Role of CD44, Fas Ligand, and Perforin in the Cytotoxicity Mediated by Natural Killer Cells

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(ABSTRACT)

Two important mechanisms of lymphocyte-mediated cytotoxicity, one perforin based and the other Fas ligand (FasL) based, have been characterized recently. It has also been shown that CD44, an adhesion molecule, can participate in signaling cytotoxic activity of cytotoxic T lymphocytes (CTLs). In the current study we tested the hypothesis that activation of natural killer (NK) or lymphokine activated killer (LAK) cells induces the expression of FasL, perforin, and CD44 which together contribute towards increased cytolytic activity. To this effect, we used wild-type mice, perforin-knockout mice, and mice lacking a functional FasL. We observed that both interleukin-2 (IL-2) and Poly I:C triggered NK/LAK cells to lyse targets through the perforin- and FasL- pathways. In addition, Fas^+ tumor targets were more susceptible to lysis by poly I:C and IL-2 activated NK/LAK cells when compared to Fas^- targets. Furthermore, Fas^- tumor cells injected subcutaneously into syngeneic mice could grow and induce tumors, whereas, Fas^+ tumors were rejected. IL-2 treatment increased the CD44 expression on NK cells, which was responsible for the lysis of endothelial cells through its ligand, hyaluronate. Upregulation of perforin and FasL in activated NK/LAK cells may explain why such cells can kill a wide variety of tumor cells efficiently. On the other hand, activated NK/LAK cells express increase increased levels of CD44 and use this molecule to mediate cytotoxicity of endothelial cells, which may account for the vascular leak seen during IL-2 therapy.

DEDICATION

I would like to dedicate this work to my parents, Edward and Judith Bradley, without whose love and support this work could not have been completed.

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LIST OF ABBREVIATIONS

Ag	antigen
Ab	antibody
ADCC	antibody dependent cell-mediated cytotoxicity
Ca	Calcium
CD	cluster of differentiation
Cr	Chromium
CTL	cytotoxic T lymphocyte
E:T	effector: target
FITC	fluoroisothiocyanate
FcR	Fc receptor
gld	generalized lymphoproliferation
HEV	high endothelial venule
HIV	human immunodeficiency virus
Ig	immunoglobulin
IFN	interferon
IL	interleukin
KIL	killer inhibitory receptors
LAK	lymphokine activated killer
LFA	lymphocyte function associated antigen
lpr	lymphoproliferative
mAbs	monoclonal antibodies
MHC	major histocompatibility complex
NK	natural killer
PMN	polymorphonuclear neutrophils
pgp-1	phagocytic glycoprotein-1
Poly I:C	polyinosinic-polycytidylic acid
TME	transformed murine endothelial
TNF	tumor necrosis factor

CHAPTER 1.0: BACKGROUND AND SPECIFIC AIMS

1.1 INTRODUCTION

Immunology, which is the study of the immune system, is responsible for the body's defense against outside invaders, whether they be bacteria, viruses, fungi, or parasites (Janeway and Travers, 1994). The body has outer defenses against pathogens such as the skin. However, infectious agents can evade these defenses and enter the body where they can live and replicate, either intracellularly or extracellularly (Roitt, *et. al*., 1996).

When an outside invader enters a body the immune system initiates an immune response. That is, the immune system must recognize the invader as foreign, and it must initiate a response to destroy it. There are two kinds of immune responses by which this can take place: either an innate immune response, or an adaptive immune response (Roitt, *et. al.,* 1996). The former does not rely on prior exposure to the pathogen, is non-specific, and involves the pathogen being digested by phagocytes (JanewayandTravers,1994) such as macrophages, monocytes and polymorphonuclear neutrophils (Roitt, *et.al.,* 1996). The latter is specific, involves lymphocytes, and the immune response against this particular pathogen will improve with each exposure. Thus, adaptive immunity can last for a lifetime (Roitt, *et. al*., 1996).

Both kinds of immune responses are not completely independent of one another. Phagocytes can process parts of a pathogen and present it to a T cell. This is known as antigen presentation. Also, lymphocytes can release cytokines which can stimulate phagocytes. In addition, antibodies secreted by B cells can coat pathogens which will enhance recognition by phagocytes.

Phagocytes are divided into the mononuclear phagocytes and polymorphonuclear neutrophils (PMN). Mononuclear phagocytes are derived from bone marrow stem cells. As mentioned earlier, their primary purpose is to internalize and digest pathogens. They include Kupffer cells found in the liver, Synovial A cells found in the synovial cavity, and monocytes found in the blood. Monocytes eventually migrate to the tissues and evolve into macrophages which in turn can present antigens to T cells. PMNs are similar to monocytes but are short lived and make up the majority of the leukocytes in the blood (Roitt, *et. al.,* 1996).

There are three kinds of lymphocytes: B cells, T cells, and natural killer cells. B cells are derived from, and mature in the bone marrow. If a B cell comes into contact with an antigen for which it is genetically programmed to express a surface receptor, it will multiply and become a plasma cell. These plasma cells, in turn, will secrete antibodies specific for the original antigen recognized by the B cell (Roitt, *et. al*., 1996).

T cells are also derived from bone marrow, however they reach maturity in the thymus. There are two types of T cells: helper T cells and cytotoxic T cells (Roitt, *et. al.,* 1996). Helper T cells express the adhesion molecule CD4 but not CD8. Thus they are referred to as $CD4^+CD8^-$. Helper T cells are activated after they come in contact with antigens bound to MHC II molecules from antigen presenting cells. Activated helper T cells secrete cytokines which can either augment or suppress immune functions. For example, they can secrete IL-2 which leads to activation and proliferation of cytotoxic T cells and NK cells (Sites and Terr, 1996).

Cytotoxic T cells are $CD4^-CD8^+$. They directly destroy cells that are recognized as foreign: either virus infected cells or allogenic cells. They recognize this by perceiving a foreign antigen in association with MHC I molecules on the target cell surface (Sites and Terr, 1996).

The final group of immune cells are natural killer (NK) cells. These are large granular lymphocytes which lyse tumor and virus infected cells in a non-specific manner. They also can kill target cells covered with specific antibodies (Roitt, *et. al.,* 1996).

1.2: NATURAL KILLER CELL ACTIVITY

Natural killer (NK) cells have been described as large granular lymphocytes (LGL) (Hori, *et. al.,* 1988) which lyse certain tumor and virus infected cells, derived from normal, unimmunized hosts. In addition, they are not restricted by their major histocompatability complex (Lanier, *et. al.,* 1986). Human NK cells are $CD16^+$, $CD56^+$, $CD3\gamma/\delta^-$, $TCR\alpha/\beta^-$, and TCR γ/δ^-. Murine NK cells are ASGM 1^+ and $NK1.1/NK2.1^+$. They're found in 5-10% of peripheral blood; up to 25% of the spleen; and in smaller quantities in the lungs, liver, GI tract, and peripheral lymphoid organs (Storkus, *et. al.,* 1991). They are present in both SCID mice and athymic nude mice (Moretta, *et. al.,* 1994). Because NK cells lack transcripts for the α and β chain of T cells (Tutt, *et. al.,* 1986), and the T cell γ chain gene they do have is not expressed (Lanier, *et. al.,* 1986), they are not

believed to be mature T cells, nor early cells committed to becoming T cells (Tutt, *et. al*., 1986). Human fetal NK cells, however, do express CD3γ,δ,ε, and ζ which they lose upon maturation (Spits, *et. al.,* 1995). It has been shown that immature $CD7^{+}3^{-}4^{-}8^{-}$ cells derived from human thymus can become either T cells or NK cells. These cells become NK cells ($CD3^{-}CD16^{+}$) when cultured with irradiated H9 leukemia cells and IL-2; thus showing that NK cell precursors are in the thymus. Similar results were obtained when lymphoid cells derived from liver were used (Moretta, *et. al.,* 1994). Natural killer cells have been shown to both augment the growth of human granulocyte-macrophage and erythroid progenitor cells *in vitro* (Gewirtz, *et al.,*. 1987).

NK cells have been shown to lyse certain targets in an MHC-unrestricted way. Sensitivity to NK cells has an inverse relationship with MHC class I molecules; for example, cells lacking class I molecules such as lymphoblasts from MHC class I deficient mice, and from bone marrow, are sensitive to NK cells. This is explained by the missing-self hypothesis which states that autologous cells with a missing or changed MHC molecule can be lysed by NK cells. One model to explain this says that unknown antigens are perceived by NK cells, and that these unknown antigens mask the class I molecules. The other model explains that engagement of certain receptors on NK cells with Class I molecules prevents lytic activity (Raulet, *et. al.,* 1992).

However, more recently it has been shown that there is a balance between receptors that activate and those that inhibit. As mentioned, NK cell target sensitivity is inversely proportional to MHC I expression. That is, the less MHC a target expresses, the more likely it is to be killed by NK cells. This is because MHC I in association with an antigen is expressed on the cell surface where it can be recognized by CTLs (Raulet and Held, 1995).

Inhibition takes place when MHC I comes in contact with its receptor, Ly-49 in mice and killer inhibitory receptors (KIR), specifically, p58 in humans (Raulet and Held, 1995: Wang, *et. al.,* 1997; Rojo, *et. al.,* 1997). Ly-49 is a type II integral membrane protein belonging to the C-type lectin family, whereas KIRs are transmembrane proteins that are of the Ig superfamily. Recently it has been found that murine NK cells express gp49B, also called gp49B1, which is a member of the Ig superfamily and is structurally homologous to KIR in humans. Thus, it appears that mouse NK cells bear two distinctive receptors that serve as coreceptors in mediating inhibition (Rojo, *et. al.,* 1997; Wang, *et. al.,* 1997).

After MHC I is engaged a signal is then sent to halt the lytic pathway. Alternatively, it has been found that NKR-P1, which is closely linked to Ly-49, can activate NK cells to destroy some tumor targets (Raulet and Held, 1995). This has been shown by the fact that HLA class I Abs and $F(ab')_2$ fragments prevent the inhibition signal from being sent from target to NK cell (Kaufman, *et. al.,* 1993). Furthermore, NK cells can lyse targets coated with antigen via the Fc receptor. Interestingly, inhibitory signals tend to override activating signals which might mean that a kind of balance is required (Raulet and Held, 1995).

In addition, resistant targets can become more sensitive to NK cells if these targets decrease their surface cell expression of MHC I. Also, NK cells can lyse normal MHC I

expressing targets if in the MHC I antigen binding cleft there is a foreign antigen, or they express alloantigens (Kaufman, *et. al.,* 1993).

In addition to lysing sensitive tumor targets, NK cells can lyse certain virus infected cells. However, it does not use the same intracellular signaling mechanism used for tumor cells, as there is no calcium signaling. Because of this, viruses may use different receptors on NK cells from tumor cells, or they may be activated by virus induced cytokines. Virus activation of NK cells has been shown to use either α-interferon independent or α–interferon dependent mechanisms (Paya, *et. al.,* 1990).

It is not clear how NK cells interact with bacteria. Activity has been shown against gram-positive and gram-negative kinds. NK cells will react with some bacteria better than others, or not at all. Tumor target lysis is enhanced in the presence of bacteria, or bacterial cell walls. Because bacterial activation of NK cells does not cause any change in the Golgi apparatus, an extracellualr mechanism of killing is suggested (Garcia-Penarruba, *et. al.,* 1989). NK cells have also been shown to present certain soluble protein antigens to T cells (Roncarolo, *et. al.,* 1991).

Cytokines and other factors have been shown to regulate NK cells. Natural Killer Cell Stimulatory Factor, now called IL-12 (Ling, *et. al.,* 1995), isolated from B lymphoblastoid cells, increases cytolytic activity in fresh NK cells. It increases adhesion molecule expression important in lysis, as well as causes NK cell proliferation in the presence of IL-2 (Robertson, *et. al.,* 1992). Culturing NK cells in the presence of IL-2 leads to NK cell proliferation and increases granule formation (Zarcone, *et. al.,* 1987). It can also cause $NK1.1^-$ cells to differentiate into $NK1.1^+$ cells (Koo, *et. al.,* 1986). Culturing with IFN-g does not affect cell size or shape, but does alter the structure of their lytic granules (Zarcone, *et. al.,* 1987), and has been shown to reduce tumor cell sensitivity to NK cells (DeFries and Golub, 1988). Several chemokines, such as lymphotactin, IL-8, MCP-1, -3, MIP-1a, and IP-10, have been shown to be chemotactic for both freshly isolated and activated NK cells (Giancarlo, *et. al.,* 1996). Histamine, in the presence of monocytes, has also been shown to increase the activity of NK cells (Hellstrand and Hermodsson, 1990). Phospholipase C can cause an increase in intracellular calcium and activates protein Kinase C. Protein Kinase C can be activated along with tyrosine phosphorylation of certain NK cell proteins, when they are cultured with IL-2, phorbol ester, and diacylglycerol (Einspahr, *et. al.,* 1990). Flurone acetic acid also increases the activity of NK cells, especially when they are added to rIL-2, IFN-α, or IFN–β (Hornung, *et. al.,* 1988). rIL-4 has no effect on resting NK cells and can inhibit the lysis of targets by stimulated NK cells. It also blocks NK cell proliferation (Nagler, *et. al.,* 1988). NK cell-mediated cytotoxicity activity has been shown to be inhibited by activation of the cAMP-dependent second messenger pathway (Windebank, *et. al.,* 1988). NK cell function has also been shown to be inhibited by prostoglandin E_2, reactive O_2 metabolites, and other heat stable factors produced by macrophages and PMNs (Thiele and Lipsky, 1985). Reduced NK cell activity is also seen in beige mutant mice. They have a disease similar to Aedichi-Higoshi Syndrome in humans, resulting in altered cell granules; hence the decrease in NK cell function.

NK cells and T cells also interact. T cells can downregulate NK activity by producing cytokines such as IL-4 and TGF-β. On the other hand, direct control between NK cells and T cells via CD56 on NK cells is required for T cell proliferation and differentiation. Without NK cells present, T cells will only proliferate (Kos and Engleman, 1996).

1.3: NK CELL ACTIVATION AND ADHESION MOLECULES

NK cells express various adhesion molecules or receptors through which they adhere to targets or transmit signals for activation. This is known as antibody dependent cell cytotoxicity (ADCC). Many of these, such as NK1.1 are expressed solely by NK cells and can be used to distinguish them from other cells (Lanier, *et. al.,* 1986). Some of these adhesion molecules such as CD16, LFA and CD2 stimulate NK cell activity (Moretta, *et. al.,* 1989; Storkus and Dawson, 1991; Vivier, *et. al.,* 1991). CD16 is the receptor for the Fc portion of IgG. IL-2 initiates NK activity by binding to the two subunit compound of p55 (CD25/Tac) and p75 (Phillips, *et. al.,* 1989). Redirected lysis is also observed by CD69, whose gene can be mapped to the NK-gene complex (Testi, *et. al.,* 1994). CD27/CD70 interaction has also been found to mediate NK cell cytotoxicity (Yang, *et. al.,* 1996). CD56 on the other hand, mediates no lysis, and therefore only aids in binding target and effector cell (Storkus and Dawson, 1991).

Also, IL-2 can either decrease or increase certain adhesion molecule expression on NK cells. For example, it has been shown that IL-2 decreases L-selectin through which NK cells bind to peripheral lymph nodes. However, IL-2 increases expression of CD44 and α_4/β_7 through which NK cells bind to mucosal HEVs (Uksila, J., *et. al.,* 1997).

Once activated by adhesion molecules or some other factor, a whole range of events can be set into motion. For example, activation through CD16 causes tyrosine phosphorylation of ζ. This in turn leads to calcium ion influx, phosphoinositol production, lymphokine gene expression, and target cell lysis (Vivier, *et. al.,* 1991; Edwards, 1992). It should be noted that these events do not occur if the tumor target is NK resistant (Kaufman, *et. al.,* 1993). CD2 also induces these events (Moretta, *et. al.,* 1989; Vivier, *et. al.,* 1991). Furthermore, activated cells can increase expression of some adhesion molecules, such as the transferrin receptor and NK1.1 (Newman, *et. al.,* 1984; Lanier, *et. al.,* 1986).

The lytic process can also be set into motion by adhesion molecules. This begins with target cell binding events. This is followed by post binding events, which include a calcium ion dependent lytic phase, and a calcium independent killer cell-independent lytic phase. Finally, there is a secretory process whereby materials are transferred from the NK cell to its target (Farram and Targan, 1982). This begins with granule and cytoskeletal rearrangement (Zarcone, *et. al.,* 1987). These granules contain perforin and granzymes.

Perforin (also called cytolysin, pore forming protein, and C9-related protein) lyses targets by forming transmembrane channels. It has been shown to mediate cytotoxicity of tumor cells and virally infected cells, as well as is seen in autoimmune reactions, and transplant rejection. It is encoded by a single gene which is homolgous among species. The perforin mRNA has a half life of two to three hours in humans and intermediate length in mice. However, its half life is reduced to about five minutes upon interaction with a target. Perforin is constitutively expressed by NK cells; this expression is not affected by IL-2, as it is with other cell types. Perforin and granzyme A are not regulated in a coordinated fashion, probably because they lack a similar promoter. It is not known if perforin and granzyme A are always regulated and secreted together (Podack, *et. al.,* 1991).

Grazyme A, also referred to as MTSP-1 and serine esterase, is one of seven such proteolytic enzymes which are genetically similar (Ebnet, *et. al.,* 1992). It cleaves BLT (N-α–benzyloxycarbonyl-L-lysine thiobenzyl ester) (Goldfarb, *et. al.,* 1992). Granzyme A is the only granzyme to have a disulfide linked homodimer (Ebnet, *et. al.,* 1992).

Perforin is glycosylated, therefore it must go through all three Golgi stacks, then it must leave through the trans Golgi network. Whereas granzyme A contains high levels of mannose carbohydrate and mannose-6-phosphate, and leaves the cis or medial stacks. The lysosomal mannose-6-phosphate dependent pathway is utilized by granzyme to get to the granule. This is not used by perforin (Podack, *et. al.,* 1991).

The granules containing perforin and granzyme A contain properties of secretory granules and lysosomes. Like lysosomes, they have an acidic pH, and mannose-6-phosphate for lysosomal targeting. Grazyme A uses the mannose-6-phosphate receptor to communicate with the endocytotic/edosomal pathway. This pathway allows membrane proteins (CD3, MHC, etc.) to go to the vesicular area of the granule to be displayed (Podack, *et. al.,* 1991).

Secretion of granules allows targeting via the vesicle associative complex, or by other membrane proteins. The perforin inserts into the membrane of the target. This is followed by polymerization of about twenty molecules. Conformational changes convert this into a tubular complex with an approximately 14nm transmembrane channel. This leads to cell lysis by osmosis and calcium influx. The target may attempt to repair itself by taking in extracellular fluid through pinocytosis. However, fluid between the target and effector cell contains lymphotoxin, TNF, and granzyme A, which contribute to DNA breakdown of the target pore formation and cytostasis (Podack, *et, al.,* 1991, Hudig, *et. al.,* 1993). Granzyme A may also break up basement membrane type IV collagen (Goldfarb, *et. al.,* 1992).

1.4: CD44 AND HYALURONATE

CD44 also known as Pgp-1, In(Lu) related p80, Hermes, ECMR III, Hutch 1 (Tan, *et. al.,* 1993), and hyaluronate receptor (Gunthert, 1993) is a 90 kDa protein involved in endothelial cell recognition, found on all mononuclear cells (Banchereau, *et. al.,* 1992). Specifically, it can be found on lymphocytes, monocytes, neutrophils, epithelial cells, glial cells, and fibroblasts (Dianzani and Malavasi, 1995). It has many isoforms with diverse molecular weights, and different functional roles (Tan, *et. al.,* 1993). In secondary lymphoid organs, lymphocytes, lymphocyte expression of CD44 is at a high density in the mantle zone and T cell areas; whereas it is less dense in the B cell germinal centers. This adhesion molecule regulates primary and metastatic tumor development *in vivo* as well as has a role in the development of B cells in bone marrow (Banchereau, *et. al.,* 1991). Antibodies against CD44 have shown a variety of results including: activation of T cells through CD2 or CD3 TCR; interference with lymphohematopoiesis in long term bone marrow cultures; production of monocyte TNF and IL-2; interfering with the adhesion of lymphoid cells; and increased CTL activity (Seth, *et. al.* 1991). In addition, mAbs to CD44 have been shown to cause homotypic aggregation; hyaluronate binding in some cell lines; and the release from monocytes of macrophage CSF, IL-2, and TNF. Possible ligands for CD44 include hyaluronic acid, extracellular matrix proteins (collagen types I and VI, and fibronectin), and mucosal addressins (Tan, *et. al.,*1993). It can also bind to MAdCAM-1 and endothelial proteoglycans. It has also been shown that T cells activated through CD44 bind to targets through LFA-1. In addition, CD44 can trigger Ca^{2+} influx (Dianzani and Malavasi, 1995).

The binding of CD44 to high endothelial venules (HEVs) can be disrupted by the addition of mAb Hermes-3 which is specific for the epitope on the membrane proximal region. Furthermore, the chondroitin sulfate side chain's ligand is fibronectin. Lastly, the NH_2-terminal region of CD44 is responsible for binding to hyaluronate (Gunthert, 1993). However, recent studies have shown that hyaluronate might not be the ligand for lymphocte CD44 to synoviocytes and endothelial cells (Brennan, *et. al.,* 1997).

Hyaluronate is part of the extracellular matrix and is a high mass polysaccharide. It plays a role in cell proliferation, migration, and differentiation, as well as it immobilizes water in the extracellular matrix. Binding to hyaluronate can induce cellular aggregation, lymphocyte activation, and secretion of cytokines (Gunthert, 1993).

CD44 has been shown to increase NK cell activity. Anti-CD44 mAbs S5 and IM7 were able to enhance NK cell activity in a dose dependent manner by recognizing a common epitope (Tan, *et, al.,* 1993). Also, activation through CD44 can increase expression of CD69, activate TNF-a secretion, and activate the protein tyrosine kinase dependent signaling pathway (Galandrini, *et. al.* 1996).

1.5: APOPTOSIS AND FAS-FAS LIGAND INTERACTION

Cell death occurs by two mechanisms: necrosis and apoptosis. The cause of necrosis is usually pathological in origin and comes about due to factors such as exposure to certain toxins, lytic viral infection, hyperthermia, hypoxia, complement attack, and injury (Nagata 1994). Apoptosis, or programmed cell death is characterized by chromatin condensation, blebs on the cell membrane, and oligonucleosome-length DNA fragmentation. The cell then shrinks in size (Berke, 1995). It loses plasma membrane microvilli (Nagata, 1994). The endoplamic reticulum dilates. And lastly, the cell breaks up into small membrane bound fragments or "apoptotic bodies" (Berke, 1995). The physiological reason for having such a system to eliminate those autoreactive cells missed in thymic selection. Apoptosis occurs during embryonic development, endocrine-dependent tissue atrophy, and regular tissue turnover. Tumor regression by NK cells, CTLs, TNF, lymphotoxin, UV irradiation, g-irradiation, and some drugs can occur by an apoptotic pathway.

