday, A. 1988. Specificity and function of T cells bearing γ/δ receptors. *Immunol. Today 9:73.*

293. Janeway, C.A. 1988. Frontiers of the immune system. *Nature 333:804.*

294. Maki, R.G., Old, L.J., Srivastava, P.K. 1990. Human homologue of murine tumor rejection antigen gp96: 5′-regulatory and coding regions and relationship to stress-induced proteins. *Proc. Natl. Acad. Sci. USA 87:562.*

295. Fisch, P., Malkovsky, M., Kovats, S., Sturm, E., Braakman, E., Klein, B.S., Voss, S.D., Morrissey, L.W., DeMars, R., Welch, W.J., Bolhuis, R.L.H. and Sondel, P.M. 1990. Recognition by human $V_\gamma 9/V_\delta 2$ T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. *Science 250:1269.*

296. Henkart, P.A. 1985. Mechanism of lymphocyte-mediated cytotoxicity. *Ann. Rev. Immunol. 3:31.*

297. Harris, D.T., Jaso-Friedmann, Devlin, R.B., Koren, H.S., and Evans, D.L. 1991. Identification of a evolutionarily conserved, function-associated molecule on human natural killer cells. *Proc. Natl. Acad. Sci. USA 88:3009.*

298. Harris, D.T., Jaso-Friedmann, Devlin, R.B., Koren, H.S., and Evans, D.L. 1992. The natural killer cell-like lytic activity expressed by cytolytic T lymphocytes is associated with the expression of a novel function-associated molecule. *Scand. J. Immunol. 35:299.*

299. Granger, G.A. and Kolb, W.P. 1968. Lymphocyte *in vitro* cytotoxicity: mechanisms of immune and non-immune lymphocyte mediated target L cell destruction. *J. Immunol. 101:111.*

300. Henkart, M.P. and Henkart, P.A. 1982. Lymphocyte mediated cytolysis as a secretory phenomenon. *Adv. Exp. Med. Biol. 146:227.*

301. Bykovskaja, S.N., Sergeev, A.V., Raushenbauch, M.O., and Bykovsky, A.F. 1980. The ultrastructure of the tubular complex in the cytoplasm of cytolytic T lymphocytes. *Scand. J. Immunol. 11:261.*

302. Henkart, P.A., Millard, P.J., Reynolds, C.W., and Henkart, M.P. 1984. Cytolytic activity of purified cytoplasmic granules from cytotoxic rat granular lymphocyte tumors. *J. Exp. Med. 160:75.*

303. Podack, E.R., Olsen, K.J., Lowrey, D.M., and Lichtenheld, M. 1988. Structure and function of perforin. *Curr. Topics Micro. Immunol. 140:11.*

304. Henkart, P. and Yue, C.C. 1988. The role of cytoplasmic granules in lymphocyte cytotoxicity. *Prog. Allergy 40:82.*

305. Tschopp, J. 1990. Perforin-mediated target cell lysis by cytolytic T lymphocytes. *Ann. Rev. Immunol. 8:279.*

306. Henkart, P.A., Berribi, G.A., Takayama, H., Munger, W.E. and Sitkovsky, M.V. 1987. Biochemical and functional properties of serine esterases in acidic cytoplasmic granules of cytotoxic T lymphocytes. *J. Immunol. 139:2398.*

307. Manyak, C.L., Norton, G.P., Lobe, C.G., Bleakley, R.C., Gershenfeld, H.K., Weissman, I.L., Kumar, V., Sigal, N.H., and Koo, G.C. 1989. IL-2 induces expression of serine protease enzymes and genes in natural killer and nonspecific T killer cells. *J. Immunol. 142:3707.*

308. Garcia-Sanz, J.A., MacDonald, H.R., Jenne, D.E., Tschopp, J., and Nabholz, M. 1990. Cell specificity of granzyme gene expression. *J. Immunol. 145:3111.*

309. Podack, E.R. 1988. Granule-mediated cytolysis of target cells. *Curr. Topics Micro. Immunol. 140:33.*

310. Hayes, M.P., Berrebi, G.A., and Henkart, P.A. 1989. Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme A. *J. Exp. Med. 170:933.*

311. Zunino, S.J., Bleackley, R.C., Martinez, J., and Hudig, D. 1990. RNKP-1, a novel natural killer-associated serine protease gene cloned from RNK-16 cytotoxic lymphocytes. *J. Immunol. 144:2001.*

