Effect of Homozygous *lpr* and *gld* Mutations on the Immune Functions and Induction of Autoimmunity

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(Abstract)

The murine *lpr* gene encodes for an aberrant form of Fas (CD95), a molecule involved in apoptosis. The mouse *gld* gene leads to the expression of a defective Fas-ligand. Mice homozygous for *lpr* or *gld* mutations develop severe lymphoproliferative and autoimmune disease characterized by the accumulation of unique $CD4^-CD8^-$ (double-negative, DN) T cells. Because of these poor functions in vitro, the nature and significance of DN T cells in the autoimmune disease process is not clear. In the current study we found that *lpr* DN T cells could mediate spontaneous lysis of certain tumor cells as well as mediate redirected lysis of various tumor targets when stimulated through the CD3/αßTCR complex and certain adhesion molecules, such as, CD44 and $gp90^{MEL\text{-}14}$. The DN T cells constitutively transcribed perforin, TNF-*α* and IFN-*γ* genes. Unlike the DN T cells from *lpr* mice, similar cells from *gld* mice failed to exhibit spontaneous cytotoxicity despite expression of similar levels of cytokines and adhesion molecules. Furthermore, *lpr* DN T cells could mediate redirected lysis of Fas^+ but not Fas^- target cells. Together, these studies suggested that lysis of target cells by DN T cells was dependent on the interaction between Fas and Fas-ligand. The fact

that lpr DN T cells can be activated via CD44 and gp-90^{MEL-14} suggested that these T cells may be able to mediate lysis of endothelial cells which bear the ligand for these adhesion molecules. Further studies revealed that the lpr DN T cells could mediate spontaneous lysis of endothelial cells and that CD44-hyaluronate interactions were important for endothelial cell lysis. Thus, interactions between DN T cells and endothelial cells *in vivo* may trigger an inflammatory response and contribute to the vasculitis seen in *lpr* and *gld* mice.

We also addressed the hypothesis that acquired immunodeficiency syndrome (AIDS) may be a consequence of destabilization of the idiotypic network. These studies demonstrated that auto- or allo-immunizations involving recognition of class II MHC antigens can trigger an anti-HIV response and such possibilities should be taken into consideration while delineating the pathogenesis of AIDS.

Dedication

I would like to dedicate this work to my husband, Bryan Patrick McKibben, my parents, William and Mary Hammond, and my grandmother, Margaret R. McHugh all of whom have taught me so much about life.

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Chapter 1: General Introduction and Specific Aims

Introduction:

Immunological research was instigated from the observation that once a person got a disease, they were protected for life from that particular disease. One of the first scientists to investigate the reasons behind this phenomenon was Edward Jenner. In 1798 he observed that milkmaids who were exposed to cowpox by the udders of infected cows got a benign pox-like disease on their hands. Furthermore, these women were immune to smallpox (Stites and Terr, 1987). This observation established the foundation of vaccines which eventually eliminated the existence of smallpox. Because infectious disease was very prevalent at the start of immunological research, pioneering immunologists such as, Pastuer, Koch, and Metchnikov focused primarily on preventing the spread of disease and investigating the mechanisms of defense against infectious agents (Stites and Terr, 1987). Modern immunology has a much broader focus of studying the immune system, including the ability to distinguish self from nonself, immune cell differentiation, autoimmunity, and immunotherapy of disease.

The immune system responds to antigens which can either be foreign agents, or self components which have been altered due to viral infection or transformation. However, the immune system, under normal conditions should not respond to unaltered self molecules. Response to antigens can be either via specific recognition through T or B cells or via nonspecific recognition via phagocytic cells including

macrophages, monocytes, and dendritic cells, or large granular lymphocytes (LGL) consisting of NK cells, eosinophils, and basophils (Golub and Green, 1991).

A nonspecific immune response involves the recognition of whole bacterial cells or other foreign solid material by phagocytic cells. The phagocytes ingest the foreign material and break it down into smaller components, some of which may be expressed on the surface of the phagocyte in association with major histocompatibility complex (MHC) molecules. MHC encoded molecules expressed on the membranes of cells enable the immune system to distinguish self from nonself. Class II MHC is expressed only on antigen presenting cells such as macrophage, monocytes, B cells, and dendritic cells and usually presents extracellular peptides to T cells. Class I MHC is expressed by all cells in the body and is responsible for presentation of intracellular antigens to T cells (Abbaas et al, 1994).

The antigenic fragments presented in association with MHC molecules are very small, only about 7-10 amino acids in size and are referred to as epitopes of the antigen. In order to generate a specific immune response, T cells must recognize the antigen in association with MHC molecules (Abbas *et al.*, 1994). However, B cells can directly recognize antigen through surface bound antibody molecules and do not require the presentation of antigen in association with MHC molecules. Each T or B cell in the body can recognize only one antigenic epitope, however within the immune system there is a great variety of T and B cells such that the normal host can respond to the vast majority of infectious agents. Diversity is generated as a result of rearrangement of TCR genes in T cells and immunoglobulin genes in B cells. T cells respond to antigen associated with MHC molecules through the T cell receptor (TCR) by either directly killing the cell presenting the antigen or by secreting cytokines to

activate other immune cells (cellular immunity). B cells respond to antigen through surface bound antibody molecules which leads to proliferation and secretion of antigen specific antibodies (humoral immunity) and cytokines to stimulate other immune cells. Antibodies bind to the antigen and enhance removal of the antigen by phagocytosis or by activation of the complement system.