Fas (APO-1, CD95) (Lynch, *et. al.,* 1995) is a 48 kd adhesion molecule that is related to the TNF receptor, and nerve growth factor family (Doherty, 1993; Nagata 1994). Like TNF, it can signal apoptosis. Fas is expressed at high levels on $CD4^{+}CD8^{+}$ thymocytes (Doherty, 1993) but not $CD4^{-}CD8^{-}$ thymocytes (Nagata, 1994). Fas is expressed at high levels on activated T and B cells; HTLV-1 transformed lymphoblastoid cells; HIV; human myeloid leukemia U937; human squamous carcinoma CHU-2; and SV40-transformed mouse macrophage BAM3 cells. INF-γ and TNF-α can upregulate Fas expression in certain cell lines (Nagata, 1994). The Fas dependent pathway is Ca^{2+} independent. Also, incubation of Fas expressing cells with mAbs to Fas results in apoptosis (Doherty, 1993). In fact, injection of mice with mAbs to Fas leads to death within 5 hours (Nagata, 1994).

The Fas ligand (FasL) has been shown to be a TNF-related type II membrane protein (Nagata, 1994). It expresses high cytotoxicity against targets expressing Fas (Nagata and Goldstein, 1995). It has been found to be expressed on activated T cells and NK cells (Arase, *et. al.,* 1995). Soluble FasL, mAbs to Fas, or direct interaction between cells expressing Fas and cells expressing FasL leads to apoptosis. Though this pathway is not Ca^{2+} dependent, recent studies indicate that Fas may signal ceramide (Nagata and Goldstein, 1995).

Fas and FasL play an important role in disease. This interaction is responsible for clonal deletion in the thymus. MRL *lpr/lpr* mice lack Fas, whereas MRL *gld/gld* mice lack a functional FasL. Mice from both of these strains accumulate lymphocytes and develop an autoimmune disease similar to systemic lupus erythmatosus. On the other hand, an overactive Fas-FasL system may be responsible for $CD4^{+}$ T cell elimination in HIV infection, as well as CTL mediated diseases of tissues that highly express Fas such as liver and lungs (Nagata and Goldstein, 1995). Recently it has been found that $CD4^{+}$ T helper cells can limit the growth of mature Ag activated $CD8^{+}$ cytotoxic T cells through Fas- FasL interactions (Piazza, *et. al.,* 1997).

1.6: SPECIFIC AIMS:

Natural killer cells constitute important effector cells of the immune system which participate in the killing of cancer cells and cells infected with viruses. Lymphokine activated killer (LAK) cells are NK/T cells activated with cytokines such as IL-2. Both NK and LAK cells mediate MHC-unrestricted lysis of target cells. Two important mechanisms of lymphocyte-mediated cytotoxicity have been discovered recently. They are the FasL based pathway and the perforin-based pathway. However it is not clear which of these pathways plays an important role in NK-mediated cytotoxicity.

Adhesion molecules either aid in the binding of the effector cell to the target cell, and or they can transmit a signal that sets into motion the lytic process. If an adhesion molecule's density on the cell surface is increased after activation, it is a good candidate for lysis activation. This may be true for CD44, which is expressed on all NK cells in addition to CTLs, for which it has been shown to activate the lytic pathway.

Incubation of NK cells *in vitro* with IL-2 has been shown to upregulate the lytic activity. Moreover, such cells mediate lysis of NK-resistant targets. In fact, this observation has been used clinically to treat cancer patients with IL-2. The exact mechanism by which IL-2 activates the NK cells is not clear. In the current study we tested the hypothesis that IL-2 may trigger FasL and/or perforin expression in NK cells which may enhance their lytic activity. We also tested the hypothesis that activation of NK cells through CD44 receptor may induce cytolytic activity in NK cells and that hyaluronate may serve as an important ligand for CD44.

Therefore in the current study we addressed the following specific aims:

1) To investigate the role of FasL and perforin in NK and LAK cell-mediated cytotoxicity using wild-type mice, FasL-mutant mice (*gld/gld*), and perforin knockout mice.

2) To investigate whether CD44, a widely distributed membrane glycoprotein, involved mainly in lymphocyte homing, participates in NK cell-mediated cytotoxicity.

CHAPTER 2.0: ROLE OF PERFORIN AND FasL IN THE LYTIC ACTIVITY OF NK CELLS

2.1: INTRODUCTION

Natural killer cells are large granular lymphocytes (Hori, *et. al.,* 1988) that lyse certain tumor and virus infected cells derived from normal unimmunized hosts. They are capable of carrying out antibody dependent cell cytotoxicity. In addition, they are MHC unrestricted. That is, NK cells are capable of lysing targets without recognizing a foreign antigen with MHC-encoded molecules (Lanier, *et. al.,* 1986). NK cells can also be activated by IL-2 and IFN-γ inducers such as poly I:C to mediate increased lysis of NK-sensitive targets, as well as kill a broader panel of target cells, including NK-resistant cells (Oehler, *et. al.,* 1978). Such cytolytic property has been designated, lymphokine-activated killer (LAK) cell activity (Rosenberg and Lotze, 1986).

There are two mechanisms of lymphocyte-mediated cytotoxicity. One is perforin-based and the other is Fas-based (Nagata and Goldstein, 1995). T cells and NK cells utilize both of these mechanisms. The first is the degranulation pathway which uses perforin possibly in combination with granzymes (Podack, 1991; Smyth and Trapani, 1995). The second is the Fas-based pathway in which the interaction between Fas-ligand (FasL) expressed on cytolytic lymphocytes and Fas on target cells triggers apoptosis and target cell death (Kagi, *et. al.,* 1996; Kojima, *et. al.,* 1994). Moreover, all cytotoxic activity measured in a 4 hour assay can be attributed to perforin and FasL (Glass, 1996; Lee, 1996).

Recently, it was demonstrated that the lytic activity of NK cells in perforin-deficient mice was dramatically impaired or nonexistent, thereby suggesting that perforin is a key effector molecule involved in NK cell-mediated cytotoxicity (Lowin, *et. al.,* 1994; Kagi, *et. al.,* 1994). However, NK cells from normal mice were shown to express FasL and possess the ability to kill target cells expressing Fas antigen (Arase, *et. al.,* 1995).

Although NK cells have been known to mediate increased lysis of target cells including those that are NK-resistant, when activated with IL-2 or IFN–γ, it is not clear whether such lytic activity results from upregulation of perforin and/or FasL-based cytotoxicity. Inasmuch as, IL-2 is used to activate LAK cells in the immunotherapy of certain types of cancer, (Rosenberg and Lotze, 1986) it is important to investigate whether such treatment augments both perforin and FasL-based pathways and therefore is effective against both Fas^{+} and Fas^{-} tumors.

In the current study we used perforin-deficient and FasL-defective mice to address the role of perforin and FasL in LAK cell-mediated cytotoxicity. Furthermore, we used Fas^{+} and Fas^{-} L1210 tumor cells to delineate the *in vivo* immunotherapeutic effect of IL-2. The data demonstrates that IL-2 and poly I:C, triggers the induction of both FasL- and perforin-based cytolytic activity. Also, normal mice could readily reject syngeneic Fas^{+} L1210 but not Fas^{-} L1210 tumor cells. However, following treatment with IL-2, the mice were able to reject Fas^{-} L1210 tumor cells.

2.2: MATERIALS AND METHODS

2.2.1: Mice

C57BL/6 +/+ (+/+, or wild-type) and DBA/2 mice were purchased from Charles River (Boston, MA). C57BL/6 *gld/gld* (*gld*) were purchased from Jackson Labs (Bar Harbor, ME) and Perforin Deficient mice were obtained from Dr. William Clark, UCLA, Los Angeles, CA, and bred in our facilities (Seth, *et. al*., 1988; Kakkanaiah, *et. al.,* 1991). Female *gld* and +/+ mice of 3-4 weeks and DBA/2 female retired breeders were used.

2.2.2: Cell Lines

The tumor cell lines used were as follows: YAC-1, a Moloney virus induced lymphoma sensitive to NK cells; P815, a mastocytoma resistant to NK cells; L1210 (Fas^{+} and Fas^{-}), an NK resistant DBA/2 derived mouse lymphoma transfected with sense and antisense Fas cDNA (Rouvier, *et. al.* 1993). All were maintained in RPMI (Sigma) supplemented by 10% fetal calf serum, as previously described (Seth, *et. al.* 1991). TME-3H3 (transformed murine endothelial), an endothelial cell line immortalized by SV40 and developed by A. Hamann (Harder, *et. al.,* 1991) was generously provided by J. Lesley, The Salk Institute, and maintained as mentioned elsewhere (O'Connell and Ediden, 1990).

2.2.3: NK cells

NK cells were purified as previously described (Nagarkatti, *et. al.,* 1988). Briefly, single cell suspensions of the spleen were prepared in RPMI-1640 medium supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA) (Hammond, *et. al.,* 1993) using an homogenizer (Stomacher, Tekmar Co., Cincinnatti, OH). Plastic adherence for one hour at 37° C, was used to deplete the macrophages. The cells were passed over nylon wool columns and the nonadherent cells were used as a source of spontaneous NK activity. To study the inducible NK activity, the nonadherent cells were cultured for approximately 48 hours with 1000 U/ml of IL-2 (kindly provided by Hoffman LaRoche, Nutley, NJ). In some experiments NK activity was induced by intraperitoneal injection of poly I:C (polyinosinic-polycytdylic acid; Sigma), at 15mg/kg suspended in PBS (Lowin, *et, al.,*1994; Kagi, *et. al.,* 1994). NK cells were activated *in vivo* by i.p. injection of 50,000 U of IL-2 b.i.d. for 5 days.

2.2.4: ^{51}Cr-Release Assay to Measure Cytotoxicity

The cytotoxicity mediated by NK cells was studied using ^{51}Cr-release assay (Seth *et. al.,* 1991). Target cells (YAC-1, P815, L1210 Fas$^+$, L1210 Fas$^-$, and TME-3H3) were labeled with100 µCi ^{51}Cr in the form of $Na_2{}^{51}CrO_4$, incubated at 37° C. washed three times then seeded in 96-well plates (Costar, Cambridge, MA) at 5 x 10^3 cells/well, along with varying numbers of effector cells. The plates were incubated at 37° C for 4 hours. Following the incubation, the plates were harvested with the Titertech collecting system (Skatron Inc., Sterling, VA). The amount of ^{51}Cr released by the target cells was measured using a g-counter (TmAnalytic, Elk Grove Village, IL). Percent cytotoxicity was calculated as: (experimental release - control release)/(total release - control release) X 100. In blocking studies, the assay was performed in the presence of EGTA (6mM) and $MgCl_2$ (3mM) or in the presence of 200ng/ml of anti-Fas mAbs (Jo2, purchased from Pharmingen, San Diego, CA). In these experiments, the control release was measured both in the presence or absence of the inhibitors to ensure that the Abs or reagents alone, did not alter the control release, which was usually less than 15%. Total release was measured by incubating target cells in the presence of 0.1% sodium dodecyl sulfate (SDS).

2.2.5: Administration of IL-2 in tumor-bearing mice

Ten DBA/2 mice were injected subcutaneously with either 1 x 10^6 L1210 Fas$^+$ or L1210 Fas$^-$ resuspended in 0.2 ml PBS. In each group, 5 were injected i.p. with 0.1 ml PBS (control) b.i.d. for 7 days. The other five mice in each group were injected i.p. with 50,000 U IL-2 (provided by Hoffman LaRoche) suspended in 0.1 PBS b.i.d. for 7 days. On day 14, the mice were sacrificed and examined for tumors. The tumors were weighed and the average weight was taken.

2.3: RESULTS

2.3.1: Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in wild-type, perforin-deficient and FasL-defective mice against YAC-1 tumor targets

Mice bearing the *gld* mutation have a functional defect in FasL (Takahashi, *et. al.,* 1994) and perforin-knockout mice are unable to produce perforin (Kagi, *et. al.,* 1994), therefore NK cells from *gld/gld* and perforin-deficient mice were compared with wild-type NK/LAK cells for spontaneous, poly i.c.-induced, and IL-2 induced lytic activity against NK sensitive YAC-1 target cells in a cytotoxicity assay (Figs. 2.1A, 2.1B, and 2.1C).

The data shown in Figs 2.1A, 2.1B, and 2.1C demonstrated that IL-2 activated NK cells showed maximum cytolytic activity against NK sensitive YAC-1 targets in all three groups of mice followed by poly I:C activated and spontaneous lytic activity. When individual cytolytic activities were compared, FasL-defective mice showed a similar degree of lysis when compared to the wild-type mice. However, perforin-deficient mice exhibited virtually no significant cytolytic activity. These data demonstrated that the spontaneous NK activity is primarily perforin based. The fact that *gld/gld* mice had similar levels of spontaneous NK activity as the wild-type mice, suggested that FasL is not critical during spontaneous lysis. When spontaneous, poly I:C, and IL-2 activated NK/LAK cell activity was compared in the three groups of mice, (Figs. 2.2A, 2.2B, and 2.2C) it was noted that the wild-type mice exhibited the highest levels of cytotoxicity followed by the *gld/gld* mice and perforin-deficient mice. These data suggested that activation with poly I:C or IL-2 triggers both FasL- and perforin-based pathways. However, the fact that perforin-deficient mice had lower levels of cytotoxic activity when compared to the *gld/gld* mice, suggested that perforin may be more effective against YAC-1 targets than FasL.

To further investigate this, other cytotoxicity assays were performed. Monoclonal antibodies (mAbs) to Fas (Jo2) were added to see if blocking would occur. Also, EGTA and $MgCl_2$ were added in some experiments as this inhibits Ca^+ influx which is necessary for the perforin dependent pathway. As seen in figs. 2.3A, 2.3B, and 2.3C, mAbs to Fas had little effect in blocking NK cells from all three strains from lysing Yac-1 tumor targets. However, the addition of EGTA and $MgCl_2$ severely inhibited all NK activity. This suggests that spontaneous lysis of NK cells is dependent on the perforin dependent pathway rather than the Fas-FasL pathway.

EGTA completely blocked the IL-2 induced NK activity from *gld/gld* mice (Fig. 2.4A), and anti-Fas antibodies blocked the cytotoxicity in perforin-deficient mice (Fig.2.4B), thereby demonstrating that these were the only two cytolytic pathways that were used by the NK cells.

2.3.2: Spontaneous, Poly I:C-activated, and IL-2 activated NK/LAK activity in wild-type, perforin-deficient, and FasL-defective mice against P815 targets

We next looked at NK activity against the NK resistant line, P815. As seen in Figs. 2.5A, 2.5B, and 2.5C, IL-2 activated NK/LAK cells from both wild-type and *gld/gld* mice lysed P815 tumor targets to a similar degree, which was greater than that seen in perforin-deficient NK cells. Furthermore, lysis of targets by poly I:C. activated NK cells and spontaneous NK cells was less than 10%. These data indicate that only IL-2 activated NK cells were able to lyse P815 NK resistant tumor targets and that P815 cells were more sensitive to perforin than FasL.

As seen with YAC-1 cells, when cytotoxicity was carried out in the presence of mAbs to Fas, lysis was slightly inhibited in wild-type and perforin-deficient NK cells, and not in *gld/gld* NK cells which was expected as *gld/gld* NK cells would not mediate FasL-based cytotoxicity (Figs. 2.6A, 2.6B, and 2.6C). Again EGTA and $MgCl_2$ inhibited lysis in all cell lines. Again these data suggests that both the perforin dependent and Fas-FasL pathway are both utilized by NK cells in the cytotoxicity of tumor targets, although perforin may play a major role.

2.3.3: NK/LAK cell-mediated cytotoxicity against Fas^+ and Fas^- tumor targets in wild-type, perforin-deficient and FasL-defective mice

To exclude the possible variations seen in target cell-susceptibility and to address the role of Fas, we used L1210 tumor cells which had been transfected with Fas-sense (Fas^+) or antisense (Fas^-). As seen in Figs. 2.7A, 2.7B, and 2.7C, NK cells from wild-type and *gld/gld* mice were able to mediate efficient lysis of L1210 (Fas^-) target cells only upon activation through IL-2. In fact, NK cells from *gld/gld* mice mediated increased cytotoxicity when compared to the wild type mice; the reason for which is not clear. No lysis was observed by perforin-deficient NK/LAK cells. Thus any cytotoxicity carried out by NK/LAK cells on these Fas^- tumor targets must be through the perforin pathway.

Both IL-2 activated wild-type and *gld/gld* NK/LAK cells were able to mediate lysis of L1210 (Fas^-) to a similar degree (Figs. 2.8A, 2.8B, and 2.8C). The addition of mAbs to Fas had no effect as L1210 does not express Fas. Since the only way for these cells to lyse their targets was through the perforin pathway, the addition of EGTA and $MgCl_2$ completely inhibited all lysis. Furthermore, perforin deficient NK cells could not lyse L1210 (Fas^-) targets at all as both the perforin dependent and Fas-FasL pathways could not be utilized.

When L1210 (Fas^+) targets were similarly tested, it was noted that they were resistant to spontaneous killing by the NK cells similar to L1210 (Fas^-) targets (Figs. 2.9A, 2.9B, and 2.9C). Interestingly, when activated with poly I:C, the Fas^+ targets became susceptible to cytotoxicity. This cytotoxicity was both FasL- and perforin-based because both *gld/gld* and perforin-knockout mice exhibited weak cytotoxicity and that wild-type mice had higher levels of cytotoxicity. Similar results were also seen following IL-2 activation of NK cells against L1210 (Fas^+) and (Fas^-) targets. When L1210 (Fas^+) and (Fas^-) targets were compared, Fas^+ targets were more susceptible to lysis following poly I:C or IL-2 activation of NK cells, thereby suggesting that activation of NK cells triggers not only the perforin-based, but also the FasL-based pathway.

2.3.4: IL-2 activated NK cells lyse endothelial cells only through the perforin-dependent pathway

We next looked at the ability of NK cells to lyse transformed murine endothelial cells (TME-3H3) (Figs 2.10A, 2.10B, and 2.10C). This was done because NK cells have been implicated as lysing endothelial cells when large doses of IL-2 are administered in immunotherapy of cancer. This leads to what is known as vascular leak syndrome (VLS). Wild-type NK/LAK cells were able to lyse TME-3H3 targets upon activation by IL-2, and to a lesser extent upon activation by poly i.c. This was also seen as the case when *gld/gld* NK/LAK cells were used. Lysis by perforin-deficient NK cells both activated by IL-2 and perforin was less than 10%. Also, there was no spontaneous lysis by NK/LAK cells. These data suggest that endothelial cells can indeed be killed by activated NK/LAK cells, especially IL-2 activated NK/LAK cells. Furthermore, this lysis appears to be dependent almost entirely upon the perforin dependent pathway.

2.3.5: In vivo administration of IL-2 upregulates both perforin- and FasL-based cytotoxicity

To further corroborate that IL-2 activation *in vivo* upregulates both perforin and FasL-based cytolytic activity, mice were injected with 50,000 U IL-2 twice a day for 5 days. NK/LAK cells from IL-2 treated wild-type, *gld/gld*, and perforin-knockout mice were tested for lytic activity against L1210 (Fas^-) (Figs. 2.11A, 2.11B, and 2.11C) and L1210 (Fas^+) (Figs. 2.12A, 2.12B, 2.12C) tumor cells. The data shown suggested that *in vivo* IL-2 activated NK/LAK were able to kill Fas^+ target cells to a greater extent than Fas^- L1210 tumor cells. Also, the L1210 (Fas^+) targets were killed by both FasL and perforin based pathways. These data indicate that *in vivo* IL-2 administration does trigger both perforin and FasL-based cytotoxicity and therefore IL-2 treatment may be more effective against Fas^+ tumor targets.

2.3.6: Effect of Immunotherapy with IL-2 against L1210 (Fas^+) and L1210 (Fas^-) tumor growth

Inasmuch as the expression of Fas triggered increased susceptibility of tumor cells to lysis by activated NK cells, we next addressed whether L1210 (Fas^+) and L1210 (Fas^-) tumor cells would exhibit differential ability to induce tumors in the syngeneic host and whether IL-2 immunotherapy would have varying effects on these tumor cell lines. To this effect, 1 X 10^6 L1210 (Fas^+) or L1210 (Fas^-) tumor cells were injected subcutaneously and were administered either with PBS as a control, or IL-2 (50,000 U b.i.d. for one week). The tumor growth was monitored and on day 14, the mice were sacrificed and the tumor size was measured. As shown in Fig. 2.13, the L1210 (Fas^+) tumor cells failed to grow and induce tumors, whereas L1210 (Fas^-) cells caused large tumors. Furthermore, upon IL-2 treatment, the L1210 (Fas^-) tumors were completely rejected by the host. These data suggested that when injected subcutaneously, the Fas^+ tumor cells trigger an immune response that is sufficient to induce tumor rejection. Furthermore, IL-2 administration which was shown to upregulate perforin-based cytotoxicity by NK/LAK cells may have been responsible for inducing the rejection of Fas^- tumor cells.

2.4: Discussion

In the current study, we demonstrated that poly I:C or IL-2 triggers NK/LAK activity by enhancing both FasL- and perforin-based cytotoxicity. Fas^+ tumor targets were found to be more sensitive to poly I:C or IL-2 induced cytotoxicity when compared to the Fas^- tumor targets. Also, L1210 (Fas^+) tumor cells were readily rejected when injected subcutaneously into syngeneic mice whereas L1210 (Fas^-) tumor cells grew and induced tumors. Moreover, IL-2 administration *in vivo* was shown to trigger both FasL- and perforin-based cytolytic activity.

The role of perforin in NK/LAK cell-mediated cytotoxicity has been well established (Trinchieri, 1989). In addition, recent studies have revealed the existence of a perforin-independent pathway in NK/LAK cell or T cell mediated cytotoxicity based on the interactions between FasL expressed on effector cells and Fas receptor on the target cells (Oshimi, *et. al.,* 1996; Lee, 1996; Yagita, *et. al.* 1995). In the current study we investigated the role of perforin and FasL during spontaneous and induced NK/LAK activity using a variety of NK sensitive and resistant targets, as well as Fas^+ and Fas^- target cells. Using NK sensitive YAC-1 targets we noted that the spontaneous cytotoxicity was mainly perforin dependent, inasmuch as, perforin-deficient mice exhibited no significant levels of cytotoxicity, and the *gld/gld* mice showed cytotoxicity comparable to wild-type mice. However, upon activation with poly I:C *in vivo* or IL-2 *in vitro*, the inducible cytotoxicity against YAC-1 and P815 targets was dependent on both perforin and FasL. Moreover, in such cytolytic activity, perforin played a more important role because the perforin-knockout mice exhibited low levels of inducible cytolytic activity when compared to the *gld/gld* mice. In addition, in blocking studies with mAbs to Fas and $MgCl_2$ to block the perforin-dependent pathway, more blocking was seen when perforin was impaired. This can be explained by the fact that YAC-1 and P815 tumor targets expressed lower levels of Fas. Moreover, not all targets expressed Fas.