312. Shi, L., Kraut, R.P., Aebersold, R., and Greenberg, A.H. 1992. A natural killer cell granule protein that induces DNA fragmentation and apoptosis. *J. Exp. Med. 175:553.*

313. Ostergaard, H.L., Kane, K.P., Mescher, M.F., and Clark, W.R. 1987. Cytotoxic T lymphocyte mediated lysis without the release of serine esterase. *Nature 330:71.*

314. Tirosh, R. and Berke, G. 1985. T-lymphocyte-mediated cytolysis as an excitatory process of the target: I. evidence that the target cell may be the site of Ca^{2+} action. *Cell. Immunol. 95:113.*

315. Berke, G. 1991. Lymphocyte-triggered internal target disintegration. *Immunol. Today 12:396.*

316. White, J., Blackman, M., Bill, J., Kappler, J., Marrack, P., Gold, D.P., and Born, W. 1989. Two better cell lines for making hybridomas expressing specific T cell receptors. *J. Immunol. 143:1822.*

317. Leo, O., Foo, M., Sachs, D.H., Samelson, L.E., and Bluestone, J.A. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA 84:1374.*

318. Goodman, T. and LeFrancois, L. 1989. Intraepithelial lymphocytes: anatomical site, not T cell receptor form, dictate phenotype and function. *J. Exp. Med. 170:1569.*

319. Farrar, J.J., Fuller-Farrar, J., Simon, P.L., Hilfiker, M.L., Stadler, B.M., and Farrar, W.L. 1980. Thymoma production of T cell growth factor (interleukin 2). *J. Immunol. 125:2555.*

320. Truitt, K.E., Larrick, J.W., Raubitschek, A.A., Buck, D.W., and Jacobson, S.W. 1984. Production of human monoclonal antibody in mouse ascites. *Hybridoma 3:195.*

321. Hoogenrad, N., Helman, T., and Hoogenrad, J. 1983. The effect of pre-injection of mice with pristane on ascites tumour formation and monoclonal antibody production. *J. Immunol. Meth. 61:317.*

322. Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth. 65:55.*

323. Meager A. 1987. Quantification of interferons by antiviral assays and their standardization, in Lymphokines and Interferons, eds. Clemens, M.J., Morris, A.G. & Gearing, A.J.H. IRL Press Oxford.

324. Spitalny, G.L., and Havell, E.A. 1984. Monoclonal antibody to murine gamma interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. *J. Exp. Med. 159:1560.*

325. Youngner, J.S., and Salvin, S.B. 1973. Production and properties of migration inhibitory factor and IFN in the circulation of mice with delayed hypersensitivity. *J. Immunol. 111:1914.*

326. Julius, M.H., Simpson, E., and Herzenberg, L.A. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol. 3:645.*

327. Brunner, K.T., Mauel, J., Cerottini, J-C., and Chapuis, B. 1968. Quantitative assay of the lytic action of immune lymphoid cells of ^{51}Cr-labelled allogeneic target cells *in vitro;* inhibition of isoantibody and by drugs. *Immunol. 14:181.*

328. Pross, H.F., Baines, M.G., Rubin, P., Shragge, P., and Patterson, M.S. 1981. Spontaneous human lymphocyte-mediated cytotoxicity against tumour target cells. IX. The quantitation of natural killer cell activity. *J. Clin. Immunol. 1:51.*

329. Ferrara, J.L.M., Guillen, F.J., van Dijken, P.J., Marion, A., Murphy, G.F., and Burakoff, S.J. 1989. Evidence that large granular lymphocytes of donor origin mediate acute graft-versus-host disease. *Transpl. 47:50.*

330. Clancy, J.Jr., Goral, J., Kovacs, E.J., and Ellis, T. 1989. Role of recombinant interleukin-2 (rIL-2) and large granular lymphocytes (LGLs) in acute rat graft-versus-host disease (GVHD). *Transpl. Proc. 21:88.*

331. Clancy, J., Mauser, L., and Chapman, A.L. 1983. Level and temporal pattern of naturally cytolytic cells during acute graft-versus-host disease (GVHD) in the rat. *Cell. Immunol. 79:1.*

332. Gartner, J.G., Merry, A.C., and Smith, C.I. 1988. An analysis of pulmonary natural killer cell activity in F_1-hybrid mice with acute graft-versus-host reactions. *Transplantation 46:879.*

333. Ortaldo, J.R., Herz, R., Pestka, S., and Herberman R.B. 1985. Stimulation of NK activity by interferons and interleukins. *J. Leuk. Biol. 38:55.*