Because B and T cells are antigen-specific, they can provide immunological memory. Upon primary exposure to an antigen, a small population of memory T and/or B cells is generated. These memory cells provide protection against subsequent exposure to the same antigen.

The development of immune cells begins in the bone marrow. B cell precursors mature in the fetal liver and bone marrow. Mature B cells express IgM on the surface. Upon antigenic stimulation, B cells undergo further differentiation into antibody secreting plasma cells. Plasma cells may secrete IgM or may undergo class switching and secrete IgG, IgA, IgD, or IgE depending on the antigen and cytokines produced by other immune cells (Virella *et al.*, 1990). Phagocytic cells, such as, monocytes mature in the bone marrow and migrate to peripheral tissues where they may undergo further differentiation into macrophages. Other nonspecific immune cells including NK cells, neutrophils, eosinophils, and dendritic cells also mature in the bone marrow and then migrate to peripheral tissues (Abbas *et al.*, 1994).

Maturation of T cells occurs in the thymus. T cell precursors migrate from the bone marrow into the cortex of the thymus. Upon arrival in the thymus, pro-T cells do not express the TCR or other T cell markers such as, CD3, CD4 or CD8. One of the first events in T cell maturation is the rearrangement of the TCR genes. There are two types of TCR expressed by T cells, $\gamma\delta$ and $\alpha\beta$. If the $\gamma\delta$ TCR genes are rearranged

and expressed, the resulting T cell does not undergo thymic positive and negative selection and is exported to the periphery. $\gamma\delta$-T cells may either be $CD4^-CD8^-$ or $CD8^+$ and are considered to be of a more primitive lineage than $\alpha\beta$-T cells as they do not require the presentation of antigen on classical MHC molecules in order to be activated. $\gamma\delta$-T cells comprise only a small percentage of the total T cell population (Boehmer, 1988).

If the $\alpha\beta$ TCR genes are rearranged and expressed, the T cell will undergo positive and negative selection. Selection in the thymus takes place when the immature T cells express $\alpha\beta$TCR, CD3, CD4, and CD8. These thymocytes are referred to as double positive (DP) as they express both CD4 and CD8. During the selection process, DP thymocytes interact with instructional cells in the thymic cortex which express high levels of MHC molecules. Those T cells expressing a TCR which is capable of recognizing foreign antigen in association with MHC are positively selected. Double positive T cells with TCRs which bind class I MHC will downregulate CD4 expression and become single positive CD8 T cells. T cells whose TCR binds to class II MHC will lose expression of CD8 and become single positive CD4 T cells. Single positive T cells are allowed to migrate to peripheral tissues and home to the lymph nodes and spleen in particular. Peripheral T cells may express CD4 or CD8, but not both (Boehmer, 1988).

DP thymocytes also undergo negative selection. It is during negative selection that autoreactive T cells are depleted. If the DP T cell has a TCR which binds with high affinity to MHC molecules presenting self peptides, that T cell is given a signal from thymic instructional cells to undergo apoptosis and is thereby deleted. The majority of T cells entering the thymus are deleted during negative selection, very few

are exported to the periphery as mature $CD4^+$ or $CD8^+$ T cells.

$\alpha\beta$-TCR^+ peripheral T cells are divided into cytotoxic/suppressor (T_c/T_s) T cells which express CD8 marker or helper cells (T_H) which express CD4. T_H cells can be further divided into T_H1 or T_H2 based on the cytokines secreted and functional activities. T_H1 cells secrete IL-2, and IFN-γ (Cherwinski *et al.*, 1987; Mosmann and Coffman, 1989) and are though to mediate delayed type hypersensitivity reactions (Cher and Mosmann, 1987). T_H2 cells produce IL-4, and IL-5 (Cherwinski *et al.*, 1987; Mosmann and Coffman, 1989) and provide stimulation to B cells in the production of antibodies during an immune response (Boom *et al.*, 1988; Coffman *et al.*, 1988).

The immune response is normally tightly regulated. Upon exposure to antigen, immune cells are activated to produce cytokines which stimulates proliferation and activation of other immune cells. Once antigen is removed from the body, immune cell activation is downregulated and many immune cells undergo apoptosis via stimulation through the Fas molecule and are thereby deleted from the periphery. A few antigen specific T and/or B memory cells will survive for long periods of time and will provide protection from future exposure to the same antigen.

The immune system must be able to distinguish self from non-self in order to function properly. T cells recognizing self antigens are deleted in the thymus. However, sometimes self tolerance by the immune system is disrupted. The mechanisms of loss of tolerance include 1) escape of autoreactive T cells from thymic negative selection and subsequent activation in the periphery; 2) activation of peripheral anergized self-reactive immune cells; 3) generation of autoreactive clones due to infection with bacteria or viruses containing antigenic epitopes which resemble self molecules. In such cases, the tight regulation of the immune system is disrupted

and autoimmune disease may develop. In autoimmune disease, the immune system loses its tolerance for self and attacks self tissues.