To further explore the role of Fas expression on target cells, we tested NK-resistant L1210 (Fas^+) and L1210 (Fas^-) transfectants for their susceptibility to induce cytotoxicity. It was interesting to note that L1210 target cells behaved like NK resistant P815 target cells since they were both resistant to spontaneous and poly I:C activated, but not IL-2 activated NK/LAK cell-mediated cytotoxicity. Furthermore, L1210 (Fas^+) target cells were more sensitive to induced NK/LAK activity when compared to L1210 (Fas^-) target cells. These data suggested that Fas expression on target cells can increase susceptibility of tumor targets to induced NK/LAK activity. The fact that perforin-deficient mice completely failed to mediate lysis of L1210 (Fas^-) targets, and that they could mediate lysis of L1210 (Fas^+) cells to a lower level than the *gld/gld* or wild-type mice suggested that perforin does play an important role in induced NK/LAK-activity. It should be noted that despite the strong expression of Fas on the L1210 tumor cells, FasL-based cytotoxicity as seen in perforin-knockout mice was lower than that seen in wild-type mice. Similarly, the perforin-based lytic activity seen in *gld/gld* mice was also less than seen in wild-type mice. Thus, the presence of both perforin- and FasL based cytotoxicity, was clearly an advantage to efficiently kill Fas^+ tumor cells. In addition the perforin pathway was blocked to a greater extent than the FasL-dependent pathway in blocking studies using anti-Fas Abs and $MgCl_2$.

To further corroborate the *in vitro* results *in vivo*, we compared the ability of L1210 (Fas^+) and L1210 (Fas^-) transfectants to grow and induce tumors in syngeneic mice, and tested whether IL-2 administration would inhibit the growth of these tumor cells. In these studies it was striking that injection of 1×10^6 Fas^+ tumor cells subcutaneously failed to induce tumors of significant size in 14 days whereas L1210 (Fas^-) tumor cells grew to induce large tumors. Also, IL-2 administration completely inhibited L1210 (Fas^-) tumor growth.

In the current study we also noted that an endothelial cell line was effectively killed by IL-2 activated NK/LAK cells. These studies demonstrated that IL-2 administration can activate FasL or perforin which in turn may cause significant damage to the endothelial cells resulting in vascular leak syndrome.

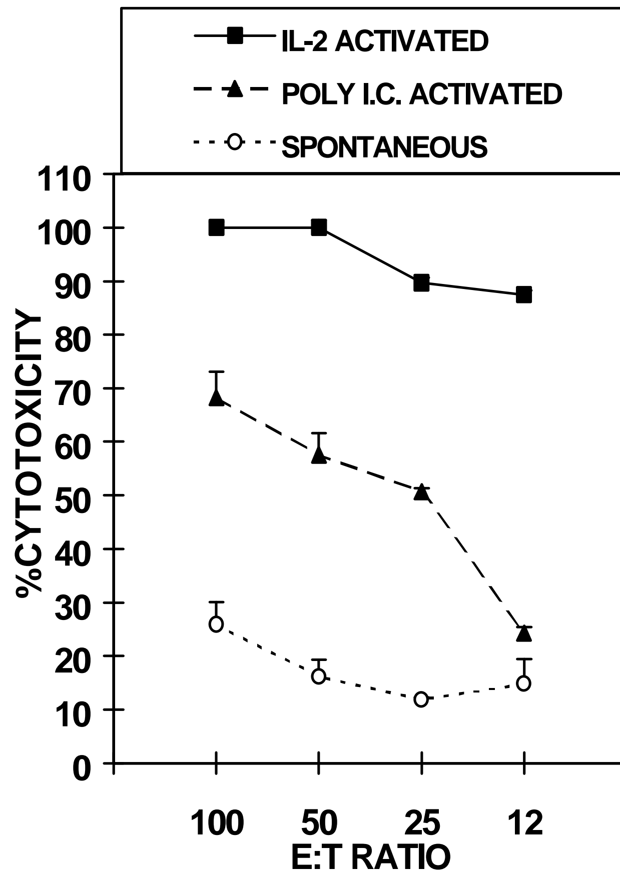

Fig. 2.1A. Spontaneous, Poly I:C activated and IL-2 activated NK/LAK activity in wild-type mice against YAC-1 tumor targets. IL-2 activated NK/LAK cells were cultured with 1000 U/ml of IL-2 for 48 hours. Poly I:C activated NK cells were harvested from the spleens of mice injected with 15 µg/kg Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured by ^{51}Cr release assay at various effector: target ratios.

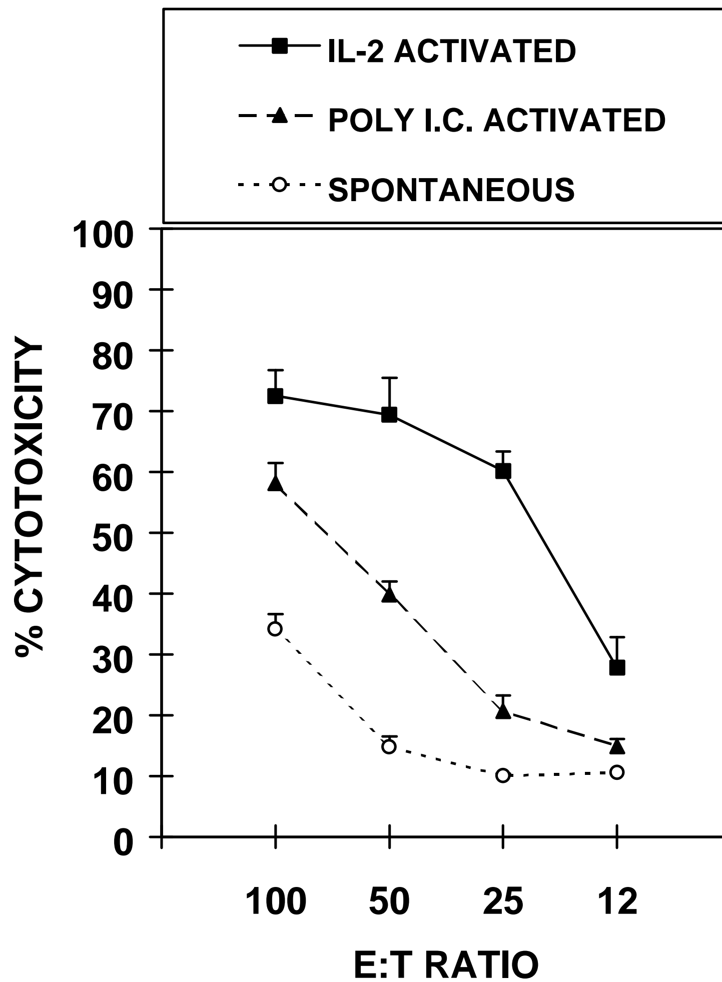

Fig.2.1B. Spontaneous, Poly I:C activated and IL-2 activated NK/LAK activity in *gld/gld* mice against YAC-1 tumor targets. IL-2 activated NK/LAK cells were cultured with 1000 U/ml of IL-2 for 48 hours. Poly I:C activated NK cells were harvested from the spleens of mice injected with 15 µg/kg Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured by ^{51}Cr release assay at various effector: target ratios.

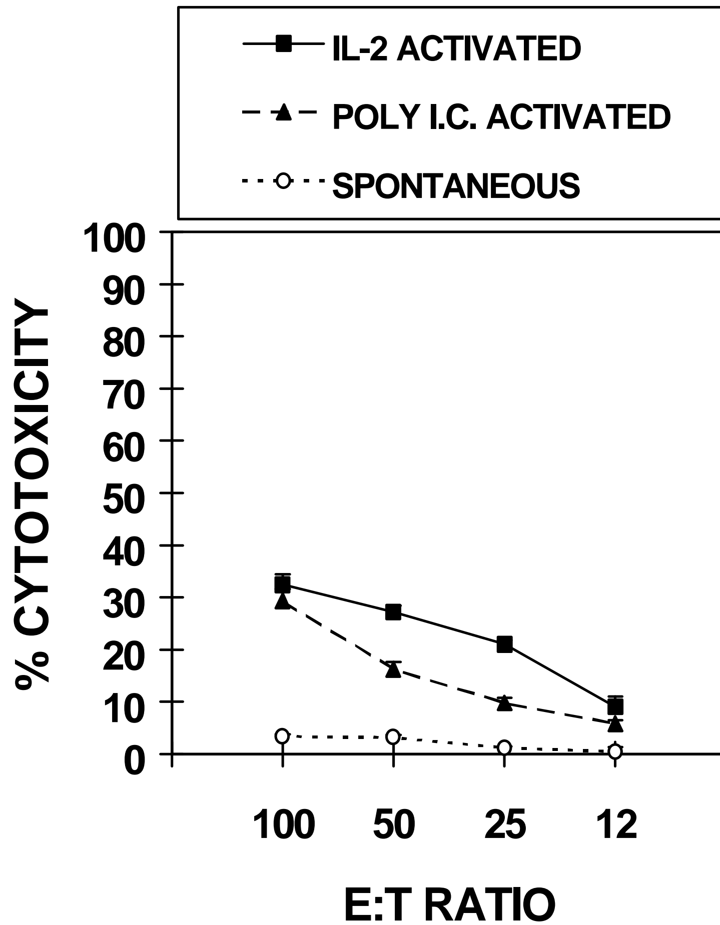

Fig. 2.1C. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in perforin-deficient mice against YAC-1 tumor cells. IL-2 activated NK/LAK cells were cultured with 1000 U/ml of IL-2 for 48 hours. Poly I:C activated NK cells were harvested from the spleens of mice injected with 15 μg/kg Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured by ^{51}Cr release assay at various effector: target ratios.

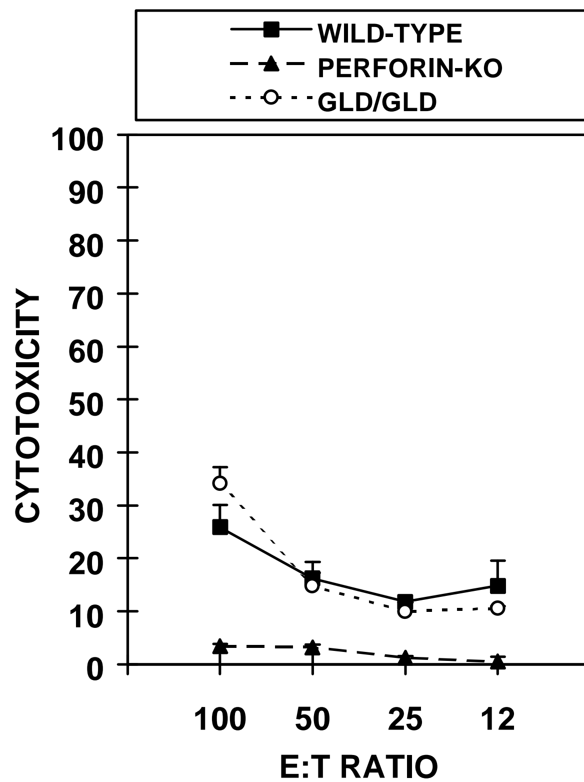

Fig. 2.2A. Spontaneous NK activity in wild-type, perforin-deficient, and *gld/gld* mice against YAC-1 tumor targets. Freshly purified NK cells were used to measure spontaneous activity. Cytotoxicity was measured using a ^{51}Cr release assay at various effector: target ratios.

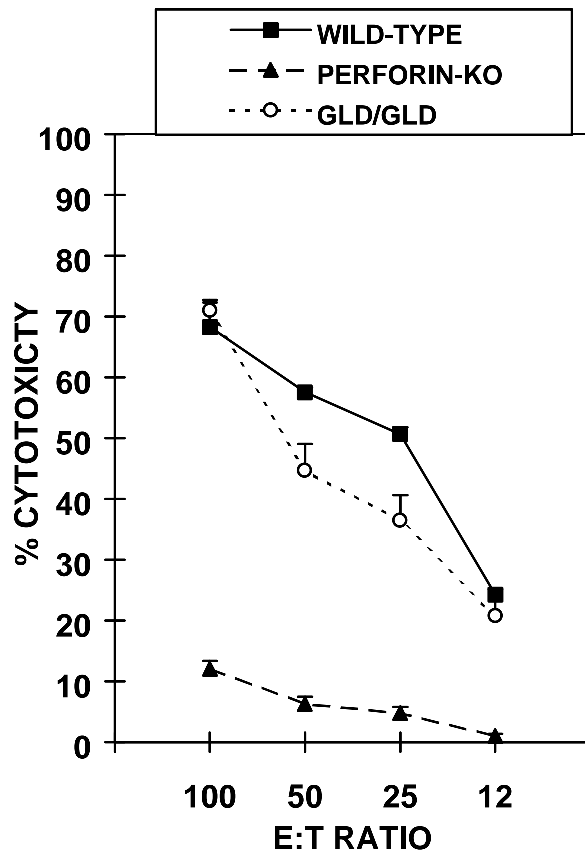

Fig. 2.2B. Poly I:C activated NK activity in wild-type, perforin-deficient, and *gld/gld* mice against YAC-1 tumor targets. Mice were injected with 15 μg/kg Poly I:C 24 hours prior to harvesting the spleens and purifying NK cells. Cytotoxicity was measured by ^{51}Cr release assay at various effector: target ratios.

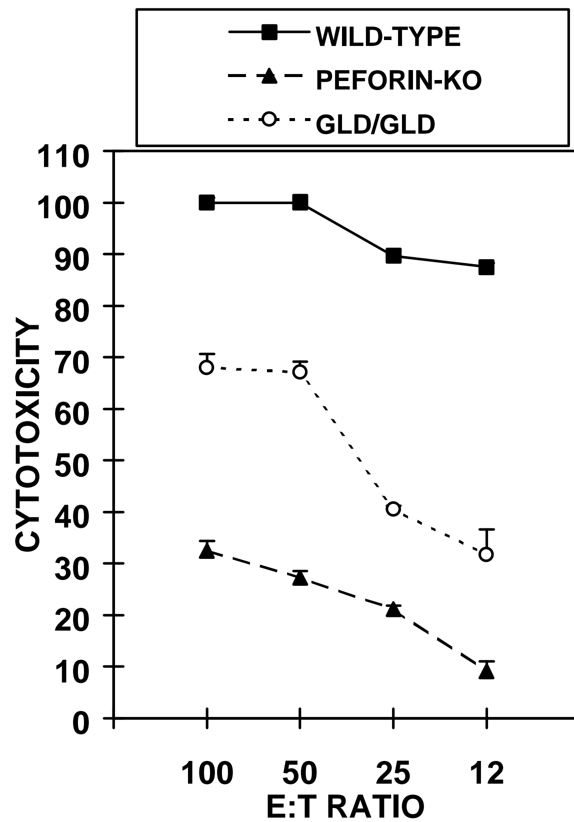

Fig. 2.2C. IL-2 activated NK activity in wild-type, perforin-deficient, and *gld/gld* mice against YAC-1 tumor targets. Nylon wool purified spleen cells were cultured *in vitro* with 1000 U/ml IL-2 for 24 hours. Cytotoxicity was measured using ^{51}Cr release assay at various effector: target ratios.

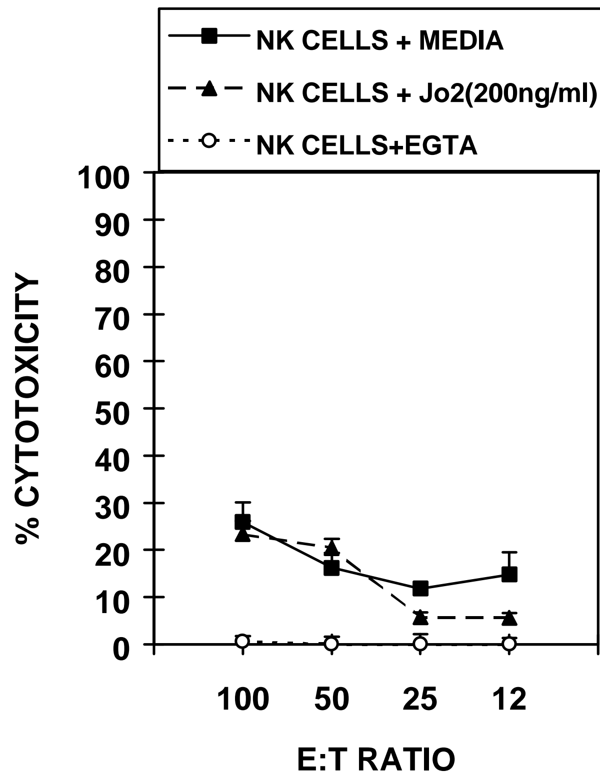

Fig. 2.3A. Spontaneous NK activity of wild-type mice against YAC-1 tumor targets is primarily perforin based. Freshly purified wild-type NK cells were measured for cytotoxicity at various effector: tumor ratios in a ^{51}Cr release assay in the presence of either media, anti-Fas mAbs (Jo2, 200 ng/ml), or EGTA and $MgCl_2$.

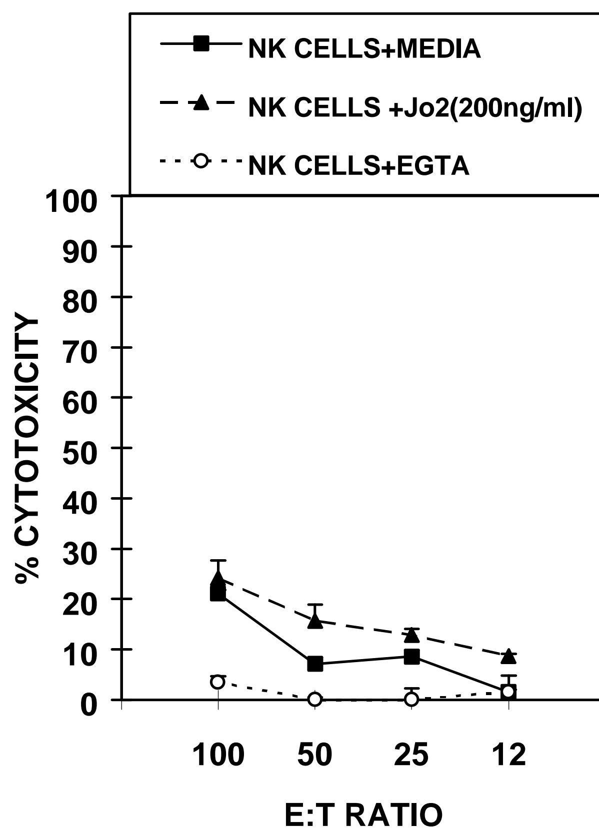

Fig2.3B. Spontaneous NK activity of *gld/gld* mice against YAC-1 tumor targets is perforin based. Freshly purified *gld/gld* NK cells were measured for cytotoxicity at various effector: target ratios in a ^{51}Cr release assay in the presence of either media, anti-Fas mAbs (Jo2, 200 ng/ml), or EGTA and $MgCl_2$.

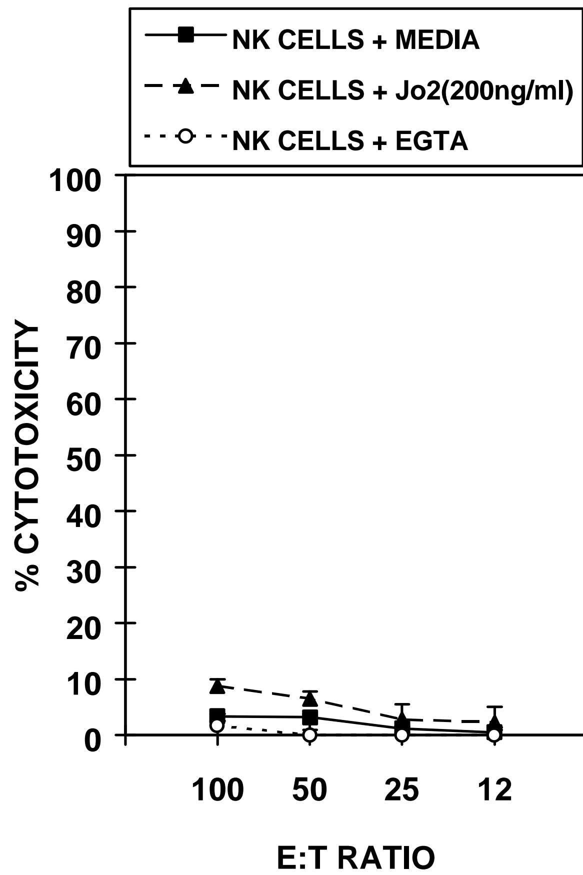

Fig. 2.3C. Spontaneous NK activity of perforin-deficient mice against YAC-1. Freshly purified perforin-deficient NK cells were measured for cytotoxicity at various effector: target ratios in a ^{51}Cr release assay in the presence of either media, anti-Fas mAbs (Jo2, 200 ng/ml), or EGTA and $MgCl_2$.

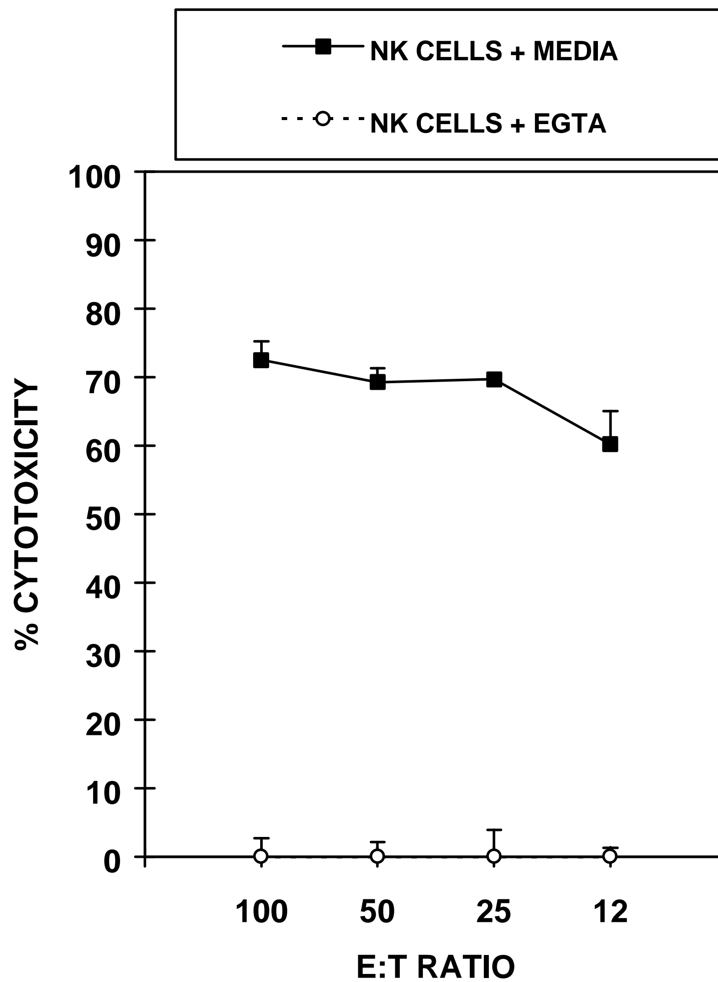

Fig. 2.4A. IL-2 activated NK/LAK activity of *gld/gld* mice against YAC-1 tumor targets is perforin based. Nylon wool purified *gld/gld* spleen cells were cultured *in vitro* for 48 hours with 1000 U/ml of IL-2. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios in the presence of media or EGTA and $MgCl_2$.