334. Rowley, D.A. and Shah, P.D. 1986. The immunological meaning of Thy-1-negative NK cells. *Immunol. Today 7:196.*

335. MacDonald, G.C. and Gartner, J.G. 1991. Natural killer (NK) cell activity in mice with acute graft-versus-host reactions: Characterization of a Thy-1^+ NK-like cell with a broadened spectrum of lytic activity in the spleen and lymph nodes. *Scand. J. Immunol. 33:553.*

336. Cipriano, D., Faanes, R.B., and Merluzzi, V.J. 1989. Protection of mice against acute lethal Graft-versus-host disease by treatment with anti-asialo GM1 antibody: Alopecia dermatitis in long term survivors. *Transpl. 47:922.*

337. McGuinness, U.M. and Baines, M.G. 1989. Augmented Natural killer cell activity during neonatal graft-versus-host disease of predominantly donor origin. *Immunobiol. 180:101.*

338. Fung, K.Y. and Sabbadini, E. 1976. Cytotoxicity in graft-versus-host reaction: II. Lysis of target cells of parental genotype by F1 hybrid macrophages. *Transpl. 22:449.*

339. Lymphocytes and effector cells, in Practical Immunology, eds. Hudson L, Hay FC. Blackwell Scientific Publications, 1989.

340. MacDonald, G.C. and Gartner, J.G. 1991. The host/donor origin of cells mediating NK and NK-like cytotoxic activity in F_1-hybrid mice with acute graft-versus-host reactions. *Transpl. 52:141.*

341. van de Griend, R., Bolhuis, R.L.H., Stoter, G., and Roozemond, R.C. 1987. Regulation of cytolytic activity in $CD3^-$ and $CD3^+$ killer cell clones by monoclonal antibodies (anti-CD16, anti-CD2, anti-CD3) depends on subclass specificity of target cell IgG-FCR. *J. Immunol. 138:3137.*

342. Stankova, J., Hoskin, D.W., and Roder, J.C. 1989. Murine anti-CD3 monoclonal antibody induces potent cytolytic activity in both T and NK cell populations. *Cell. Immunol. 121:13.*

343. Steeg, P.S., Moore, R.N., Johnson, H.M., and Oppenheim, J.J. 1982. Regulation of murine macrophage Ia antigen by a lymphokine with immune interferon activity. *J. Exp. Med. 156:1780.*

344. Phillips, J.H., Weiss, A., Gemlo, B.T., Rayner, A.A., and Lanier, L.L. 1987. Evidence that the T cell antigen receptor may not be involved in cytotoxicity mediated by γ/δ and $\alpha/ß$ thymic cell lines. *J. Exp. Med. 166:1579.*

345. Pollard, M., Chang, C.F., and Srivastova, K.K. 1976. The role of microflora in the development of graft-versus-host disease. *Transpl. Proc. 8:533.*

346. van Bekkum, D.W., Roodenburg, J., Heidt, P.J., and van der Waaij, D. 1974. Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. *J. Natl. Cancer Inst. 52:401.*

347. Jones, J.M., Wilson, R., and Bealmear, P.M. 1971. Mortality and gross pathology of secondary disease in germfree mouse radiation chimeras. *Rad. Res. 45:577.*

348. van Bekkum, D.W. and Knaan, S. 1977. Role of bacterial microflora in development of intestinal lesions from graft-versus-host reaction. *J. Natl. Cancer Inst. 58:787.*

349. Storb, R., Prentice, R.L., Buckner, C.D., Clift, R.A., Applebaum, F., Deeg, J., Doney, K., Hansen, J.A., Mason, M., Sanders, J.E., Singer, J., Sullivan, K.M., Witherspoon, R.P., and Thomas, E.D. 1983. Graft-versus-host disease and survival in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. Beneficial effect of a protective environment. *New Engl. J. Med. 308:302.*

350. Ortaldo, J.R., Mason, A.T., Gerard, J.P., Henderson, L.E., Farrar, W., Hopkins III, R.F., Herberman, R.B., and Rabin, H. 1984. Effects of natural and recombinant IL 2 on regulation of IFNγ production and natural killer activity: lack of involvement of the TAC antigen for these immunoregulatory effects. *J. Immunol. 133:779.*

351. Doherty, P.C., Allan, W., and Eichelberger, M. 1992. Roles of α/ß and γ/δ T cell subsets in viral immunity. *Ann. Rev. Immunol. 10:123.*