Systemic Lupus Erythematosus (SLE) is a debilitating autoimmune disease which predominantly affects females. Symptoms of SLE include severe skin rash, high levels of circulating anti-DNA antibodies, vasculitis, and glomerulonephritis which results in renal failure (Stites and Terr, 1987). The cause of SLE is not know but extensive research on the disease has been possible by using murine models of the disease. One such model is MRL-*lpr/lpr* which develops lymphadenopathy due to the accumulation of large numbers of abnormal T cells which lack both CD4 and CD8 expression and are referred to as double-negative (DN) T cells. The characterization of these abnormal T cells and their role in the autoimmune disease of *lpr* mice is the focus of the current studies.

Specific Aims

The function and origin of the abnormal DN T cells which are found in large numbers in *lpr* or *gld* mice is not known. Many researchers believe that *lpr/gld* DN T cells are anergic, without demonstrating any function. However, recent studies suggested that these T cells may play an active role in the autoimmune disease of *lpr/gld* mice. The aims of the present study are to investigate the nature of DN T cells and their potential for actively participating in autoimmune disease. The specific aims are as follows:

1) To investigate the helper/cytotoxic functions of *lpr* or *gld* DN T cells and to study their ability to get activated through adhesion molecules other than the T cell Receptor (TCR), such as CD44 and $gp90^{MEL-14}$ which participate in the interaction of lymphocytes with endothelial cells.

2) To study the ability of DN T cells to damage endothelial cells and potentially contribute to the vasculitis seen in *lpr* mice and to investigate the mechanism of such vasculitis.

3) To determine whether the interaction between activated normal cytotoxic T cells and endothelial cells which bear ligands for the adhesion molecules CD44 and $gp90^{MEL-14}$ would lead to lysis of endothelial cells and whether this phenomenon accounts for the induction of vascular leak syndrome (VLS) seen in mice administered with high doses of IL-2 during immunotherapy.

4) To examine the nature of antibodies against gp120 of Human Immunodeficiency Virus (HIV) found in HIV non-infected autoimmune mice.

Chapter 2: Review of Literature

I. *lpr* and *gld* Double Negative (DN) T cells:

The murine strain MRL-*lpr*/*lpr* develops symptoms synonymous with the human disease Systemic Lupus Erythematosus (SLE) and has a 50% mortality rate at the age of six months. Characteristics of the autoimmune disease in these mice include severe lymphadenopathy, splenomegaly, high levels of anti-DNA antibodies, vasculitis, and hypergammaglobulinemia (Cohen and Eisenberg, 1991). The major cause of death in *lpr* mice is glomerulonephritis which results in renal failure.

The *lpr* (lymphoproliferation) mutation has been identified as a defect in the Fas gene (Wu *et al.*, 1993). Wu *et al.* have determined that there is a 5.3 Kb insertion of DNA in the second intron of Fas gene, located on mouse chromosome 19, of *lpr*/*lpr* mice (Wu *et al.*, 1993). Fas is a member of the CD40, nerve growth receptor, and tumor necrosis factor receptor (TNFR) family of proteins and is involved in apoptosis, or programmed cell death (Nagata and Suda, 1995). Fas is important not only in thymic selection of T cells, but also in the regulation of the immune response. It is expressed in the thymus, spleen, heart, ovary and activated peripheral T and B lymphocytes (Cheng *et al.*, 1995).

Lymphadenopathy in *lpr* mice is caused by the accumulation of an unusual T cell subset with the phenotype $\alpha\beta TCR^+$, $CD4^-$, $CD8^-$, $B220^+$ (CD45R). Because these cells lack both CD4 and CD8, they are referred to as double-negative (DN) T cells. Lymph nodes of *lpr* mice contain approximately 90% DN T cells, the origin and function of which is not known. DN T cells are present in normal (MRL + / +) mice, but constitute only 3-5% of the total T cell population.

Mice with the *gld* (generalized lymphoproliferative disease) mutation lack Fas-ligand expression (Ransdell *et al.*, 1994).. Mice homozygous for the *gld* mutation develop autoimmune disease which is very similar to *lpr/lpr* mice, including the accumulation of DN T cells. Because of a defect in fas expression, *lpr* and *gld* lymphocytes are not capable of Fas-mediated apoptosis. Normally, antigen-activated immune cells in the periphery undergo Fas-mediated apoptosis upon elimination of antigen. However, in *lpr* and *gld* mice the antigen activated cells accumulate in the periphery, resulting lymphadenopathy (Gillete-Ferguson and Sidman, 1994).

The origin of *lpr* DN T cells has not been conclusively determined. Many researchers believe that DN T cells originate in the thymus. Evidence to support this theory includes the fact that neonatal thymectomy eliminates the accumulation of DN T cells in *lpr* mice (Smathers *et al.* 1984). It has also been shown that DN T cells have undergone negative selection (Kotzin *et al.*, 1988; Singer *et al.*, 1989) which is only known to take place in the thymus. However, other investigators believe DN T cells to originate in the liver. Huang *et al.* (Huang *et al.*, 1994) have isolated $\alpha\beta TCR^+$ DN T cells expressing B220, which is characteristic of *lpr* DN T cells, in the liver of normal mice. These cells were found to be actively dividing and undergoing apoptosis in normal mice. The theory of Huang *et al.* is that normal liver is a major site for T cell

destruction and that the Fas defect in *lpr* mice results in a failure of the peripheral deletion process resulting in the leakage of $B220^+$ DN T cells to peripheral organs.