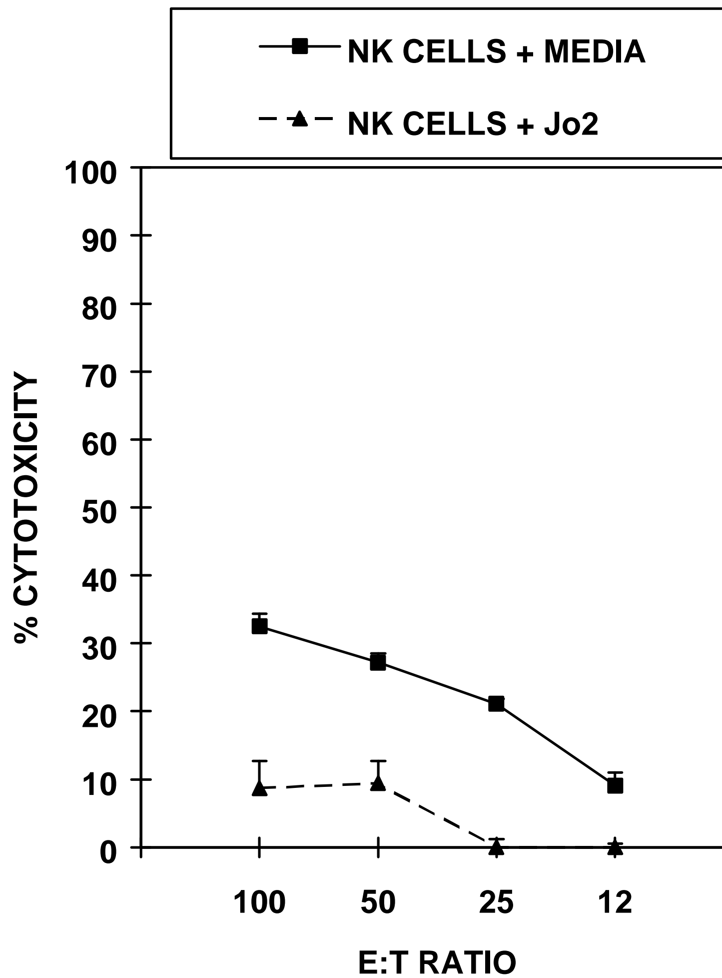

Fig. 2.4B. IL-2 activated NK/LAK activity of perforin-deficient mice against YAC-1 tumor targets is FasL dependent. Nylon wool purified spleen cells from perforin-deficient mice were cultured *in vitro* for 48 hours with 1000 U/ml IL-2. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios in the presence of media or EGTA and $MgCl_2$.

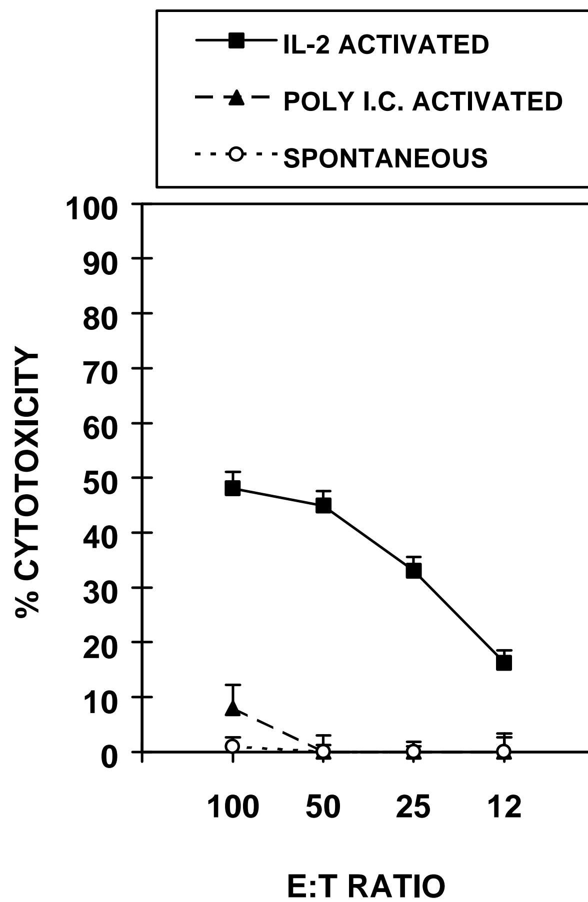

Fig. 2.5A. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in wild-type mice against P815 tumor targets. IL-2 activated NK/LAK cells were cultured *in vitro* with 1000 U/ml IL-2 for 48 hours. Poly I:C activated NK cells were purified from the spleens of C57BL/6 mice injected with 15 µg/kg Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

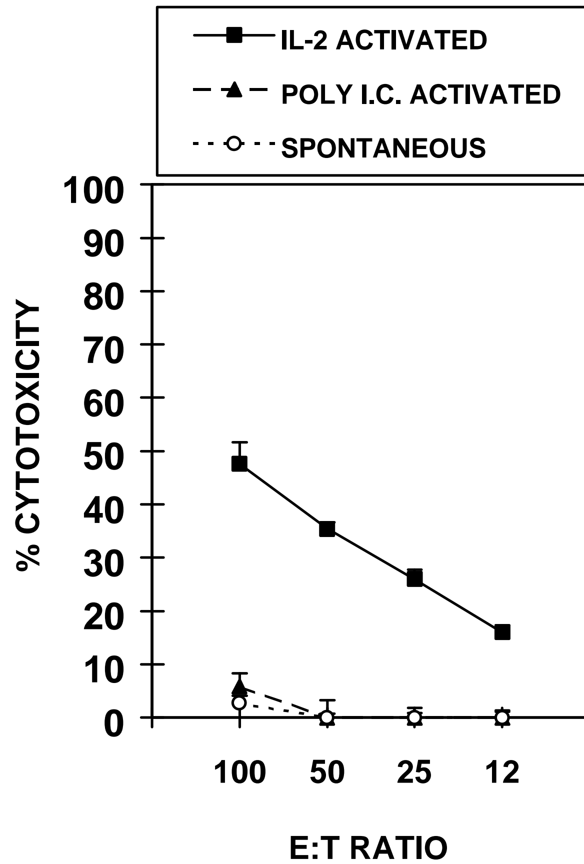

Fig. 2.5B. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in *gld/gld* mice against P815 tumor targets. IL-2 activated NK/LAK cells were cultured *in vitro* with 1000 U/ml IL-2 for 48 hours. Poly I:C activated NK cells were purified from the spleens of *gld/gld* mice injected with 15 µg/kg Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

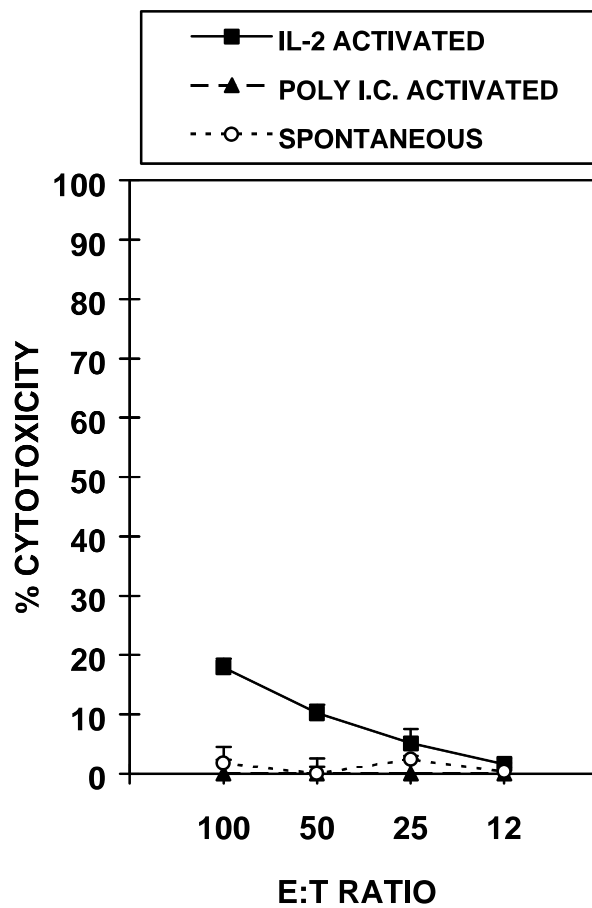

Fig. 2.5C. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in perforin-deficient P815 tumor targets. IL-2 activated NK/LAK cells were cultured *in vitro* with 1000 U/ml IL-2 for 48 hours. Poly I:C activated NK cells were taken from the spleens of perforin-deficient mice injected with µg/kg Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

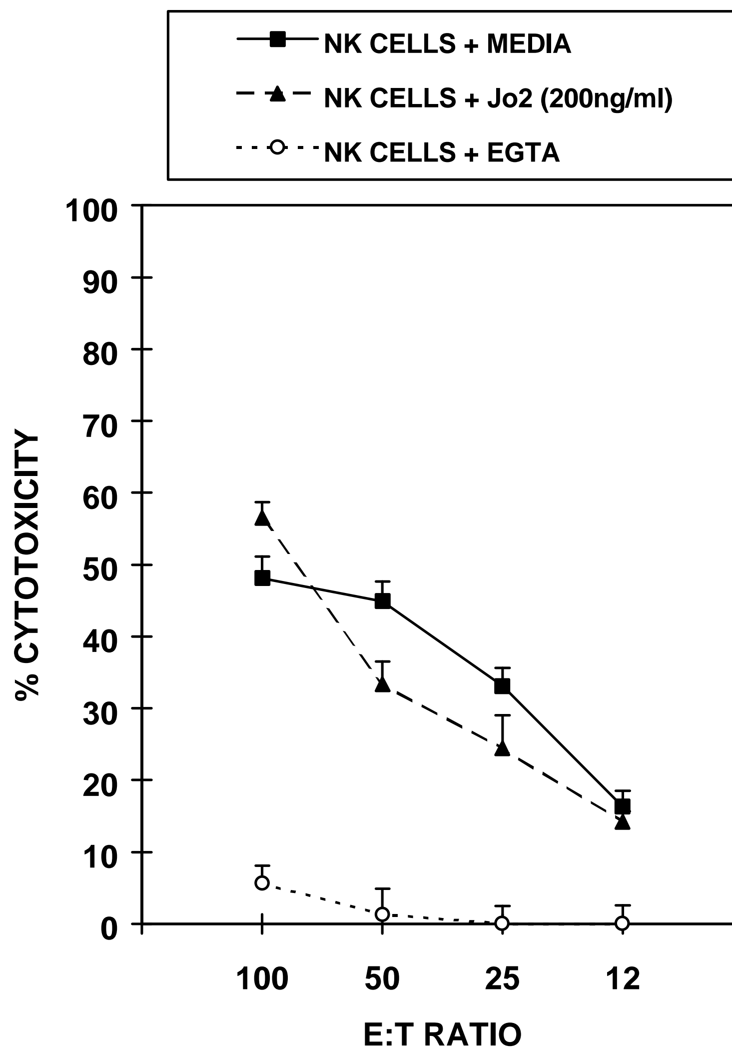

Fig.2.6A. NK/LAK activity of wild-type mice against P815. Nylon wool purified spleen cells from C57BL/6 mice were cultured *in vitro* for 48 hours with 1000 U/ml IL-2. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios in the presence of either media, anti-Fas mAbs (Jo2, 200 ng/ml), or EGTA and $MgCl_2$.

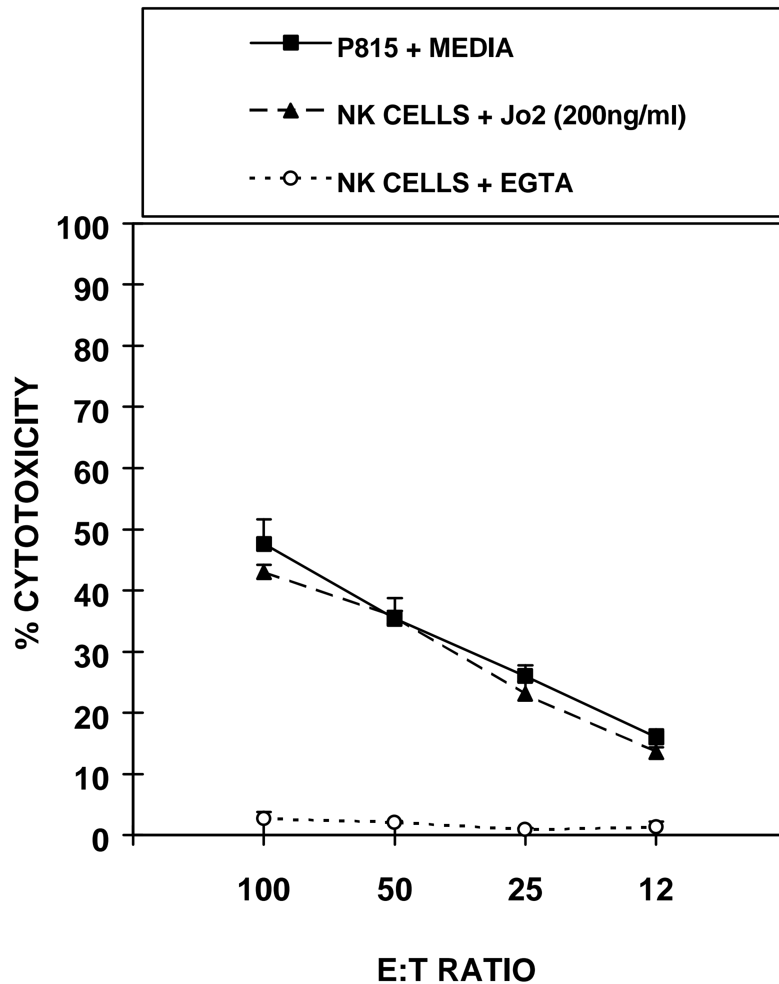

Fig. 2.6B. IL-2 activated NK/LAK activity of *gld/gld* mice against P815. Nylon wool purified spleen cells from *gld/gld* mice were cultured *in vitro* for 48 hours with 1000 U/ml IL-2. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios in the presence of either media, anti-Fas mAbs (Jo2, 200 ng/ml), or EGTA and $MgCl_2$.

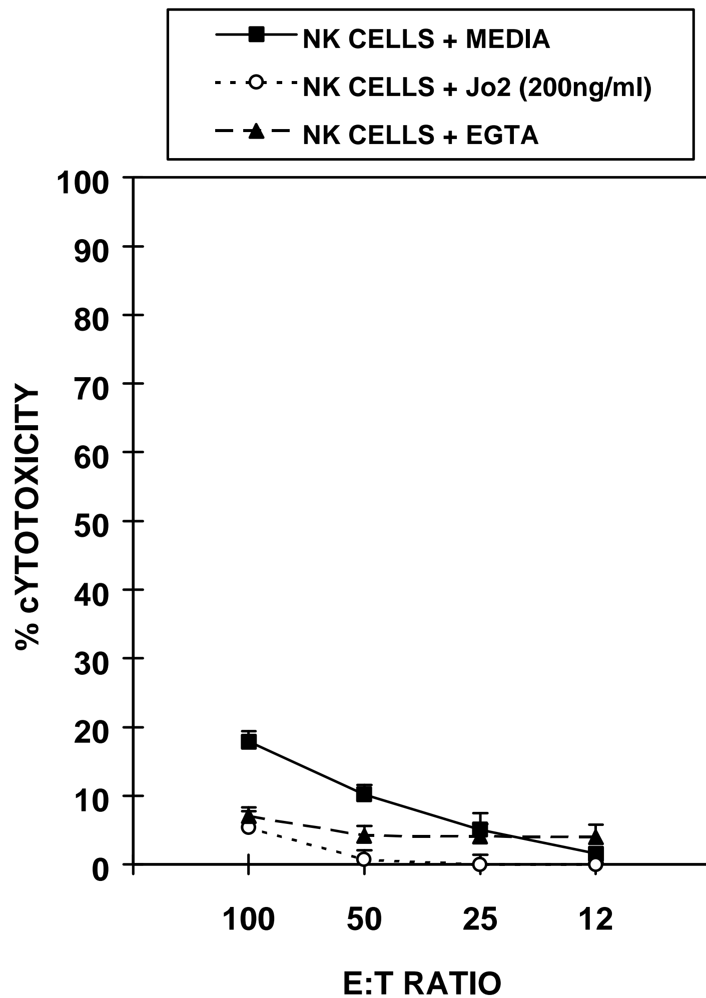

Fig. 2.6C. IL-2 activated NK/LAK activity of perforin-deficient mice against P815. Nylon wool purified spleen cells from perforin-deficient mice were cultured *in vitro* for 48 hours with 1000 U/ml IL-2. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios in the presence of either media, anti-Fas mAbs (Jo2, 200 ng/ml) or EGTA and $MgCl_2$.

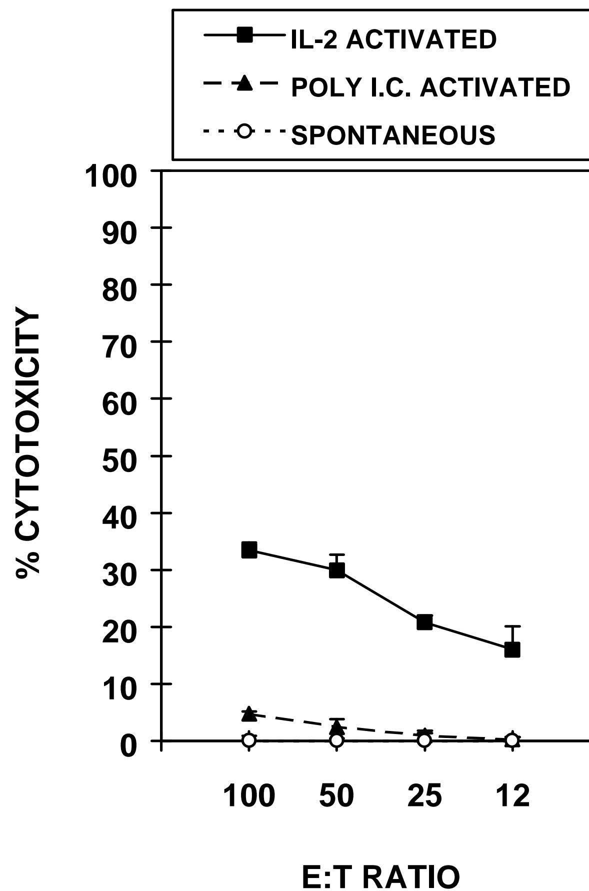

Fig. 2.7A. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in wild-type mice against L1210 (Fas$^-$) tumor targets. IL-2 activated NK/LAK cells were cultured *in vitro* with 1000 U/ml IL-2 for 48 hours. Poly I:C activated NK cells were purified from the spleens of mice injected with 15 μg/g Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

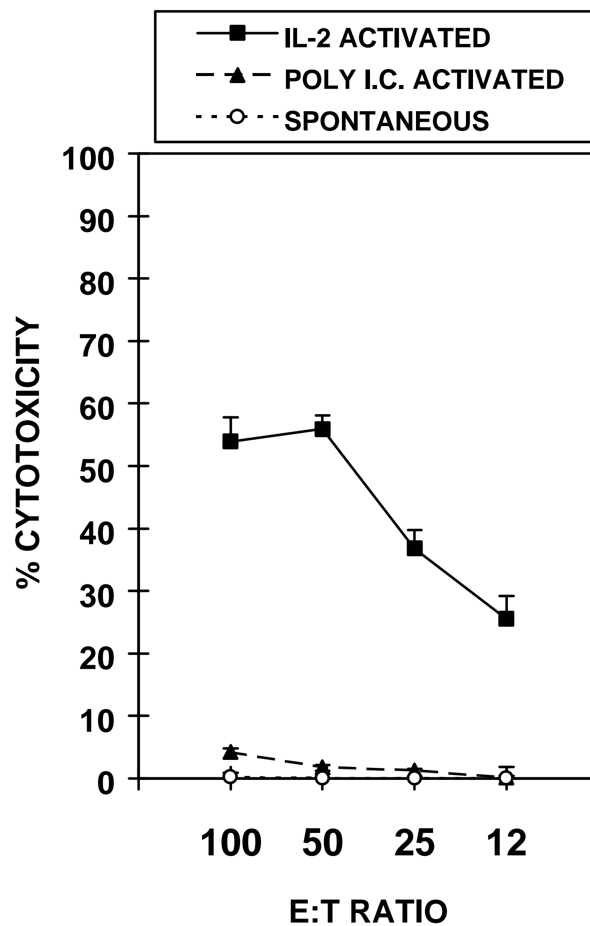

Fig. 2.7B. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in *gld/gld* mice against L1210 (Fas$^-$) tumor cells. IL-2 activated NK/LAK cells were cultured *in vitro* with 1000 U/ml IL-2 for 48 hours. Poly I:C activated NK cells were purified from the spleens of *gld/gld* mice injected with 15 µg/kg Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

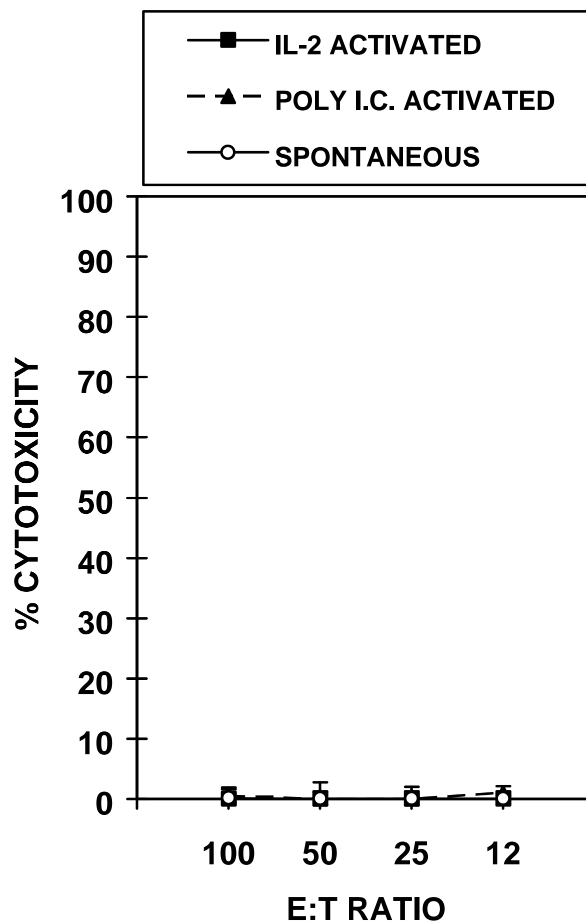

Fig. 2.7C. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in perforin-deficient mice against L1210 (Fas^-) tumor cells. IL-2 activated NK LAK cells were cultured *in vitro* with 1000 U/ml IL-2 for 48 hours. Poly I:C activated NK cells were purified from the spleens of perforin-deficient mice injected with 15 µg/kg Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

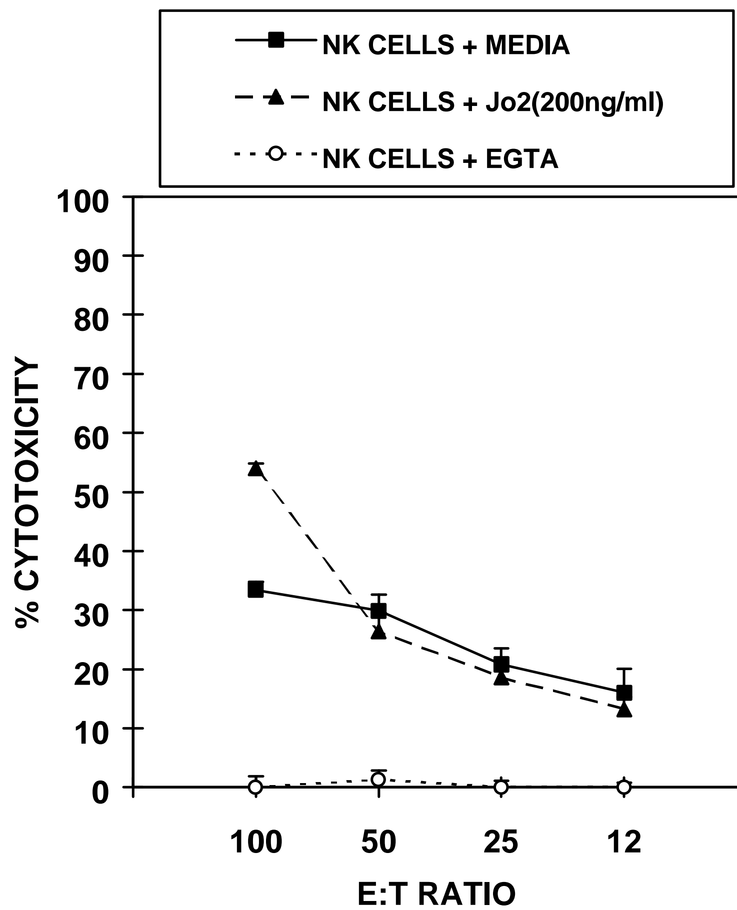

Fig. 2.8A. IL-2 activated NK/LAK activity of wild-type mice against L1210 (Fas^-) target cells. Nylon wool purified spleen cells from C57BL/6 mice were cultured *in vitro* for 48 hours with 1000 U/ml IL-2. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios in the presence of either media, anti-Fas mAbs (Jo2, 200 ng/ml) or EGTA and $MgCl_2$.

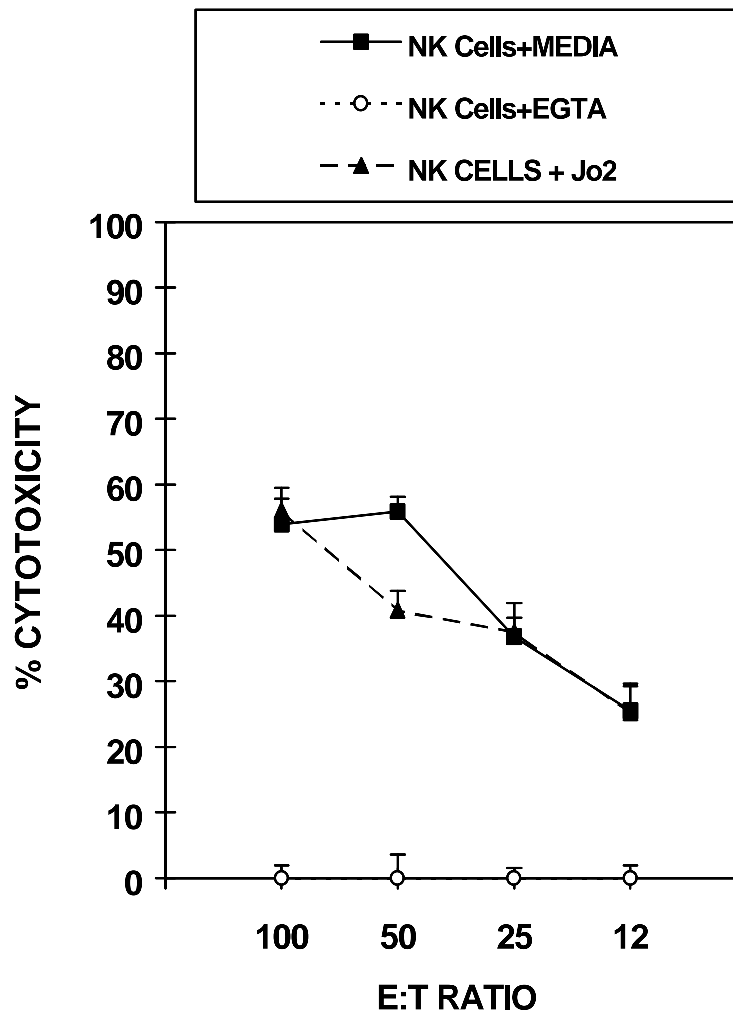

Fig. 2.8B. IL-2 activated NK/LAK activity of *gld/gld* mice against L1210 (Fas^{-}) tumor targets. Nylon wool purified spleen cells from *gld/gld* mice were cultured *in vitro* for 48 hours with 1000 U/ml IL-2. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios in the presence of either media, anti-Fas mAbs (Jo2, 200 ng/ml), or EGTA and $MgCl_2$.

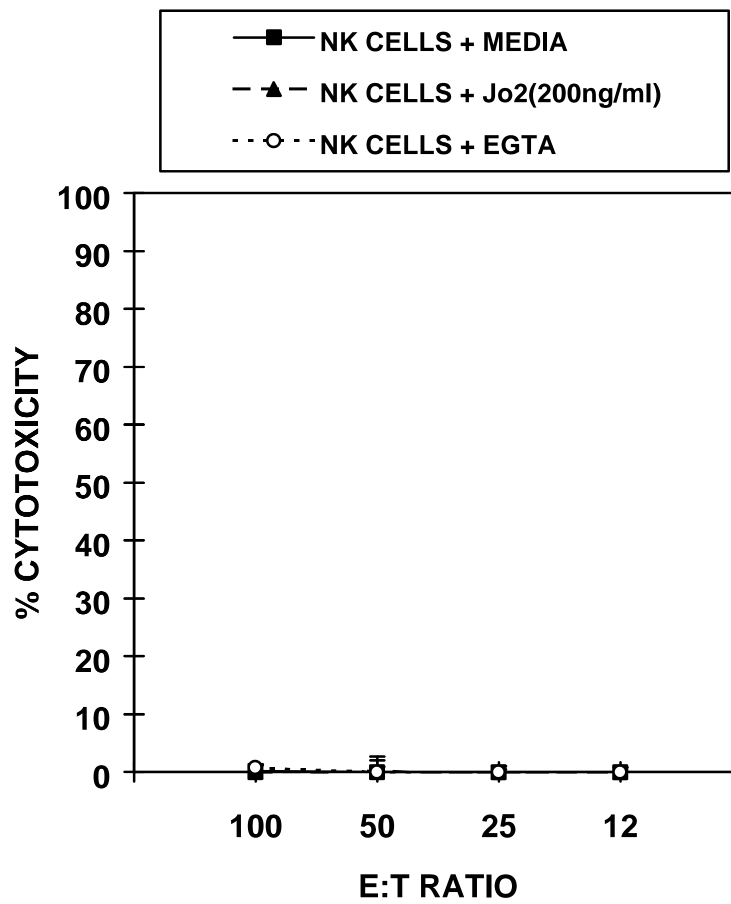

Fig. 2.8C. IL-2 activated NK/LAK activity of perforin-deficient mice are unable to lyse L1210 (Fas^-) tumor targets. Nylon wool purified spleen cells from perforin-deficient mice were cultured *in vitro* for 48 hours with 1000 U/ml of IL-2. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios in the presence of either media, anti-Fas mAbs (Jo2, 200 ng/ml), or EGTA and $MgCl_2$.

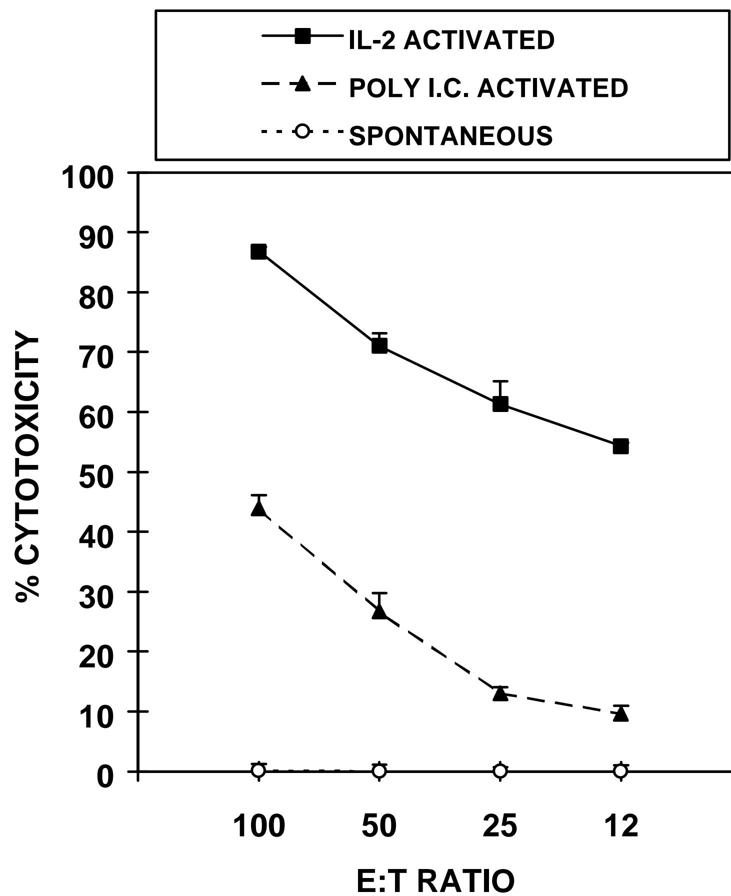

Fig. 2.9A. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in wild-type mice against L1210 (Fas^{+}) tumor cells. IL-2 activated NK/LAK cells were cultured with 1000 U/ml of IL-2 for 48 hours. Poly I:C activated NK cells were purified from the spleens of C57BL/6 mice injected with 15 μg/kg of Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

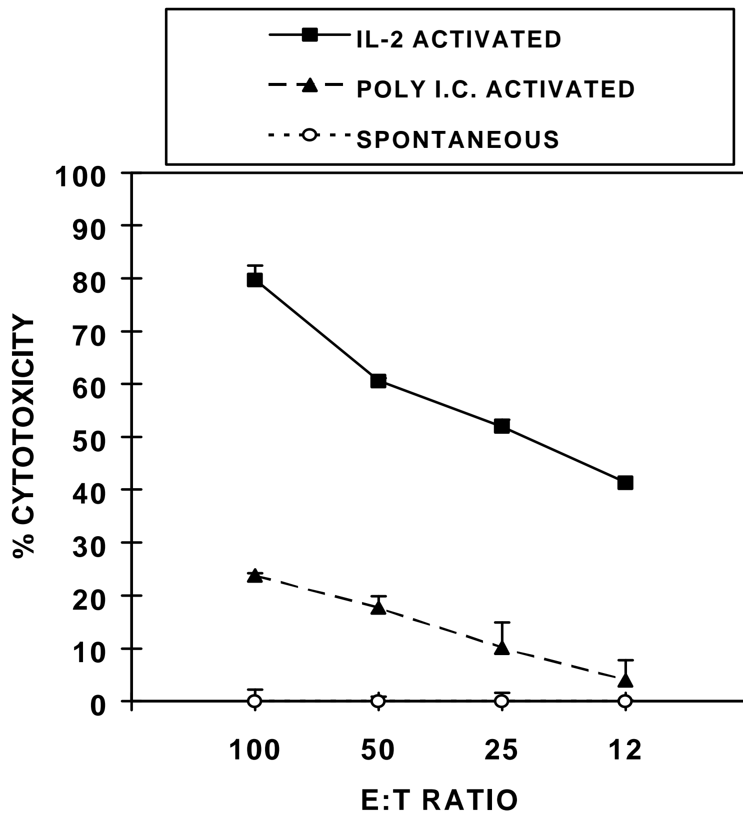

Fig. 2.9B. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in *gld/gld* mice against L1210 (Fas^+) tumor cells. IL-2 activated NK LAK cells were cultured with 1000 U/ml of IL-2 for 48 hours. Poly I:C activated NK cells were purified from the spleens of mice injected with 15 µg/kg of Poly I:C prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

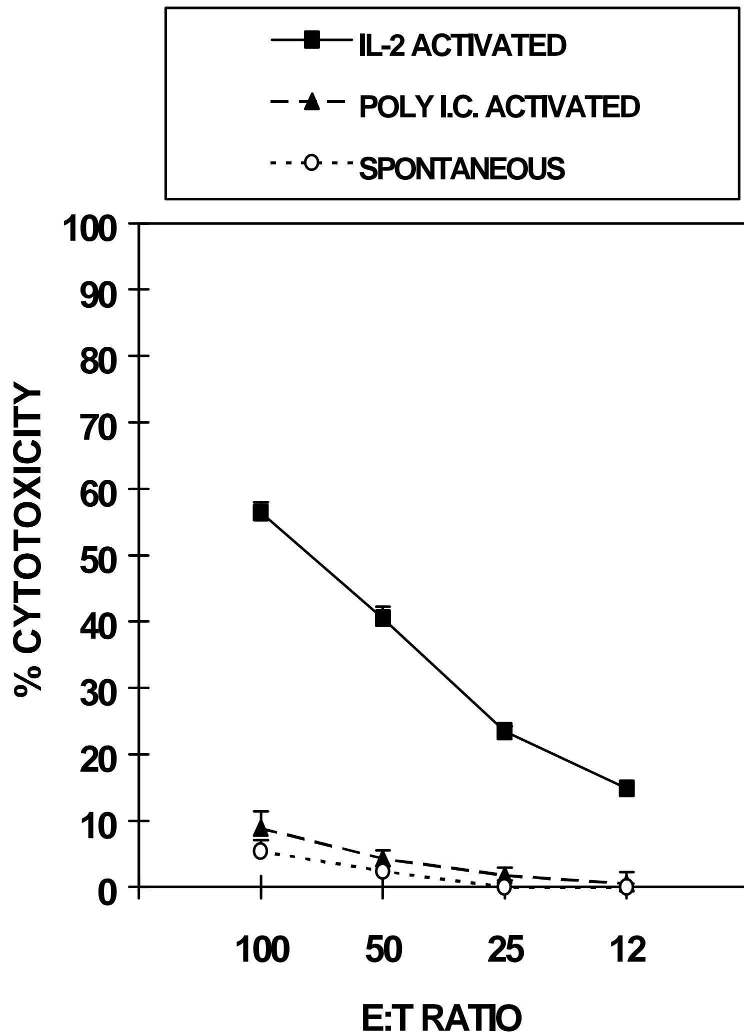

Fig. 2.9C. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in perforin deficient mice against L1210 (Fas^+) tumor cells. IL-2 activated NK/LAK cells were cultured with 1000 U/ml of IL-2 for 48 hours. Poly I:C activated NK cells were purified from the spleens of perforin deficient mice injected with 15 µg/kg Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

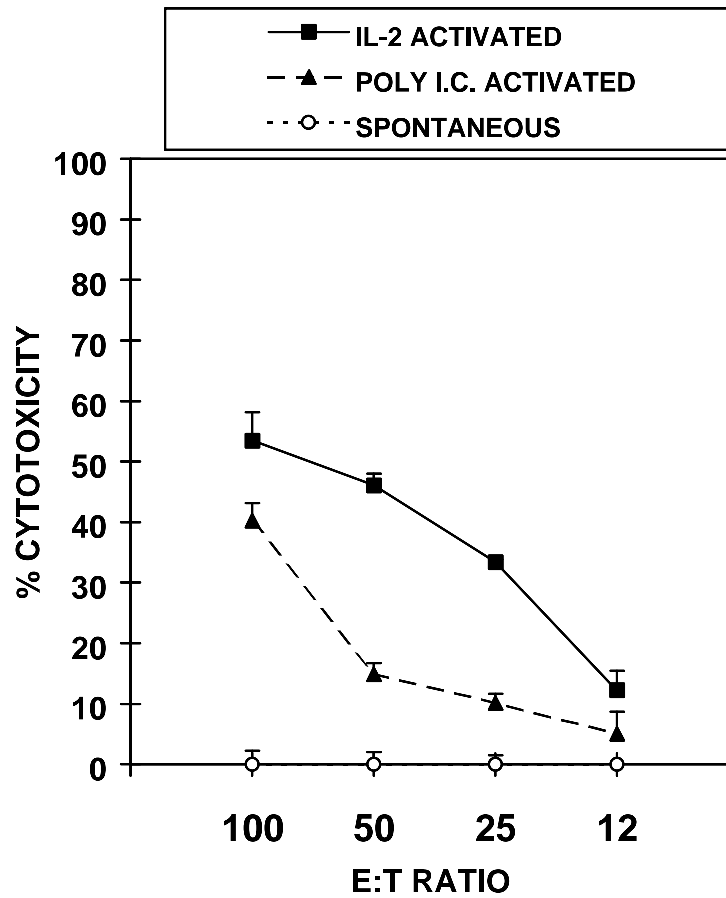

Fig. 2.10A. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in wild-type mice against TME-3H3 cells. IL-2 activated NK/LAK cells were cultured with 1000 U/ml of IL-2 for 48 hours. Poly I:C activated NK cells were purified from the spleens of mice injected with 15 µg/kg of Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

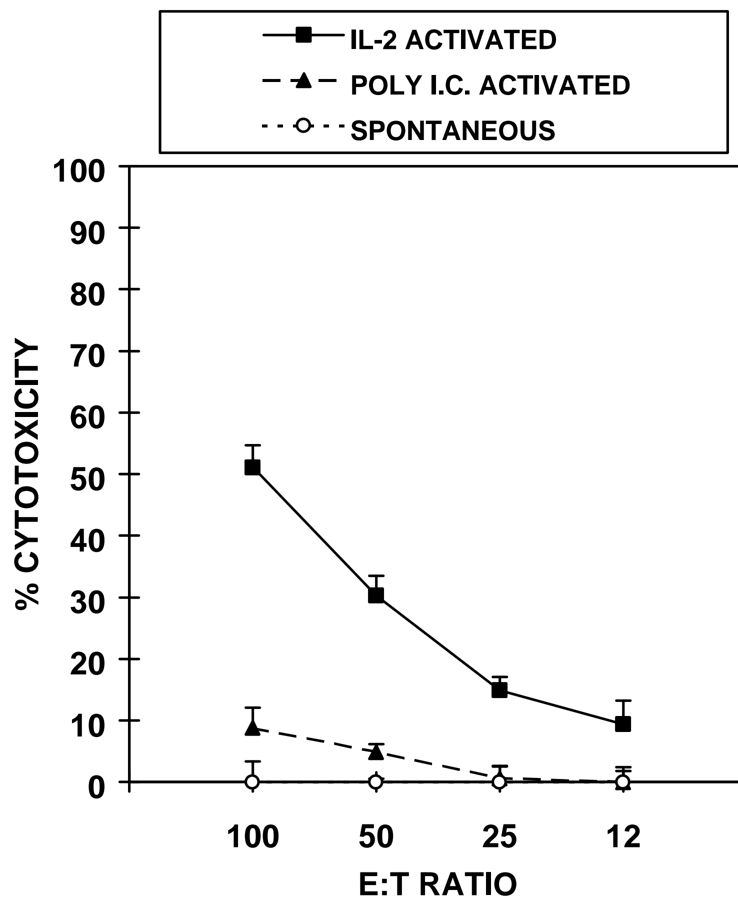

Fig. 2.10B. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in *gld/gld* mice against TME-3H3 cells. IL-2 activated NK/LAK cells were cultured with 1000 U/ml of IL-2 for 48 hours. Poly I:C activated NK cells were purified from the spleens of mice injected with 15 μg/kg of Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

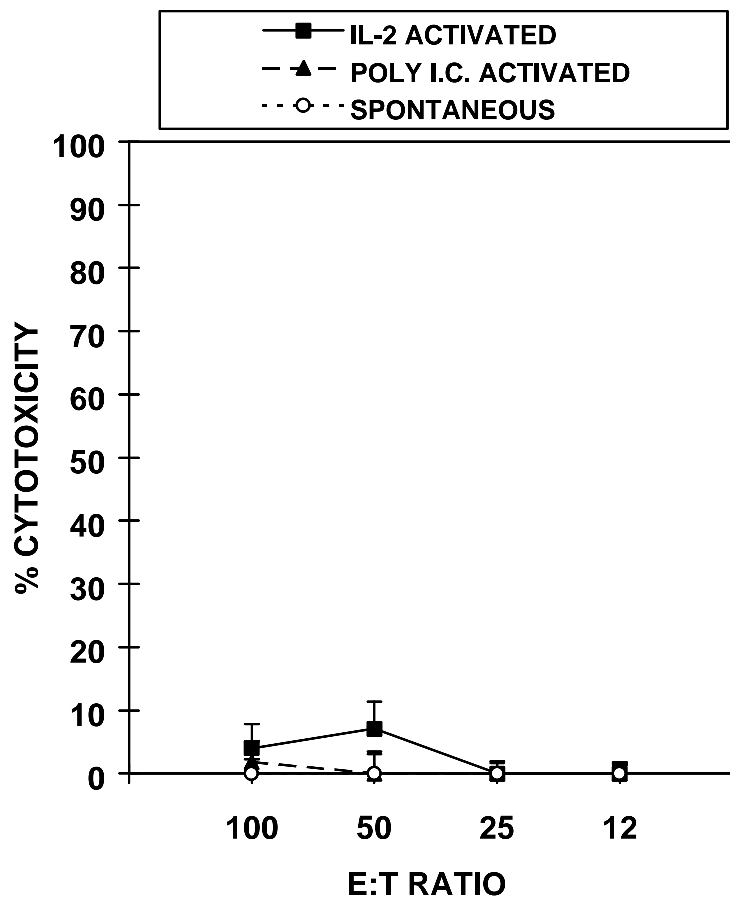

Fig. 2.10C. Spontaneous, Poly I:C activated, and IL-2 activated NK/LAK activity in perforin-deficient mice against TME-3H3 cells. IL-2 activated NK/LAK cells were cultured with 1000 U/ml for 48 hours. Poly I:C activated NK cells were purified from the spleens of perforin-deficient mice injected with 15 μg/kg of Poly I:C 24 hours prior to harvesting. Spontaneous activity was measured from freshly purified NK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