It is not clear whether DN T cells arise from $CD4^+CD8^+$, double positive (DP) thymocytes or from a distinct lineage of T cells. Studies in which *lpr* mice were continuously treated with anti-CD4 antibodies (Santoro *et al.*, 1988), or anti-CD8 antibodies (Geise and Davidson, 1994) demonstrated a drastic decrease in the accumulation of DN T cells. DN T cells have also been shown to be positively selected on MHC class I molecules (Herron *et al.*, 1993) which is known to occur at the $CD4^+CD8^+$ (DP) stage of thymocyte differentiation. This data, combined with the fact that the CD8 gene in DN T cells is demethylated (Wadsworth *et al.*, 1990) indicate that DN T cells once expressed CD4 and/or CD8. Therefore, DN T cells may arise from DP thymocytes or single positive CD4 or CD8 cells which downregulate the expression of these molecules upon entering the periphery. Whether the DN T cells found in *lpr/gld* mice represent an abnormally expanded population of the DN cells found in +/+ mice, or whether they stem from a distinct differentiation process has not yet been determined.

DN T cells are unique in several ways. The surface marker B220 is normally found only on bone marrow stem cell precursors and B cells, however, *lpr* DN T cells have also been shown to express this marker. There is substantial evidence to prove that DN cells are of the T and not B cell lineage including: lack of rearrangement of immunoglobulin genes; the presence of the TCR and CD3 on the surface (Nemazee *et al.*, 1985); and the fact that neonatal thymectomy in *lpr* mice eliminates the accumulation of DN T cells (Kotzin *et al.*, 1988).

Though DN T cells express the TCR and CD3, flow cytometric analysis of lymph

node cells of MRL-*lpr*/*lpr* mice shows a dull expression of these molecules, whereas control MRL-+/+ lymph nodes stain brightly positive for the TCR and CD3 (Morse *et al.*, 1982). Not only do DN cells have a decreased expression of the TCR, but the signal transduction associated with the TCR appears to be faulty. Cell proliferation studies have shown *lpr* lymph node cells to be unresponsive to stimulation by concanavalin A and anti-CD3 monoclonal antibodies (mAb), both of which induce strong proliferation of MRL-+/+ lymph node cells (Davignon *et al.*, 1988). DN cells fail to express functional Interleukin-2 receptors (IL-2R) and secrete IL-2 upon mitogenic stimulation through the CD3/TCR complex (Davignon *et al.*, 1985).

Recent studies have shown defects in DN T cell signalling pathways including dysfunctional Ca^{2+}-ATPase pump (Wang *et al.*, 1993), inefficient coupling of the CD3/TCR complex with functional G proteins (Coggeshal and Altman, 1989), and constitutive phosphorylation of CD3-ζ chain (Samelson *et al.*, 1986). Thus, DN T cells are thought by some investigators to be anergic T cells. Partial restoration of DN T cell stimulation can be achieved by the addition of phorbol myristate acetate (PMA) in combination with Ca^{2+} ionophore (Kakkanaiah *et al.*, 1990), or by the addition of antibodies to the costimulatory molecule CD28 in combination with antibodies against the TCR/CD3 complex (Clements *et al.*, 1993). It has been questioned whether the large number of DN T cells in *lpr* mice result from the accumulation of cells or from the active proliferation. Because of the general unresponsiveness of DN cells to normal T cell growth factors and cell cycle analysis studies which showed that only 5% of DN cells are actively dividing at any given time (Mountz *et al.*, 1988), lymphadenopathy in *lpr* mice is believed to be caused by the accumulation of cells rather than proliferation.

Though the activation of DN T cells through the TCR is faulty and it is believed that these T cells are anergic, a number of studies have shown that DN T cells may play an active role in murine and human autoimmune disease. Studies in which *lpr/lpr* mice were treated with cyclosporin A (CsA) (30), anti-CD8 antibodies (Geise and Davidson, 1994), anti-CD4 antibody (Santoro *et al.*, 1988), anti-CD3 antibody (Henrickson *et al.*, 1994), anti-Thy-1 (Wofsy *et al.*, 1985), or MEL-14 antibody (Mountz *et al.*, 1988) demonstrated a significant reduction in numbers of DN T cells and a decrease in the severity of autoimmune symptoms. Mice treated with CsA demonstrated a marked reduction in the severity of arthritis and glomerulonephritis (Mountz *et al.*, 1987). Furthermore, a substrain of MRL-*lpr/lpr* mice, designated *lpr*-II (long-lived) has been shown to have a limited expansion of DN T cells (Fossati *et al.*, 1993). *lpr*-II have a survival time twice that of *lpr* and a delayed onset of glomerulonephritis. Thus results from the above experiments suggest that the accumulation of DN T cells coincides with the onset and severity of autoimmune disease of *lpr* mice.

It is not known what role DN T cells have in the autoimmune disease of *lpr* mice, but DN T cells have been shown to infiltrate sites of inflammation in some autoimmune diseases. DN T cells were found in substantial numbers in the pericardial infiltrate of rats with autoimmune myocarditis (Hanawa *et al.*, 1993). This study also revealed DN T cells actively infiltrating the myocardial tissue. Renal lesions in *lpr* mice have also been shown to contain significant numbers of DN T cells. Furthermore, DN T cells in these lesions proliferate exclusively in response to renal tissue *in vitro* (Diaz-Gallo and Kelley, 1993). These studies demonstrate that DN T cells may play an active role in autoimmune disease of animal models.

Though human SLE is not characterized by lymphadenopathy to the extent that is found in *lpr* mice, recent studies have shown that some patients with SLE have increased numbers of DN T cells in the peripheral blood. Sneller *et al.* (Sneller *et al.*, 1992) describe two SLE patients with severe lymphadenopathy disorder similar to that seen in *lpr* and *gld* mice. These patients exhibited large numbers of $\alpha\beta TCR^+$ DN T cells in peripheral blood and lymph nodes. Phenotypic analysis and functional studies revealed that these DN T cells are very similar to those found in *lpr* and *gld* mice. Though this syndrome is rare in human SLE, another study by Shivakumar *et al.* (Shivakumar *et al.*, 1989) demonstrated a significant increase in $\alpha ßTCR^+$ DN T cells in the peripheral blood of 12 patients with active SLE and 8 patients with inactive SLE compared to control individuals. It was discovered that patients with active lupus had a more prominent increase in DN T cells than those in remission. Thus the increase in DN T cells in human SLE in the patients of this study correlates with the autoimmune disease state, similar to that in *lpr* mice. In addition, $\alpha ßTCR^+$ DN T cell clones have been isolated from SLE patients which augment the production of autoantibodies of autoreactive B cells *in vitro* (Shivakumar *et al.*, 1989). These studies demonstrate that DN T cells may play an active role in human autoimmune disease.

Flow cytometric analysis, performed in our lab and by others (Dumont *et al.*, 1988; Haynes *et al.*, 1989), of DN T cells from *lpr* mice show these cells to be highly positive for the adhesion molecules $gp90^{MEL\text{-}14}$ and CD44. Adhesion molecules are important in mediating cell-to-cell contact as well as the distribution of lymphocytes to lymphoid organs. Lymphocytes preferentially home to specific organs via cell surface markers, such as, $gp90^{MEL\text{-}14}$. Specialized blood vessels called high endothelial venules (HEV) are found in lymphoid organs. These blood vessels are lined with

endothelial cells bearing the ligands for the adhesion molecules found on lymphocytes. Lymphocytes bearing adhesion molecules specific for the ligand on the endothelial cells will bind to the endothelial cell and are allowed to pass through the blood vessel, into the lymphoid organ. $gp90^{MEL-14}$ has been shown to be the homing receptor to the lymph nodes, however, the ligand on endothelial cells for $gp90^{MEL-14}$ has not yet been discovered.

CD44, also known as Pgp-1 (phagocytic glycoprotein-1), is involved in lymphopoiesis, lymphocyte homing and cell activation. The expression of CD44 appears to be dependent on the maturation stage of the T cell. The most immature thymocytes which are $TCR^-CD3^-CD8^-CD4^-$ express a high density of CD44 on the surface. However, as thymocytes mature into $TCR^+CD3^+CD4^+CD8^+$ double positive cells, the expression of CD44 is lost. In the periphery, CD44 expression is low or not expressed on resting lymphocytes and is upregulated upon activation (Lesley *et al.*, 1993). Approximately 80-90% of prothymocytes express CD44, compared to only about 20% of normal adult thymocytes. It is believed that the small percentage of peripheral lymphocytes constitutively expressing high levels of CD44 are memory T or B cells (Lesley *et al.*, 1993). *lpr* lymph node cells are about 90% positive for CD44, which may suggest that DN T cells are either immature or activated, however, the expression of the TCR in these cells indicates that they are mature T cells. Thus, based on CD44 expression, it would appear that DN T cells are activated *in vivo*.

Recent studies indicate CD44 is involved in cell activation. *In vitro* studies have revealed that the binding of anti-CD44 antibodies causes an increase in proliferation upon mitogenic stimulation of peripheral T cells via the CD2 and CD3 surface molecules (Galandrini *et al.*, 1993). Stimulation through CD44 leads to tyrosine

posphorylation of several proteins in a pattern similar to that of activation through the TCR/CD3 complex (Galandrini *et al.*, 1993). In addition to proliferation, activation through CD44 has been shown by our lab and others to stimulate cytolytic activity in cytotoxic T lymphocytes (Seth *et al.*, 1991; Galandrini *et al.*, 1993). Galandrini *et al.* demonstrated that activation through CD44 leads to exocytosis of lytic proteins from cytotoxic T lymphocytes (CTL) at a level that is comparable to stimulation through the TCR/CD3 complex (Galandrini *et al.*, 1993). Recent studies in our lab have revealed that CTL are capable of lysing nonspecific Fc-*γ* Receptor positive (FcγR$^+$) tumor targets when stimulated through the $\alpha\beta$TCR/CD3 complex as well as through the adhesion molecules CD44 and MEL-14 (Seth *et al.*, 1991). The method of detecting this lytic ability involves the use of redirected lysis assays in which a radio-labelled FcγR$^+$ tumor target is cultured with the CTL in the presence of antibodies against the TCR or CD3. The antigen binding portion of the antibody (Fab) will bind to the appropriate molecule on the CTL and the Fc portion will bind to the tumor target, thereby bridging the two cells together and triggering lysis of the target cell.