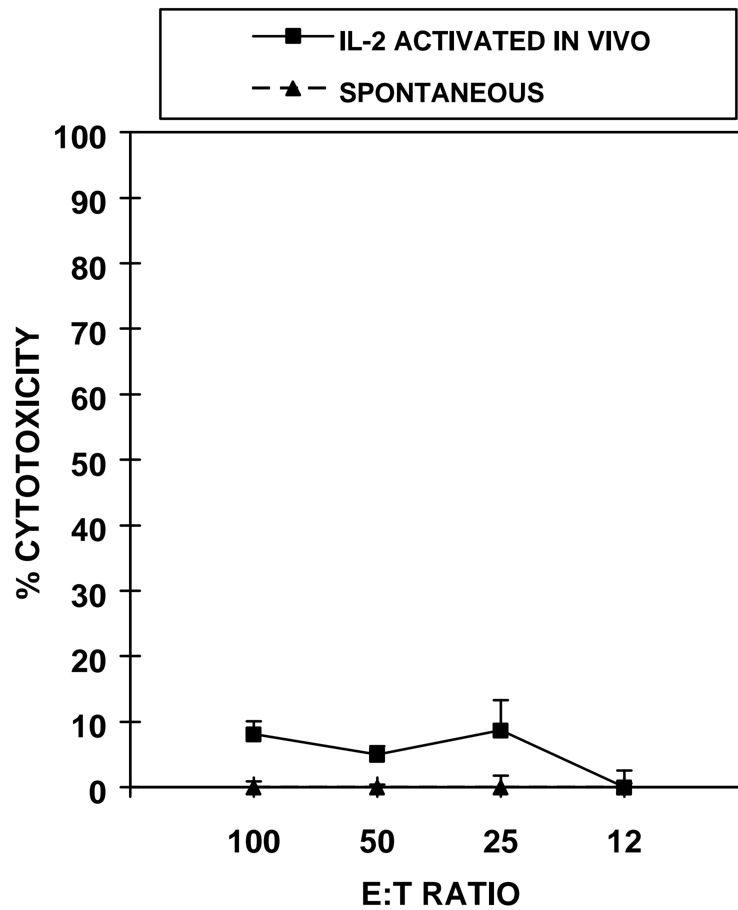

Fig. 2.11A. ***In vivo*** **IL-2 activated NK/LAK activity in wild-type mice against L1210 (Fas^{-}) tumor cells.** C57BL/6 mice were injected with 50,000 U of IL-2 i.p. twice a day. On day 5 the spleens were harvested and the cells run through nylon wool columns to purify NK/LAK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

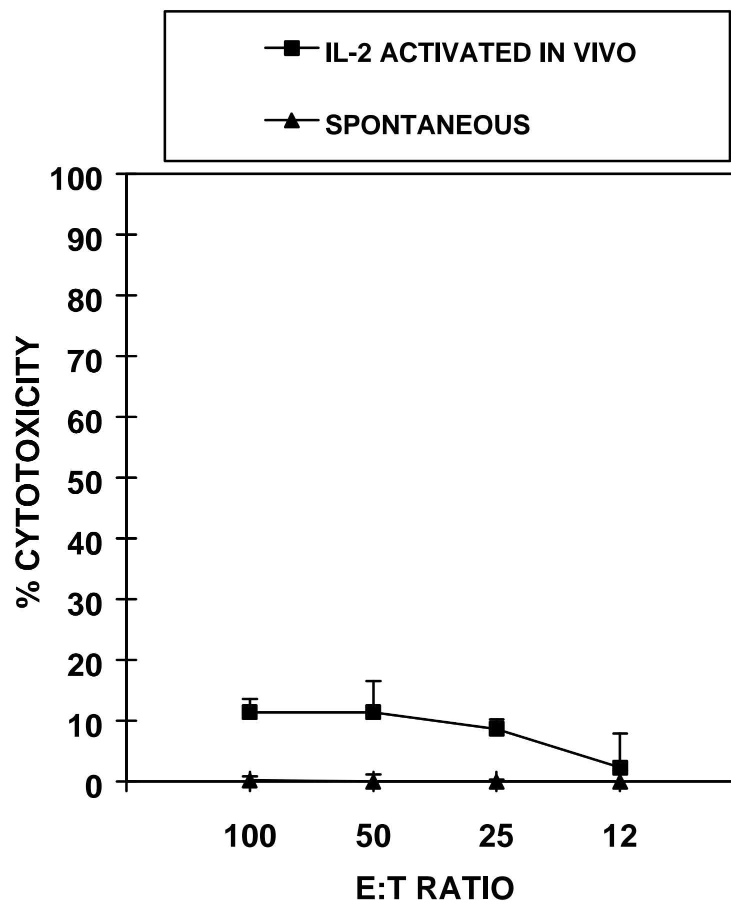

Fig. 2.11B. ***In vivo*** **IL-2 activated NK/LAK activity in** ***gld/gld*** **mice against L1210 (Fas^-) tumor cells.** *gld/gld* mice were injected with 50,000 U of IL-2 i.p. twice a day. On day 5 the spleens were harvested and the cells run through nylon wool columns to purify NK/LAK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

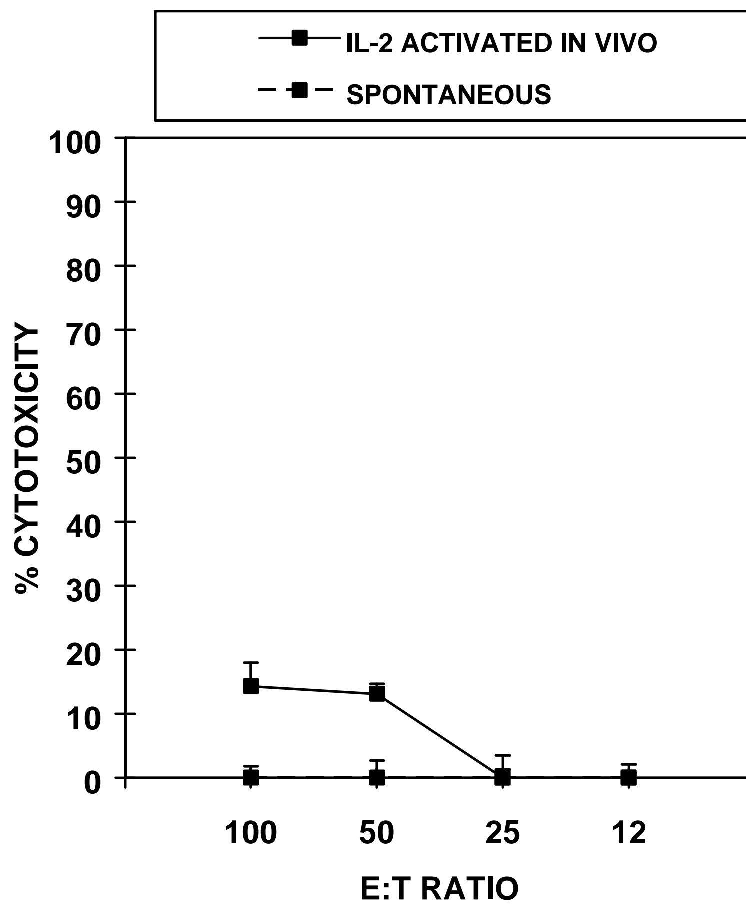

Fig. 2.11C. *In vivo* activated NK/LAK activity in perforin-deficient mice against L1210 (Fas^-) tumor cells. Perforin-deficient mice were injected with 50,000 U of IL-2 i.p. twice a day. On day 5 the spleens were harvested and the cells run through nylon wool columns to purify NK/LAK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

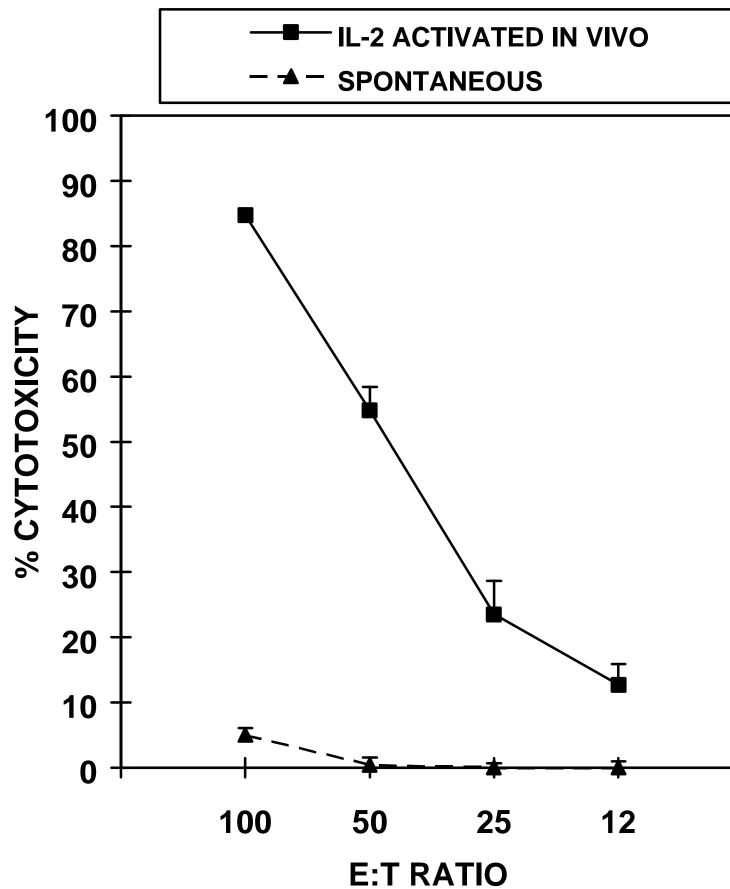

Fig. 2.12A. ***In vivo*** **activated NK/LAK activity in wild-type mice against L1210 (Fas^+) tumor cells.** C57BL/6 mice were injected with 50,000 U of IL-2 i.p. twice a day. On day 5 the spleens were harvested and the cells run through nylon wool columns to purify NK/LAK cells. Cytotoxicity was measured in ^{51}Cr release assay at various effector: target ratios.

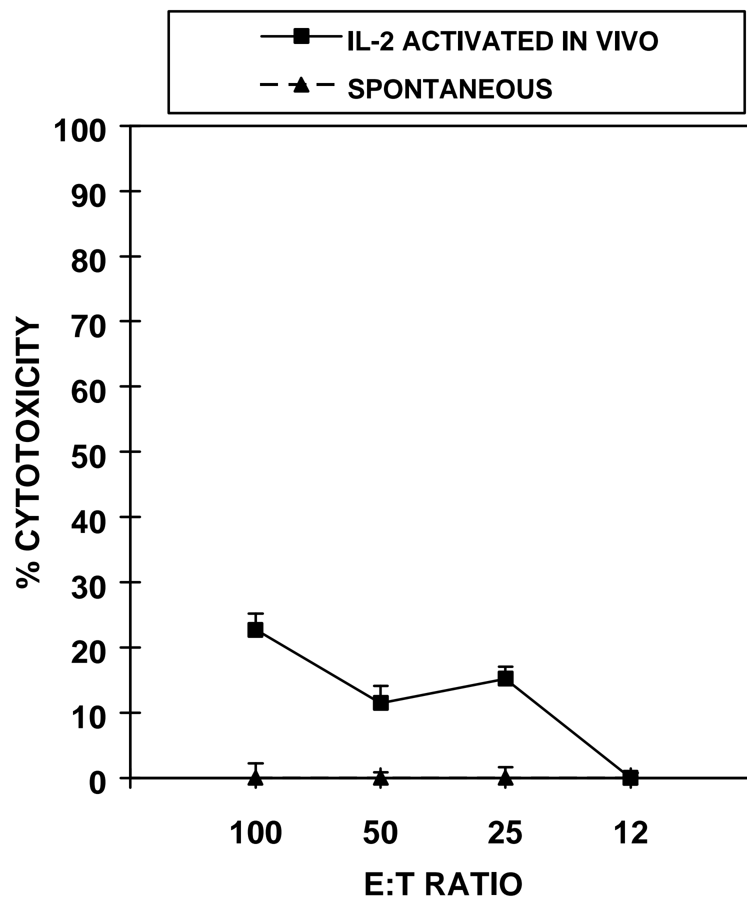

Fig. 2.12B. *In vivo* activated NK/LAK activity in *gld/gld* mice against L1210 (Fas^+) tumor cells. *gld/gld* mice were injected with 50,000 U of IL-2 i.p. twice a day. On day 5 the spleens were harvested and the cells run through nylon wool columns to purify the NK/LAK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

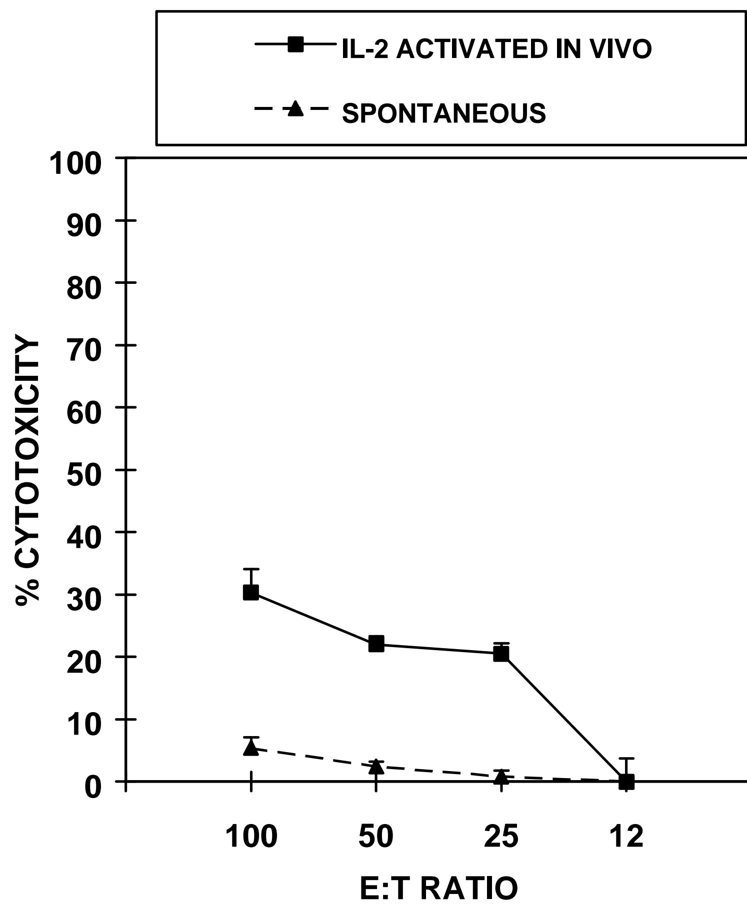

Fig. 2.12C. ***In vivo*** **activated NK/LAK activity in perforin-deficient mice against L1210 (Fas^+) tumor cells.** Perforin-deficient mice were injected with 50,000 U of IL-2 i.p. twice a day. On day 5 the spleens were harvested and the cells run through nylon wool columns to purify NK/LAK cells. Cytotoxicity was measured in a ^{51}Cr release assay at various effector: target ratios.

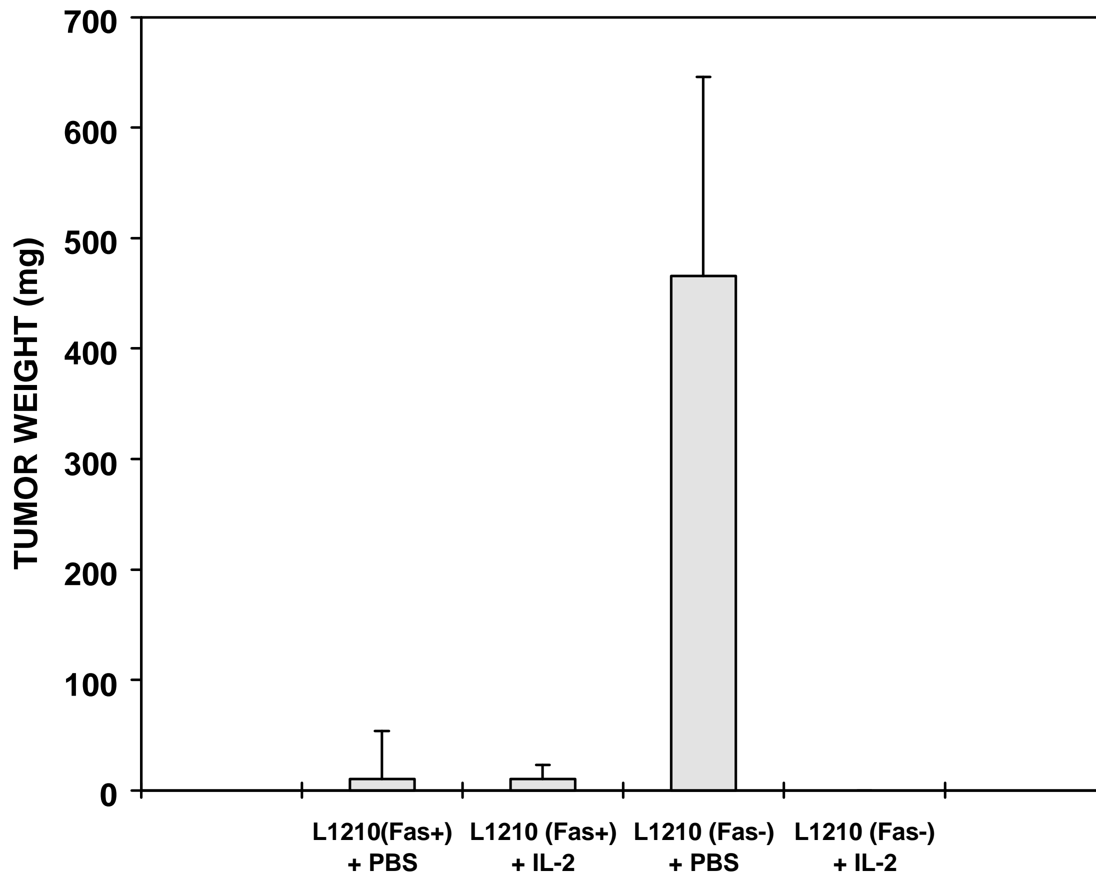

Fig. 2.13. Effect of immunotherapy with IL-2 against L1210 (Fas^+) and L1210 (Fas^-) on tumor growth. 10 DBA/2 mice were injected subcutaneously with either 1 x 10^6 L1210 (Fas^+) or 1 x 10^6 L1210 (Fas^-) tumor cells. 5 mice from each group were injected i.p. with 0.2 ml of PBS twice daily. The other 5 mice were injected i.p. with 50,000 U IL-2 twice daily. On day 7 all mice were sacrificed and examined for tumors. The average tumor size for each group was taken.

CHAPTER 3.0: ACTIVATION OF NATURAL KILLER CELLS THROUGH CD44

3.1: INTRODUCTION

Natural killer cells are large granular lymphocytes (Hori, *et. al.,* 1988) that lyse tumor and virus infected cells without prior exposure to these cells in an MHC II unrestricted manner (Lanier, *et. al.,* 1986). NK cells are also activated by such cytokines as IL-2 and IFN–γ. Upon activation, NK cells are better able to lyse sensitive targets, as well as NK resistant targets (Oehler, 1978). This is known as lymphokine activated killer activity (Rosenberg and Lotze, 1986).

NK cells express various adhesion molecules or receptors through which they adhere to targets or transmit signals for activation. Thus, they are capable of antibody dependent cell cytotoxicity (ADCC) (Lanier, *et. al.,* 1986). Cytokines have been shown to regulate adhesion molecule expression on NK cells. For example, IL-2 can down regulate L-selectin through which NK cells bind to peripheral lymph nodes. In addition, IL-2 can increase CD44 through which NK cells bind to high endothelial venules (HEVs) (Uksila, *et. al.,* 1997).

CD44 also called Pgp-1, In(Lu) related p80, Hermes, ECMR III, Hutch 1 (Tan, *et. al.,* 1993), and hyaluronate receptor (Gunthert, 1993) is a 90 kDa protein involved in endothelial cell recognition found on all mononuclear cells. This adhesion molecule regulates primary and metastatic tumor development *in vivo*, as well as has a role in the development of B cells in bone marrow (Banchereau, 1992). Possible ligands for CD44 include hyaluronic acid, extracellular matrix proteins (collagen types I and IV, and fibronectin), and mucosal addressins (Tan *et. al.* 1993). It can also bind to MAdCAM-1 and endothelial glycans. It has also been shown that T cells activated through CD44 bind to targets through LFA-1. In addition, CD44 can trigger Ca^{2+} influx (Dianzani and Malavasi, 1995).

Hyaluronate is part of the extracellular matrix and is a high mass polysaccharide. It plays a role in cell proliferation, migration, and differentiation; as well as it immobilizes water in the extracellular matrix. Binding to hyaluronate can induce cellular aggregation, lymphocyte activation, and secretion of cytokines. The NH_2-terminal region of CD44 is responsible for binding to CD44 (Gunthert, 1993).

Inasmuch as it has been shown that binding of NK cells to HEVs can be disrupted by the addition of mAb Hermes-3 (Gunthert, 1993) and mAbs S5 and IM7 can increase NK activity in a dose dependent manner (Tan, *et. al.,* 1993), we investigated the ability of NK cells to mediate lysis of different tumor targets as well as endothelial cells through CD44 and its receptor, hyaluronate. Also, previous studies from our laboratory demonstrated that CTL and $CD4^{-}CD8^{-}$

(double negative) T cells can mediate efficient lysis of tumor targets when activated through CD44 (Seth,*et. al.,* 1991; Hammond, *et. al.* 1993).

3.2: MATERIALS AND METHODS

3.2.1: Mice

C57BL/6 +/+ mice were purchased from Charles River (Boston, MA). C57BL/6 *gld/gld* mice purchased from Jackson Labs (Bar Harbor, ME). Perforin-deficient mice were kindly provided from William Clark, UCLA, Los Angeles, CA, and bred in our facilities (Seth, *et. al.,* 1988; Kakkanaiah, *et. al.,* 1991). In all experiments, mice were 3-4 weeks of age and female.

3.2.2: Cell Lines

The tumor cell lines used were YAC-1, a Moloney virus induced lymphoma sensitive to NK cells; and P815, a mastocytoma resistant to NK cells. All were maintained in RPMI (Gibco) supplemented by 10% fetal calf serum, as previously described (Seth, *et. al.,* 1991). TME-3H3 (transformed murine endothelial), an endothelial cell line, immortalized by SV40 developed by A. Hamann (Harder, *et. al.,* 1991) was generously provided by J. Lesley, The Salk Institute, and maintained in tissue culture medium (O'Connell and Ediden, 1990).

3.2.3: NK/LAK Cells

NK cells were purified as previously described (Nagarkatti, *et. al.,* 1988). Briefly, single cell suspensions of the spleen were prepared in RPMI-1640 medium supplemented by 10% fetal calf serum (Atlanta Biologicals, Norcross, GA) (Hammond, *et. al.,* 1993) using an homogenizer (Stomacher, Tekmar, Cincinnatti, OH). Plastic adherence for one hour at 37° C, was used to deplete the macrophages. The cells were passed over nylon wool columns and the nonadherent cells were cultured for approximately 48 hours with 1000 U/ml of IL-2 (kindly provided by Hoffman LaRoche, Nutley, NJ).

3.2.4: Antibodies

The mAbs used were culture supernatants from hybridomas and concentrated as previously described (Seth, *et. al.,* 1991). The following mAbs were used: GK1.5 directed against CD4; 3.155 directed against CD8; H57-597 directed against TCR-ab; 145.2C11 directed against CD3; MEL-14 directed against $gp^{mel\text{-}14}$; PK136 directed against NK1.1; 9F3 directed against CD44; and KM201 (purchased from Southern Biotechnologies). CD44 receptor globulin (CD44 Rg) (generously provided by Dr. Ivan Stamenkovic, Massachusettes General Hospital, Boston, MA) was derived from the transfection of COS cells as described (Aruffo, *et. al.,* 1990)

The supernatants were concentrated and purified using Centriprep centrifugation concentrator tubes (Amicon).

3.2.5: Flow Cytometry

Staining was carried out as previously described (Seth, *et. al.* 1991). NK cells were first depleted of any contaminating T cells by treating the cells with mAbs directed against CD4, CD8, CD3, and TCR-αβ on ice for 30 minutes. The cells were then washed three times and treated with rabbit complement (Cedarlane Laboratories, Westbury, NY) for 30 minutes at 37° C. The primary antibody was then added and incubated on ice for 30 minutes. A FITC labeled secondary antibody (Fab fragments) was added and the cells again incubated on ice for 30 minutes. The cells were washed and fluorescence was measured by a flow cytometer.

3.2.6: ^{51}Cr-Release Assay To Measure Cytotoxicity

The cytotoxicity mediated by LAK/NK cells was studied using a ^{51}Cr-release assay (Seth, *et. al.,* 1991). Target cells (YAC-1, P815, and TME-3H3) were labeled with 100 μCi ^{51}Cr in the form of $Na_2{}^{51}CrO_4$, incubated at 37° C, washed three times and then seeded in 96 well plates (Costar, Cambridge, MA) at 5 x 10^3 cells/well, along with varying numbers of effector cells. The plates were incubated at 37° C for 4 hours. Following the incubation, the plates were harvested with the Titertech collecting system (Skatron Inc., Sterling, VA). The amount of ^{51}Cr released by the target cells was measured using a g-counter (TmAnalytic, Elk Grove Village, IL). Percent cytotoxicity was calculated as: (experimental release - control release)/(total release - control release) x 100. In some studies, the assay was performed in presence of hyaluronate (Sigma) at a concentration of 0.5 mg/ml or 0.25 mg/ml). mAbs to CD44 (KM20 and 9F3) were also used in some assays to study redirected cytotoxicity.

3.3: RESULTS

3.3.1: NK cells express CD44

It has been shown that NK cells express CD44 (Tan *et al.,*), and that culturing NK cells with IL-2 increases proliferation, activation (Podack, *et. al.,* 1991), and adhesion molecule expression (Storkus, *et. al.,* 1991). In the current study we wished to check the purity of NK cells and corroborate the expression of CD44 on NK cells from wild-type mice following culture with high concentrations of IL-2. As seen in Fig. 3.1, purified NK cells did not express CD3 and TCR-αβ which are expressed on T cells. However, they did express NK1.1 which is an adhesion molecule specific for natural killer cells. They also expressed gp^{mel-14}, an adhesion molecule

involved in lymhocyte homing. Furthermore, all NK cells expressed CD44 at a high density. This suggested that CD44 may be involved in NK cell mediated cytotoxicity upon activation with cytokines such as IL-2.

3.3.2: Monoclonal Antibodies to CD44 and soluble Hyaluronate are able to inhibit lysis of YAC-1 tumor targets by NK/LAK cells

We next wanted to address whether mAbs to CD44 could inhibit lysis of NK/LAK cells derived from wild-type mice against the NK sensitive target YAC-1. Thus we performed a cytotxicity assay with spontaneous and IL-2 activated NK/LAK cells in the presence of mAbs against CD44. Inasmuch as, YAC-1 cells are FcR-negative, the Abs did not participate in redirected lysis (Seth, *et. al.,* 1991). As seen in Figs. 3.2 and 3.3, KM201 and 9F3 (mAbs against CD44) were able to inhibit lysis significantly in both spontaneous and IL-2 activated NK/LAK cells. These data demonstrated that CD44 played a role in cytotoxicity of NK/LAK cells against YAC-1 targets.

KM201 showed more inhibition than 9F3 in IL-2 activated NK/LAK cells. Inasmuch as, KM201 is specific for hyaluronate binding region of CD44, we next investigated whether the addition of soluble hyaluronate to the cytotoxicity assay would also inhibit the lytic activity of NK/LAK cells. As seen in Fig. 3.4, hyaluronate was also able to inhibit lysis of YAC-1 tumor targets by wild-type NK/LAK cells. These data suggested that hyaluronate may serve as a ligand through which CD44 binds to YAC-1 and mediates lysis.

3.3.3: Monoclonal antibodies to CD44 and soluble Hyaluronate are able to inhibit lysis of P815 by NK/LAK cells

We also looked at whether soluble hyaluronate could inhibit lysis of P815 tumor targets as it did for YAC-1. In this experiment we used wild-type, *gld/gld,* and perforin-deficient mice. *gld/gld* mice have a defect in their Fas ligand (FasL) and are thus incapable of carrying out FasL-mediated apoptosis. Perforin-deficient mice are knockout mice incapable of producing perforin, a lytic molecule secreted by cytotoxic cells to kill target cells. As seen in Fig. 3.5, hyaluronate significantly decreased lysis of P815 by wild-type NK/LAK cells. NK/LAK cells derived from *gld/gld* mice were also inhibited from lysing P815 by soluble hyaluronate (Fig. 3.6). However, perforin-deficient NK/LAK cells were not inhibited by the addition of hyaluronate (Fig. 3.7). This may be because CD44 activates a perforin-dependent pathway which the perforin-deficient mice would be incapable of carrying out.

3.3.4: Monoclonal antibodies to CD44 and soluble hyaluronate are able to inhibit the lysis of endothelial cells by NK/LAK cells

Because adoptive immunotherapy of cancer using LAK cells, IL-2 and LAK cells, IL-2, alone can lead to vascular leak syndrome (Rosenberg, *et. al.,* 1987), a disease characterized by increased vascular permeability which might be caused by lymphocytes lysing endothelial cells (Ohkikubo, *et. al.* 1991), we wanted to see if IL-2 activated NK/LAK cells would lyse endothelial cells in the same manner that they lysed tumor cells, and if so, we wished to address whether this lysis was mediated through CD44.

In the first set of experiments we tested whether wild-type naive or IL-2 activated NK/LAK cells could lyse endothelial cells. As seen in Fig. 3.8, naive NK cells failed to mediate lysis of endothelial cells. However, upon IL-2 activation the NK/LAK cells were able to mediate enhanced lysis of endothelial cells. These data suggested that IL-2 increases the lytic activity of NK/LAK cells to mediate efficient lysis of endothelial cells.

To further corroborate the role of CD44, we repeated the cytotoxicity assay again. However, this time in the presence of soluble CD44 (CD44 Rg). As seen in Fig. 3.9, soluble CD44 is able to significantly decrease lysis of cells by activated NK/LAK cells.

Finally, we addressed whether soluble hyaluronate would inhibit lysis of endothelial cells by NK/LAK cells as it did for YAC-1 and P815. As seen in Fig. 3.10, soluble hyaluronate completely inhibited lysis of endothelial cells by IL-2 activated LAK/NK cells. This further implicates CD44-hyaluronate interaction in the lysis of endothelial cells.

3.4: DISCUSSION

In the current study, we demonstrated that IL-2 activated NK cells expressed increased levels of CD44 and that IL-2 activated NK/LAK cells used this adhesion molecule to mediate lysis of tumor targets. Furthermore, we showed that CD44 uses hyaluronate to bind to these target cells. Moreover, NK/LAK cells were able to mediate lysis of endothelial cells and that this lysis was mediated by the CD44-hyaluronate pathway.

Previous studies have shown that cytotoxic T cells utilize CD44 to mediate lysis of tumor targets (Seth, *et. al.,* 1991) and that NK cells from dogs could mediate lysis of target cells through CD44 (Tan, *et. al.,* 1993). It has also been suggested that hyaluronate is the receptor for CD44 and that the binding of NK cells to HEVs can be disrupted by certain mAbs (Gunthert, 1993; Uksila, *et. al.,* 1997). In the current study we investigated the role of IL-2 activated NK/LAK activity using sensitive and resistant targets, as well as endothelial cells; and whether lysis of such targets is mediated through the CD44-hyaluronate pathway. Using NK sensitive

YAC-1 we found that mAbs to CD44, specifically KM201 and 9F3 were able to inhibit lysis in both spontaneous as well as IL-2 activated LAK/NK cells. Because KM201 is a mAb specific for the epitope of CD44 that binds to hyaluronate on target cells, we added soluble hyaluronate to see if it could block the lysis of the tumor targets. Soluble hyaluronate was able to inhibit NK cell-mediated lysis significantly. This suggests that NK/LAK cells use CD44 and its ligand, hyaluronate, to mediate lysis of YAC-1 tumor targets.

To further explore the role of CD44 and hyaluronate in LAK/NK mediated lysis of tumor targets, we tested the NK resistant tumor cell line P815. Soluble hyaluronate was again able to inhibit lysis of LAK/NK cells showing that CD44 plays a role in NK/LAK mediated lysis of P815 as in YAC-1. In addition, hyaluronate inhibited lysis of NK/LAK cells derived from FasL defective mice. However, hyaluronate was not able to inhibit lysis in NK/LAK cells derived from perforin-deficient mice. This indicates that CD44 mediated lysis uses a perforin-dependent pathway.

To further investigate the role of CD44, we tested whether NK/LAK cells could mediate lysis of endothelial cells and if so, whether CD44 was involved. NK/LAK cells were shown to lyse endothelial cells to a significant degree. Interestingly, following incubation with IL-2, the lysis of endothelial cells increased significantly. Furthermore, soluble CD44 was able to inhibit this lysis as seen in tumor targets. Even more significant, is the fact that hyaluronate completely blocked the lysis of endothelial cells. These data demonstrate that hyaluronate expressed on tumor cells or endothelial cells, may serve as a ligand for CD44 in the induction of cytotoxicity. These data are consistent with recent studies from our laboratory in which it was shown that hyaluronate could activate B cells to proliferate and differentiate following incubation with CD44 (Rafi, *et. al.,* 1997).

Thus NK/LAK cells use CD44 and its ligand to kill tumor targets as well as endothelial cells. This demonstrates that IL-2 administration can not only activate NK/LAK cells to fight cancer, but may also cause damage to endothelial cells leading to vasular leak syndrome.

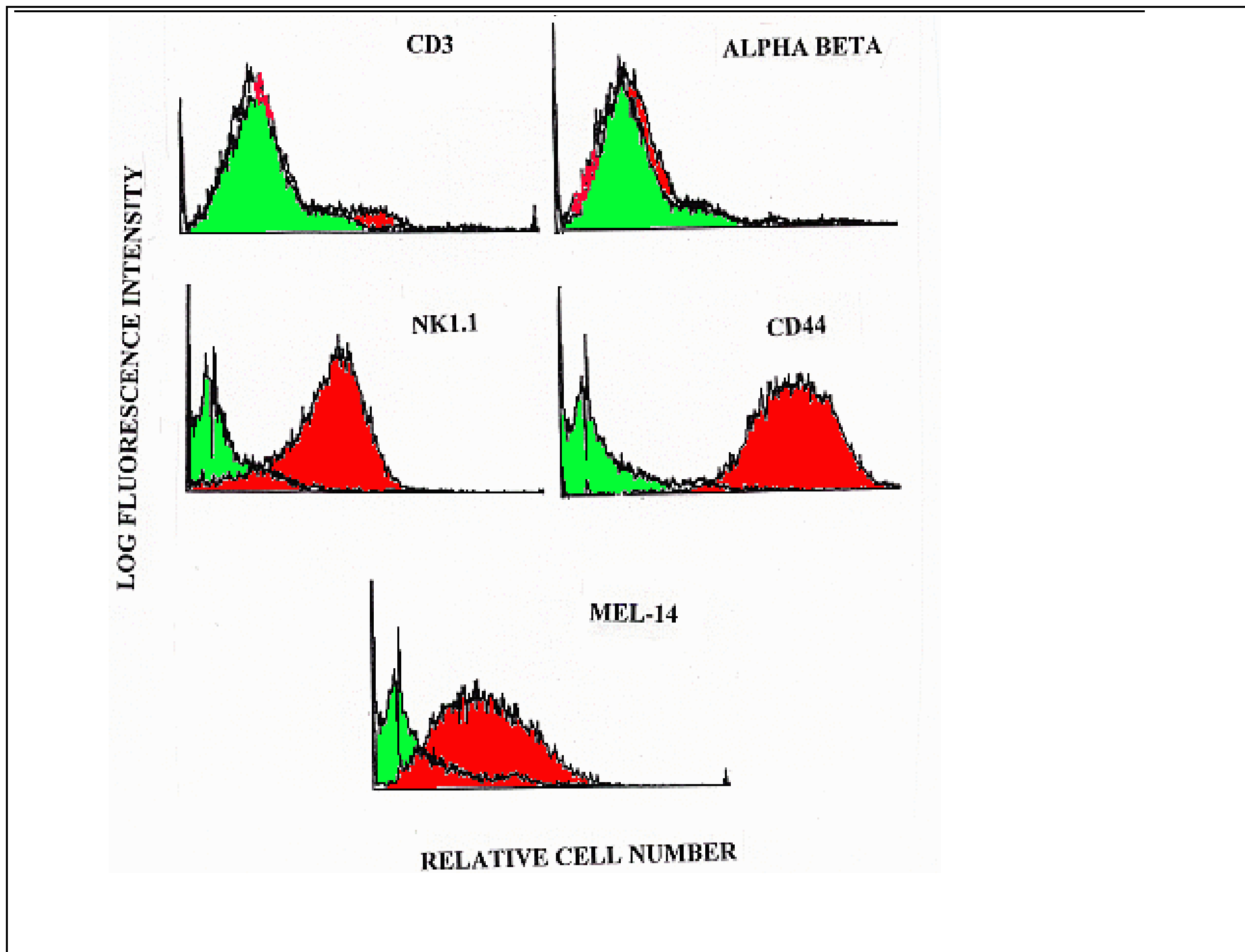

Fig. 3.1. IL-2 activated NK cells express CD44 at a high density. NK cells were derived and purified from the spleens of C57BL/6 mice and activated with 1000U/ml of IL-2 for 48 hours. They were then stained for various adhesion molecules with mAbs and analyzed by flow cytometry.

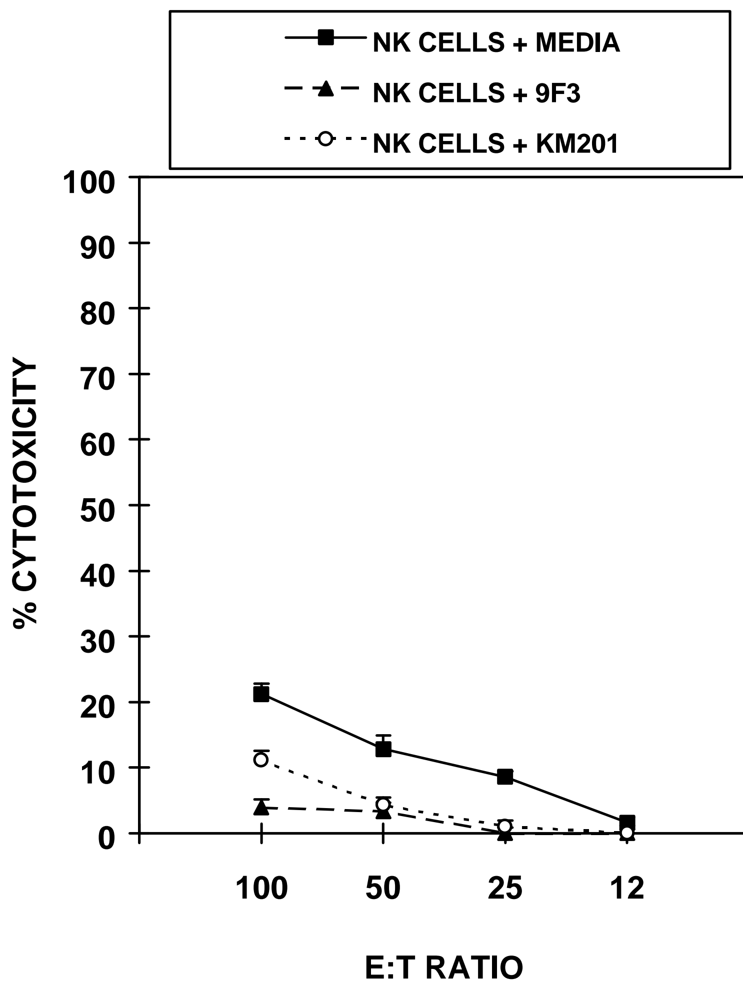

Figure 3.2. Monoclonal antibodies to CD44 are able to inhibit lysis by spontaneous NK cells of YAC-1. Nylon wool purified spleen cells from C5BL/6 mice were used. Cytotoxicity was measured in a ^{51}Cr-release assay in the presence of either media or anti-CD44 mAbs (9F3 and KM201, 100 ng/ml).

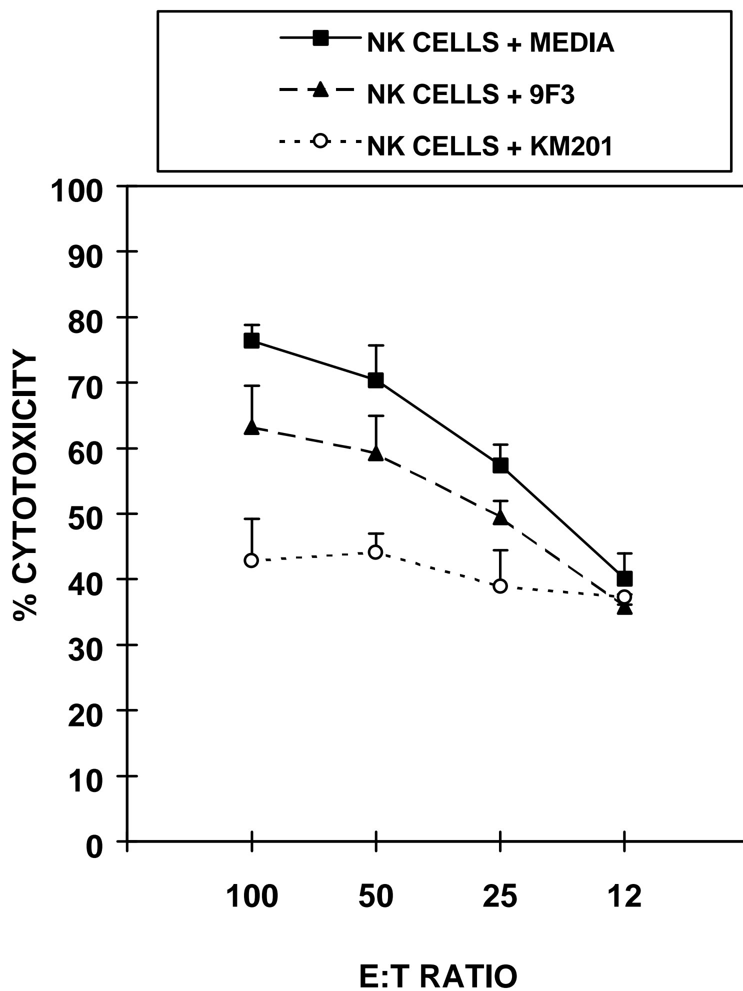

Fig. 3.3. Monoclonol antibodies to CD44 are able to inhibit lysis by IL-2 activated NK cells of YAC-1. Nylon wool purified spleen cells from C57BL/6 mice were cultured *in vitro* for 48 hours with 1000 U/ml of IL-2. Cytotoxicity was measured in a ^{51}Cr release assay in the presence of either media or anti-CD44 mAbs (KM201 and 9F3, 100 ng/ml).

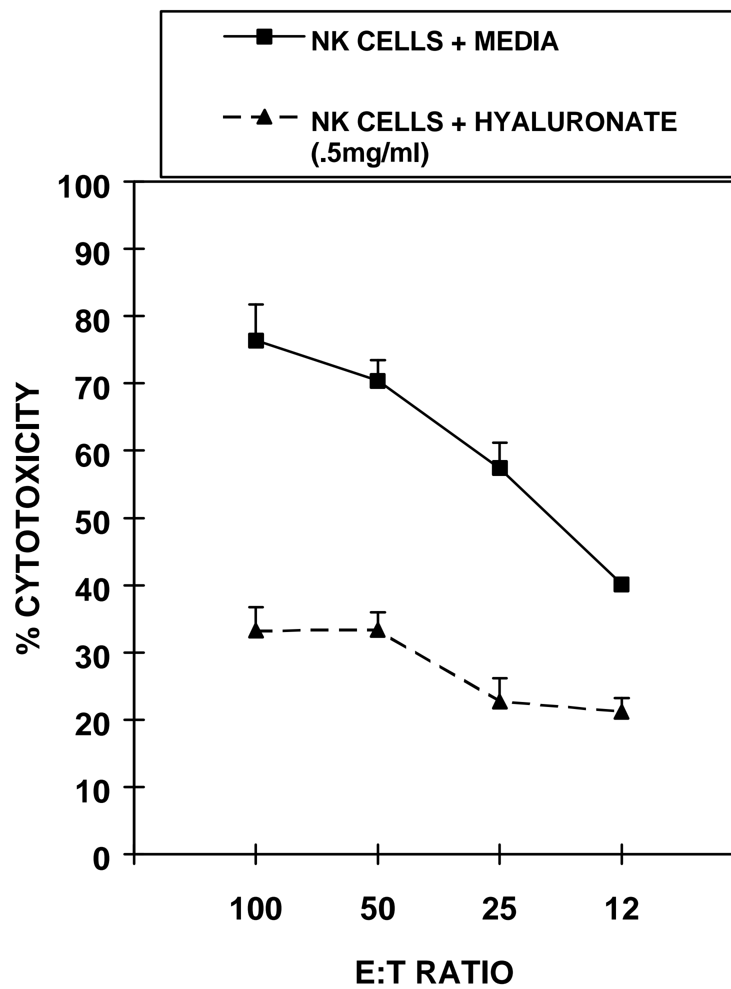

Fig. 3.4. Soluble hyaluronate is capable of inhibiting lysis of YAC-1 by IL-2 activated NK cells. Nylon wool purified spleen cells from C57BL/6 mice were cultured *in vitro* for 48 hours with 1000 U/ml of IL-2. Cytotoxicity was measured in a ^{51}Cr release assay in the presence of media or hyaluronate (0.5 mg/ml).