Lymphocytes circulate through the vascular system, enter the lymph system, migrate to the lymph nodes, and return to the vascular system through the thoracic duct. Normally, very few lymphocytes are present in peripheral tissues, unless there is a site of antigen challenge. At the site of antigen challenge, lymphocytes must be able to enter the tissues via the vascular system. The selective passage of lymphocytes through blood vessel walls is mediated by the adhesion molecules lymphocyte functional antigen-1 (LFA-1) and intracellular adhesion molecule-1 (ICAM-1). LFA-1 is expressed on all lymphocytes, whereas ICAM-1 is the ligand for LFA-1 and is found on most vascular endothelial cells and macrophages (Springer, 1990a).

ICAM-1/LFA-1 interaction plays a major role in lymphocyte adhesion to endothelial and macrophages. Patients lacking adhesion molecules have impaired inflammatory reactions as well as adhesion mediated cellular immunity (Springer, 1990b). Expression of ICAM-1 on endothelial cells is enhanced by cytokines involved in the inflammatory response, such as, IL-1, TNF-α, and IFN-γ (Springer, 1990; Springer *et al.*, 1987).

Endothelial cells are the link between the transport of lymphocytes and the localization of lymphocytes in areas on antigen challenge. Upregulation of ICAM-1 and other adhesion molecules, such as, CD44 causes an increased adhesion of lymphocytes to endothelial cells in the region of antigen challenge, and an accumulation of lymphocytes in the affected area.

Damage to endothelial cells causes an increase in vascular permeability and excessive loss of fluid from the blood into peripheral tissues--a disorder known as Vascular Leak Syndrome (VLS). Rosenstein *et al.* (Rosenstein *et al.*, 1986) have attempted to treat certain types of cancer by injecting Lymphokine Activated Killer (LAK) cells which have been stimulated by high doses of IL-2, along with high doses of IL-2 into patients with advanced cases of cancer. These patients soon developed VLS as a result of LAK/IL-2 therapy. Endothelial cells bear ligands for CD44 and $gp90^{MEL-14}$ and the expression of these molecules on T cells is enhanced upon activation. Thus the VLS seen in the patients from Rosenberg's studies (Rosenstein *et al.*, 1986; Rosenberg *et al.*, 1986; Rosenberg *et al.*, 1987) may result from activation of cytotoxic T cells via adhesion molecules on endothelial cells, leading to endothelial cell damage. These results imply that CD44 is important for transducing signals which stimulate various T cell functions.

Preliminary studies in our lab have shown that *lpr* DN T cells can directly and spontaneously lyse the Natural Killer (NK)-sensitive YAC-1 tumor cell line (Kakkanaiah *et al.*, 1990) and thus have cytotoxic capabilities. This, combined with the fact that DN T cells express abnormally high levels of the adhesion molecules CD44 and $gp90^{MEL-14}$ and constitutively express TNF-α and IFN-γ (Murray and Martens, 1989) indicates that the DN T cells are constitutively activated *in vivo*. Because normal CTL are capable of mediating lysis when stimulated through adhesion molecules and DN T cells have cytolytic activity, it is possible that DN T cells could bind to adhesion molecules on endothelial cells, become activated, and damage endothelial cells *in vivo*. Thus DN T cells may play and active role in the autoimmune disease of *lpr* mice.

While DN T cells are thought to be without function, recent studies have shown that these cells may play an active role in autoimmune disease in mice and humans (Shivakumar *et al.*, 1989; Fossati *et al.*, 1993; Hanawa *et al.*, 1993; Diaz-Gallo and Kelley, 1993). Preliminary studies in our lab have shown DN T cells to have cytolytic capabilities. The purpose of the present studies is to characterize the activation of DN T cells and to determine their potential for mediating autoimmune disease.

II. Nature of anti-HIV antibodies in autoimmune mice:

Jerne proposed that the antigen binding site on antibody molecules determines a set of immunogenic regions called idiotypes in the variable region of the immunoglobulin molecule (Jerne, 1974). The idiotype not only interacts with the

antigenic site (epitope) but also reacts with anti-antibodies bearing anti-idiotypes. Thus such an interaction can result in a network of ever increasing size. Furthermore, the immune response is a measure of the balance that may exist at any time between active stimulus and active suppression. The network concept of the immune system proposes that idiotypes and anti-idiotypes play a central role in determining the ultimate size of the immune response repertoire as well as furnishing a mechanism for the internal regulation of these responses (Silverstein, 1986).

Kion and Hoffmann made a surprising finding that normal mice exposed to allogneic cells as well as some autoimmune strains of mice, such as MRL-*lpr/lpr* made antibodies against gp120 of the Human Immunodeficiency Virus (HIV) (Kion and Hoffman, 1991), though these mice have never been exposed to HIV. Furthermore, anti-anti-self MHC antibodies were detected in autoimmune and alloimmune mice. They called the anti-anti-self MHC antibodies MHC-image (MI) and concluded that production of MI and anti-MI may be responsible for the pathogenesis of autoimmune mice. In the current study we investigated the nature and origin of anti-gp120 antibodies in autoimmune and alloimmune mice.