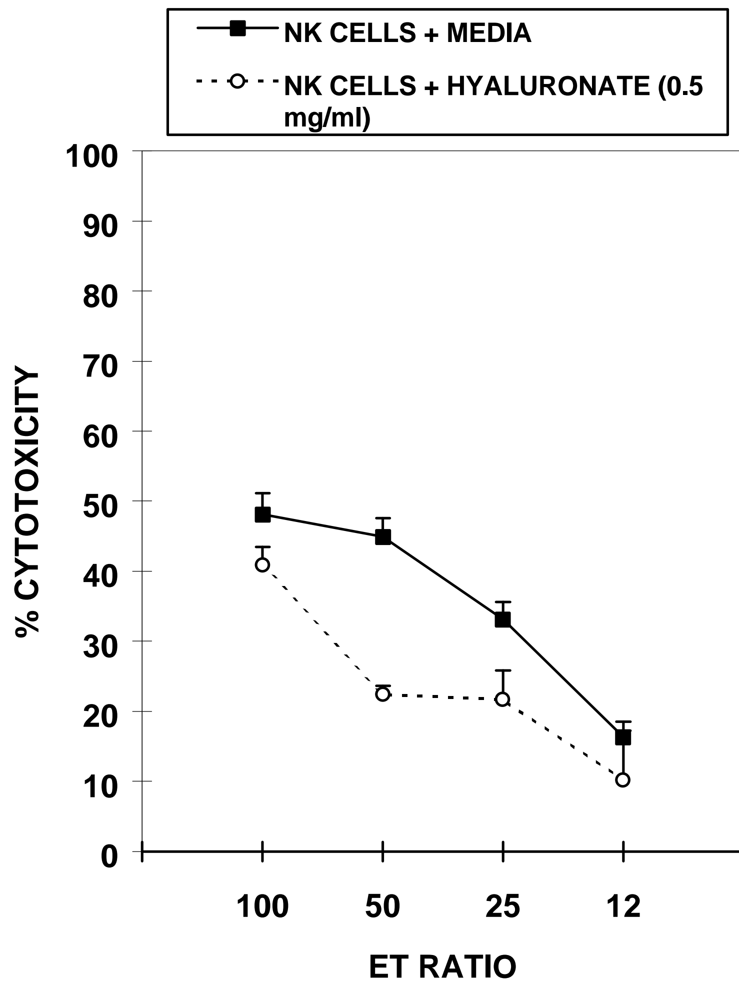

Fig. 3.5. Hyaluronate inhibits lysis of P815 by IL-2 activated wild-type NK cells. Nylon wool purified spleen cells from C57BL/6 mice were cultured *in vitro* for 48 hours with 1000 U/ml of IL-2. Cytotoxicity was measured in a ^{51}Cr release assay in the presence of media or hyaluronate (0.5 mg/ml).

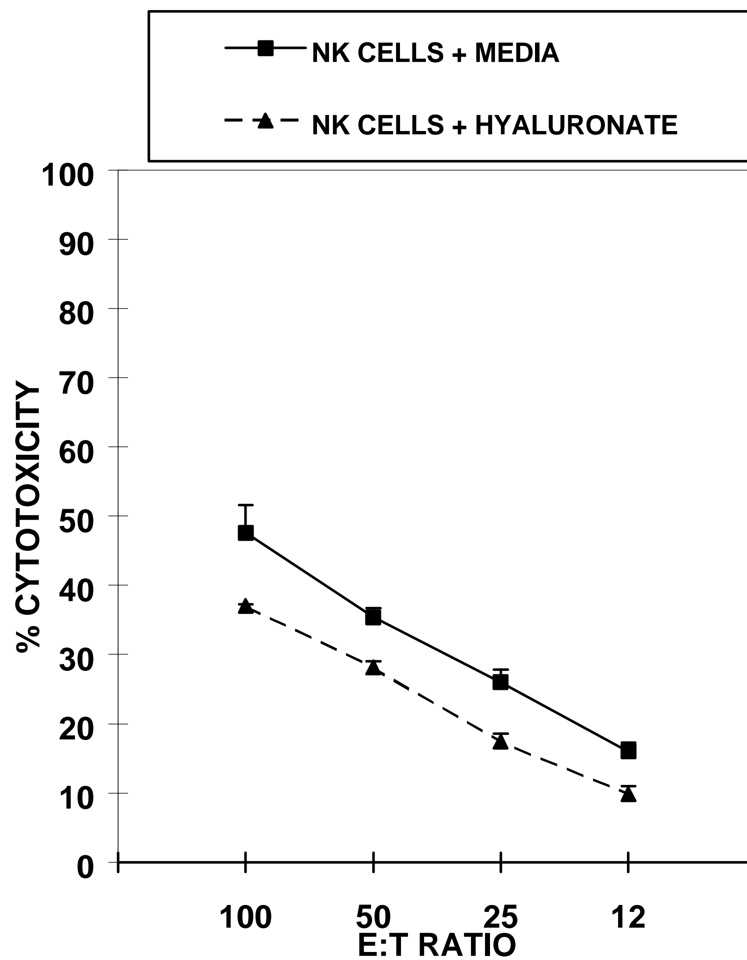

Fig. 3.6. Hyaluronate inhibits lysis of P815 by IL-2 activated *gld/gld* NK cells. Nylon wool purified spleen cells from C57BL/6 mice were cultured *in vitro* for 48 hours with 1000 U/ml of IL-2. Cytotoxicity was measured in a ^{51}Cr release assay in the presence of either media or hyaluronate (0.5 mg/ml).

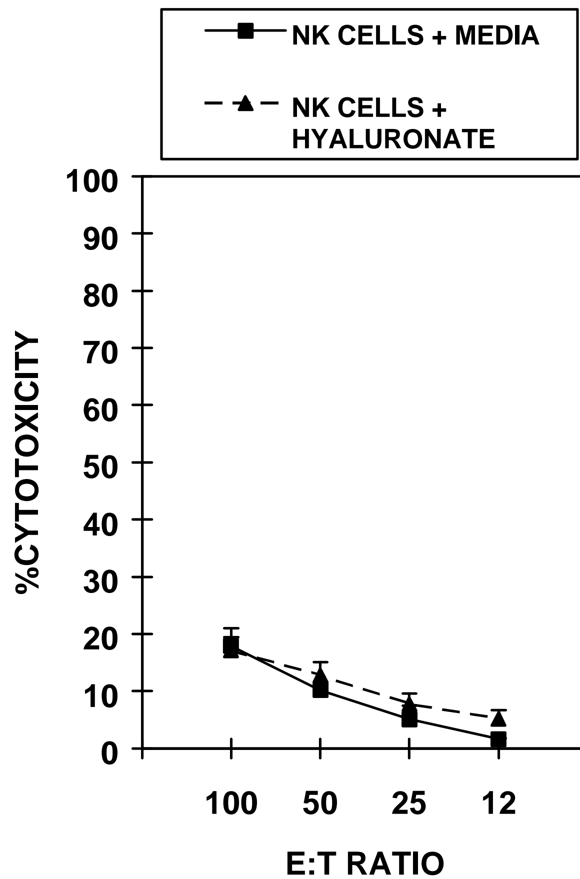

Fig. 3.7. Hyaluronate inhibits lysis of P815 by IL-2 activated perforin-deficient NK cells. Nylon wool purified spleen cells from C57BL/6 mice were cultured *in vitro* for 48 hours with 1000 U/ml IL-2. Cytotoxicity was measured in a ^{51}Cr release assay in the presence of either media or hyaluronate (0.5 mg/ml).

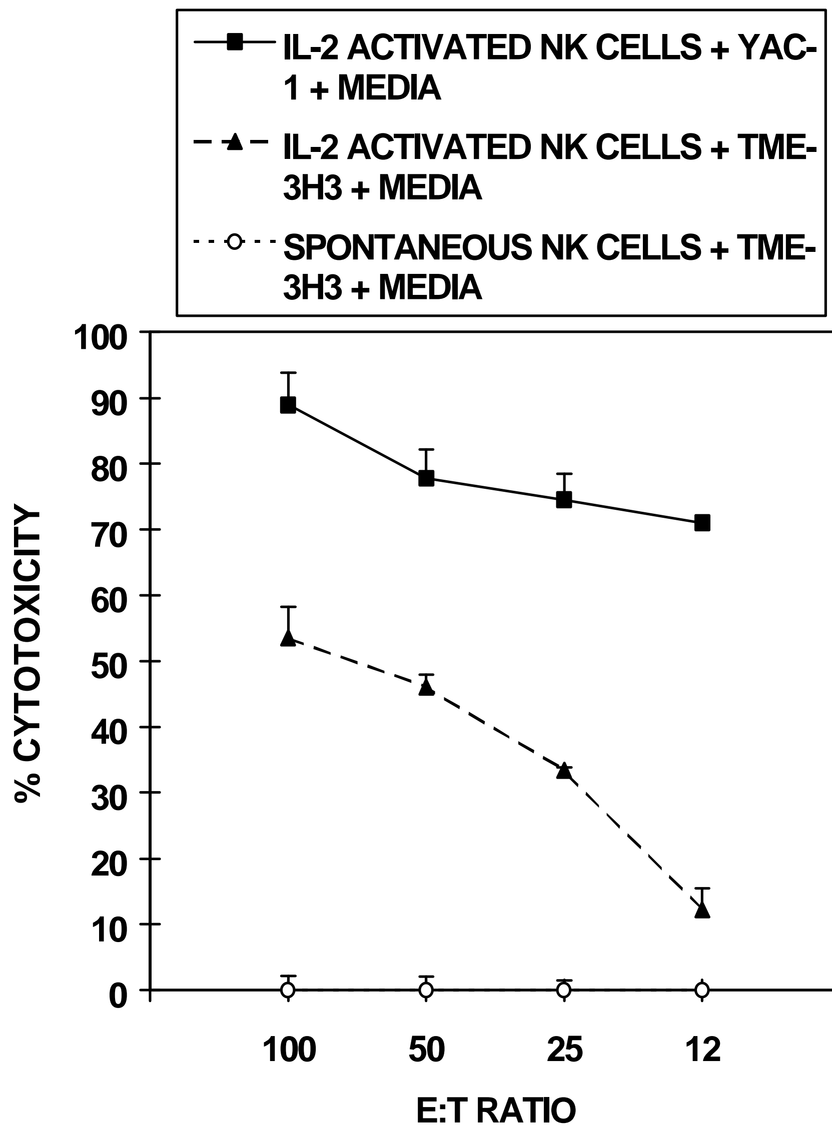

Fig. 3.8. NK/LAK cells are capable of lysing TME-3H3 endothelial cells. Nylon wool purified spleen cells from C57BL/6 mice were spontaneously tested for cytotoxicity or tested after culture *in vitro* for 48 hours with 1000 U/ml of IL-2. Cytotoxicity was measured in a ^{51}Cr release assay.

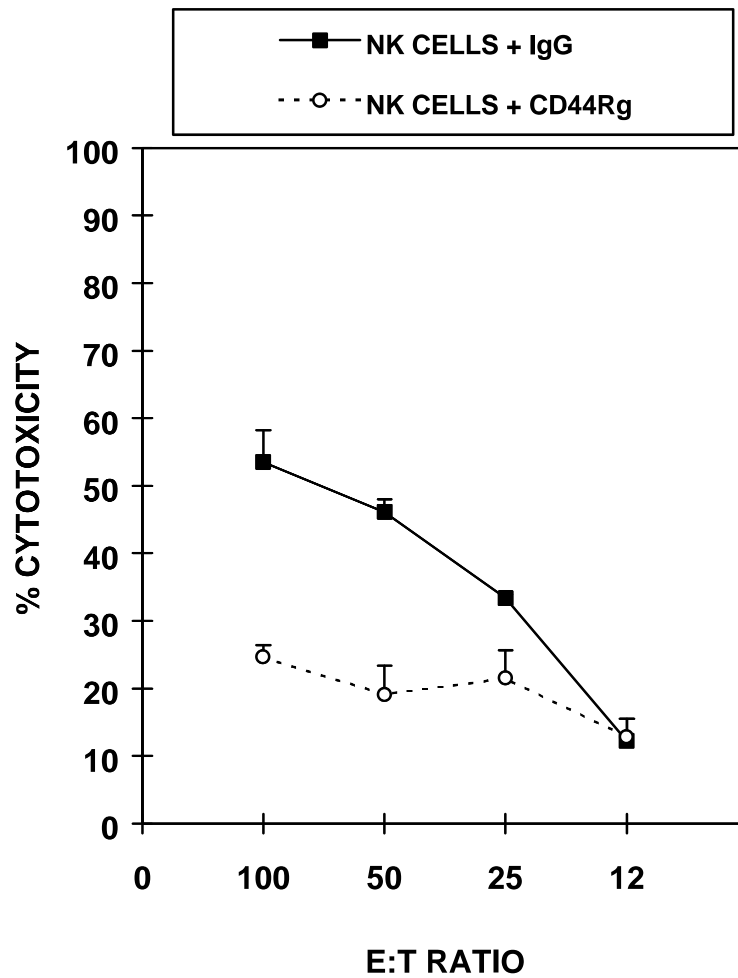

Fig. 3.9. Soluble CD44 inhibits lysis of TME-3H3 endothelial cells by IL-2 activated NK cells. Nylon wool purified spleen cells from C57BL/6 mice were cultured *in vitro* for 48 hours with 1000 U/ml of IL-2. Cytotoxicity was measured in a ^{51}Cr release assay in the presence of either media or soluble CD44 (CD44 Rg, 100 ng/ml).

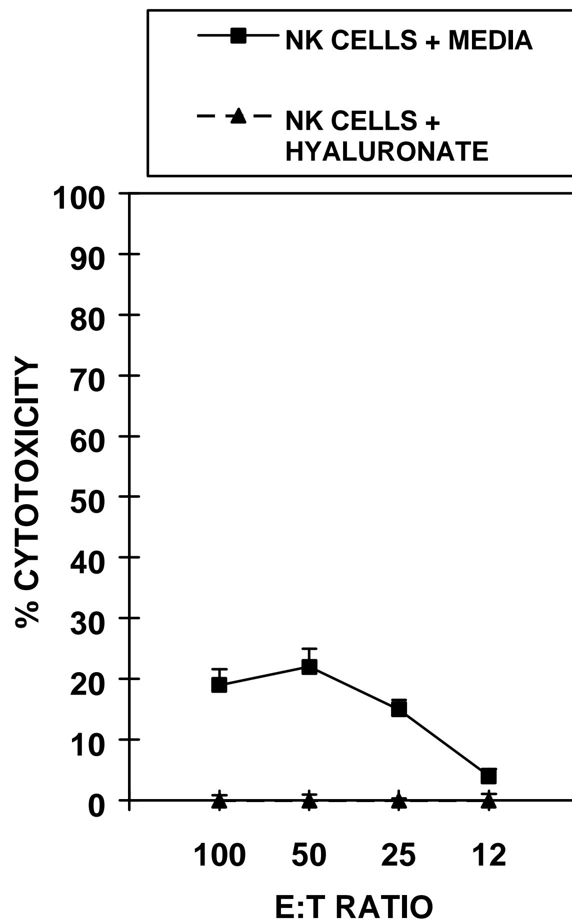

Fig. 3.10. Hyaluronate can inhibit lysis of TME-3H3 endothelial cells by IL-2 activated NK cells. Nylon wool purified spleen cells from C57BL/6 mice were cultured *in vitro* for 48 hours with 1000 U/ml of IL-2. Cytotoxicity was measured in a ^{51}Cr release assay in the presence of either media or hyaluronate (0.5 mg/ml).

CHAPTER 4.0: CONCLUSION AND SIGNIFICANCE OF THE CURRENT STUDY

In conclusion, we have demonstrated, using a variety of tumor targets and strains of mice, that NK/LAK cells can be activated by either poly I:C or IL-2 to utilize both the perforin- and FasL-based pathways to mediate efficient lysis of target cells. This was shown by activating NK cells both *in vivo* and *in vitro.* Furthermore, activated NK/LAK cells lysed Fas^+ tumor targets better than Fas^- targets. In addition, Fas^+ tumor targets were easily rejected by the syngeneic host when compared Fas^- targets. These data suggested that Fas^+ tumor cells are more immunogenic. Thus transfection of Fas-deficient tumor cells with Fas cDNA and their use *in vivo* may provide a useful approach to develop a vaccine or immunize against the wild-type tumor. We also showed that NK/LAK cells use the perforin pathway to a greater extent. These data are important because it shows that deficiency in perforin may lead to increased susceptibility to cancer. However, upon activation, NK cells readily kill tumor targets, using both perforin and FasL-based pathways. Thus, during immunotherapy of cancer using IL-2, Fas^+ tumor targets may be more susceptible to anti-tumor immunity than Fas^- tumors.

We have also shown that IL-2 activated NK/LAK cells express the adhesion molecule CD44 to a high degree and use this molecule to kill a variety of tumor targets. Moreover, hyaluronate serves as a ligand for CD44 on both tumor cells and endothelial cells. Thus, NK/LAK cells utilize CD44 in homing, binding, and mediating lysis of target cells. Inasmuch as, ligands for CD44 such as hyaluronate, are expressed both on endothelial cells as well as on tumor cells, IL-2 therapy may lead to anti-tumor immune response as well as nonspecific killing of endothelial cells triggering vascular leak syndrome. Thus IL-2 therapy may constitute a double-edged sword mediating the killing of both tumor cells as well endothelial cells. Further studies to identify whether different CD44 isoforms are involved in homing, adhesion, and killing of target cells should provide useful information on the possibility of selectively neutralizing the toxicity associated with IL-2 therapy while retaining the anti-tumor efficacy.

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CURRICULUM VITAE

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EDUCATION

Masters of Science, Biology (Immunology), May 1997
Virginia Polytechnic Institute and State University, Blacksburg, VA
Thesis: Activation of Natural Killer Cells Through Adhesion Molecules
Advisors: Dr. Mitzi Nagarkatti and Dr. Prakash Nagarkatti
Q.C.A.: 3.45

Bachelor of Science, Biology (Microbiology/Immunology option), May 1992
Minors: Chemistry and History
Virginia Polytechnic Institute and State University, Blacksburg, VA
Q.C.A.: 3.14; GRE Verbal: 590 GRE Quantitative: 620

TEACHING INTERESTS

Taught Principles of Biology Lab and General Biology Lab

- Prepared and presented prelab lectures
- Created handouts, quizzes, and practicals
- Graded quizzes, practicals, and term papers
- received 3.2-3.45/4.00 overall rating on teaching evaluations

PRESENTATIONS AT MEETINGS

Bradley, Michael J., Lisa Gote, and Mitzi Nagarkatti. Activation of Natural Killer Cell Mediated Cytotoxicity Through Adhesion Molecules CD44 and gp^{mel-14}. VAS meeting, May 20, 1993.

Bradley, Michael J., and Mitzi Nagarkatti. Natural Killer Cell Activation and Cytotoxicity Mediated Through Adhesion Molecules CD44 and gp^{mel-14}. ASM meeting, Nov. 13, 1993.

Bradley, Michael J., Lisa Gote, Prakash Nagarkatti, and Mitzi Nagarkatti. Role of CD44 and CD95(Fas) in the Regulation of Natural Killer Cell Mediated Cytotoxicity. FASEB meeting, April 13, 1995.

Bradley, Michael J., Prakash Nagarkatti, and Mitzi Nagarkatti. Perforin But Not FasL Plays a Critical Role in the Spontaneous and IL-2 Induced Lytic Activity by NK Cells: Evidence From FasL Mutant and Perforin Knockout Mice. ACS meeting, March 29, 1996.

Bradley Michael J., Mitzi Nagarkatti, and Prakash Nagarkatti. Fas Ligand is Not Critical for Spontaneous and Il-2 Induced Lytic Activity by NK Cells: Evidence From Fas Ligand-Mutant *gld* mice. AACR meeting, April 22, 1996.

GRANTS/SCHOLORSHIPS/AWARDS

Role of Adhesion Molecules in Successful Immunotherapy of Cancer, from Sigma Xi, $300; matched by Biology Department.

Role of Natural Killer Cell Adhesion Molecule Interaction in Successful Immunotherapy of Cancer, $400; matched by Biology Department.

Instructional Tuition Fees Scholarship, $1500, Fall 1994

Graduate Teaching Assistantship, Spring 1993-Fall 1995

ABSTRACTS PUBLISHED

Bradley, Michael J., Lisa, Gote, and Mitzi Nagarkatti. Activation of Natural Killer Cells Mediated Through Adhesion Molecules CD44 and gp^{mel-14}. Virginia Journal of Science, Vol. 44, No. 2., 1993.

Bradley, Michael J., Lisa Gote, Prakash Nagarkatti, and Mitzi Nagarkatti. Role of CD44 and CD95(Fas) in the Regulation of Natural Killer Cell Mediated Cytotoxicity. FASEB Journal. Vol. 9, No. 4., 1995.

Bradley, Michael J., Prakash Nagarkatti, and Mitzi Nagarkatti. Perforin But Not FasL Plays a Critical Role in the Spontaneous and IL-2 Induced Lytic Activity by NK Cells: Evidence From FasL Mutant and Perforin Knockout Mice. Published in the American

Cancer Society, 16th Annual Seminar of Cancer Researchers in Virginia Symposium booklet. March 29, 1996.

MANUSCRIPTS SUBMITTED

Bradley, Michael J., Mitzi Nagarkatti, and Prakash Nagarkatti. Fas Ligand is Not Critical for Spontaneous and IL-2 Induced Lytic Activity by NK Cells: Evidence From Fas Ligand-Mutant *gld* Mice. Submitted to The European Journal of Immunology.

PROFESSIONAL MEMBERSHIPS

Virginia Academy of Science
American Society for Microbiology
American Association of Immunologists
Sigma Xi Grants-in-Aid, the Scientific Research Society
American Association for Cancer Research

LABORATORY SKILLS

- Set up and ran numerous assays while researching the activation of NK cells
- Work with both *in vivo* and *in vitro* assays
- Proficient in ^{51}Cr release cytotoxicity assays
- Performed ^{3}H uptake proliferation assays
- Carried out BLTE colorimetric assays
- Cell staining and flow cytometric analysis
- Familiar with various tissue culture techniques
- Maintained tumor cell lines
- Performed cryogenic freezing techniques
- Responsible for ordering laboratory supplies

HONORS AND ACTIVITIES

Academic Dean's List: Fall, 1990 and Spring 1992

Phi Sigma Society (National Biological Honor Society)

Alpha Epsilon Delta (National Pre-Medical Honor Society)
President 1991-1992

Virginia Tech Well Baby Clinic 1990-1992

Student Volunteer Coordinator 1990-1992

Virginia Tech and Montgomery Regional Hospital Preceptorship Program
Preceptorships with Dr. M. Langebeck (Pathology) and Dr. D. Zedalis (Allergy/Immunology)
Preceptorship Committee Member, 1991-1992

Alpha Phi Omega (National Co-ed Service Fraternity)
Bloodmobile Chairman, 1990-1991
M. Buford Blair Award of Merit for Outstanding Service, Spring, 1991
SHARE (Self Help and Resource Exchange) Chairman, 1994-1995
Chapter Program Planning Conference Chairman, 1995-1996
M. Buford Blair Award of Merit for Outstanding Service, Fall, 1996
J. Kenneth Robinson Best Brother Award Distinguished Service Key, 1997
Average of 100 service hours performed each semester (over 1100 to date)