Chapter 3: Double-negative T Cells from MRL-*lpr/lpr* Mice Mediate Cytolytic Activity when Triggered through Adhesion Molecules and Constitutively Transcribe Perforin Gene

Introduction:

MRL-*lpr/lpr* (*lpr*) mice develop massive lymphadenopathy, hypergammaglobulinemia, and autoantibody production leading to premature death (Andrews *et al.*, 1978). Lymphadenopathy is characterized by an age-related, accumulation of $CD4^-CD8^-$ (double negative [DN]) T cells that are $CD3^+$ and TCR-$\alpha\beta^+$ and bear an unusual phenotype by expressing high densities of many markers that are not expressed by normal resting T cells such as J11d, Ly6C, CD45R, and CD44 (for a review see reference Cohen and Eisenberg, 1991). The *lpr* phenotype results from defects in Fas gene (Wu *et al.*, 1993; Watanabe-Fukunaga *et al.*, 1992).

Despite extensive research, the nature and significance of *lpr* DN T cells are not clear. It has been shown that *lpr* DN T cells are refractory to stimulation with mitogens or Abs against the CD3-TCR complex as measured by their ability to proliferate, acquire IL-2 receptors, and produce IL-2 (Davignon *et al.*, 1988; Davignon *et al.*, 1985; Kakkanaiah *et al.*, 1990; Wofsy *et al.*, 1981; Kakkanaiah *et al.* 1991). Such studies have suggested that *lpr* DN T cells are inactive or anergic T cells whose

role in autoimmune disease remains unknown.

Recently it was demonstrated that CTL can be activated independent of the TCR, via adhesion molecules such as CD44 and gp90^{MEL-14}, to mediate redirected lysis (Seth *et al.*, 1991). This, combined with our earlier studies that DN T cells from *lpr* mice exhibit spontaneous NK-like cytotoxicity (Kakkanaiah *et al.*, 1990), prompted us to undertake studies to investigate whether *lpr* DN T cells would exhibit cytotoxic potential when activated via a variety of adhesion molecules. We report a surprising finding that mAbs against the CD3-TCRα/ß, as well as against certain adhesion molecules such as CD44 and gp90^{MEL-14}, can activate the lytic potential of *lpr* DN T cells. Furthermore, unstimulated DN T cells constitutively transcribed transcripts for perforin gene. Our studies demonstrate that *lpr* DN T cells may represent activated cytotoxic T cells *in vivo* and that such a functional characteristic may contribute to the pathogenesis of autoimmune disease.

Materials and Methods:

Mice. MRL-*lpr*/*lpr* (*lpr*) and MRL +/+ mice were bred in our animal facility (Seth *et al.*, 1988).

Antibodies. The mAbs used were in culture supernatants and were from the following hybridomas: 9F3 directed against CD44; MEL-14 against lymphocyte homing receptor for endothelium (gp90^{MEL-14}); 2.4G2 against FcR; H57-597 against TCR-α/ß; 145.2C11 against CD3; 6B2 against CD45R; M17/4 against LFA-1; and

53.6.72 against CD8. All hybridomas were procured and mAbs were purified as described elsewhere (Kakkanaiah *et al.*, 1990; Seth *et al.*, 1991; Kakkanaiah *et al.*, 1991; Seth *et al.*, 1988).

DN T cells. The DN T cells were purified as described elsewhere (55), by treating LN cells twice with anti-CD4 and anti-CD8 Abs followed by complement. Next, the viable cells were isolated by density gradient centrifugation over histopaque (Sigma Chemical Co., St. Louis, MO). The purity of the DN T cells was >95% as determined by flow cytometry as described (Seth *et al.*, 1988).

Cytotoxicity Assay. Redirected cytotoxicity was determined by the capacity of DN T cells to lyse FcγR$^+$ tumor target cells in the presence of mAbs directed against adhesion molecules expressed by the DN T cells. The cytotoxicity was studied by using a ^{51}Cr-release assay as described (Seth *et al.*, 1991). Tumor targets or hybridoma cells were labeled with ^{51}Cr and seeded in 96-well plates at 5 x 10^3 cells/well, along with varying numbers of effector cells and mAb supernatants. The plates were incubated at 37°C for 4 hours. The amount of ^{51}Cr released by target cells was measured with a gamma counter (TM Analytic, Elk Grove Village, IL). Percent cytotoxicity was calculated from the ^{51}Cr release as follows: 100 x [(Experimental release - control release)/(total release - control release)].

Detection of N-α-benzyloxycarbonyl-L-lysine Thiobenzyl Esterase (BLTE or Granzyme A) in DN T cells. The total cellular content of BLTE in T cells was determined as described by Lancki *et al.* (Lancki *et al.*, 1991). Briefly, varying numbers

of purified DN T cells were lysed with 1% Triton X-100. Of this lysate, 20 μl was added to microtiter wells containing 180 μl of assay solution consisting of PBS, pH 7.2 with 2.2 x 10^{-4} M 5,5'-thio-bis(2-nitro)-benzoic acid (Calbiochem-Novabiochem Corp. La Jolla, CA) and 2 x 10^{-4} M BLT (Sigma Chemical Co.). After 30 minutes incubation at room temperature, the absorbance was read in an ELISA reader at λ = 410 nm. In assays involving activation of DN T cells through adhesion molecules, DN T cells were added to Ab-coated plates and incubated for 4-6 hours at 37°C before the BLTE assay.

PCR Analysis of IL-2 and Perforin Gene Expression in DN T cells. PCR method was employed to study whether the *lpr* DN T cells spontaneously transcribed perforin gene as described by others (Lu *et al.*, 1992) and modified as follows. Purified DN T cells or other cells were lysed using a buffer containing 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris chloride, pH 8, 0.5% NP-40, and 1 mM 2-ME. The total RNA was isolated by digesting the cell lysate at 37°C for 35 minutes with proteinase K (50 μg/ml) in a digestion buffer containing 0.2 M Tris chloride, pH 7.6, 25 mM EDTA pH 8, 0.3 M NaCl, and 2% SDS. Next, proteins were extracted in a phenol/chloroform 1:1 mixture. The nucleic acid from aqueous phase was precipitated in ethanol at -80°C overnight. The concentration of recovered RNA was measured by UV absorption spectrophotometry, and the RNA was reverse transcribed into cDNA as described in the Perkin-Elmer Cetus (Norwalk, CT) protocol. Reverse transcription was performed at 42°C for 45 minutes followed by denaturation of reverse transcriptase at 99°C for 5 minutes. The resulting cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers. The primers for ß-actin, IL-2, and perforin cDNA were selected using the Genetic Computing Group program assisted search from

GenBank sequences. The PCR was run at 94°C for 2 minutes followed by 60 cycles of denaturing at 94°C for 1 minute, annealing at 50°C for 2 minutes, and extension at 72°C for 2 minutes. The primer sequences were as follows: For ß-actin 5'-TATCCTGACCCTGAACTACCCCATT and 3'- AGCACAGCTTCTCTTTGATGTCACG; for IL-2, 5'- ATGTACAGCATGCAGCTCGCATC and 3'-GGCTTGTTGAGATGATGCTTTGACA; and perforin was 5'-GGTCAGAATGCAAGCAGAAGCACAA and 3'- TTGAAGTCAAGGTGGAGTGGAGGTT. 5 μl of the PCR product was electrophoresed on a 1.5% agarose gel stained with ethidium bromide. The demonstration of a single 502-, 464-, or 499-bp band was considered to be indicative of the expression of IL-2, ß-actin, and perforin genes, respectively.

Results and Discussion:

We investigated whether DN T cells would mediate lysis of target cells when activated via the CD3-TCR-α/ß complex. Inasmuch as the ligand for the DN TCR is unknown, we used "redirected" lysis to address this, by employing mAbs against the CD3-TCR complex and P815 target cells because these cells were FcγR$^+$ and were resistant to direct lysis by the DN T cells (Kakkanaiah *et al.*, 1990; Seth *et al.*, 1991). The data presented in Fig. 3.1 suggested that purified DN T cells from *lpr* mice lysed YAC-1 targets but not P815 target cells as demonstrated before (Kakkanaiah *et al.*, 1990). Interestingly, in the presence of mAbs against CD3 or TCR-α/ß, the DN T cells

mediated efficient lysis of P815. In contrast, mAbs against TCR-γ/δ failed to evoke cytotoxicity. These data demonstrated that cells mediating cytotoxicity were TCR-$\alpha/ß^+$ T cells. Furthermore, depletion of DN T cells using mAbs against CD44, CD45R, and J11d plus complement, virtually abolished the redirected cytotoxicity of P815 cells in the presence of anti-CD3 or anti-$\alpha/ß$ Abs (data not shown), thereby further confirming that the cytotoxicity was mediated by the DN T cells.

We had demonstrated earlier that naive normal T cells would not mediate spontaneous lysis in a redirected assay (Seth *et al.*, 1991). However, further studies were carried out to exclude the possibility that the cytotoxicity seen with *lpr* DN T cells may have resulted from contaminating $CD4^+$ or $CD8^+$ T cells. In this experiment, we used lymph node (LN) cells from MRL +/+ mice as a negative control and a TCR-$\alpha/ß^+$ CTL clone designated PE-9 (Seth *et al.*, 1991) as a positive control. The LN cells from 4-month old MRL-+/+ mice failed to mediate significant redirected lysis of P815 target cells in the presence of anti-CD3 mAbs (Fig. 3.2). In contrast, MRL-/pr/*lpr* LN cells exhibited marked lysis of P815 targets in the presence of anti-CD3 mAbs. The fact that MRL-*lpr*/*lpr* but not MRL-+/+ LN cells mediated lysis of P815 cells in the presence of anti-CD3 mAbs suggested that the cytotoxicity can be attributed to the unique DN T cells found in *lpr* mice and not to the normal $CD4^+$ or $CD8^+$ T cells.

CD44 is a broadly expressed glycoprotein implicated in leukocyte-endothelial cell binding and may direct lymphocyte homing to certain peripheral lymphoid microenvironments (Haynes *et al.*, 1989). Several recent studies (Haynes *et al.*, 1989) have demonstrated that after activation naive T cells express high density CD44 which plays an important role in T cell activation. In addition, $gp90^{MEL-14}$, a selection which is structurally distinct from CD44, also recognizes high endothelial venules and is

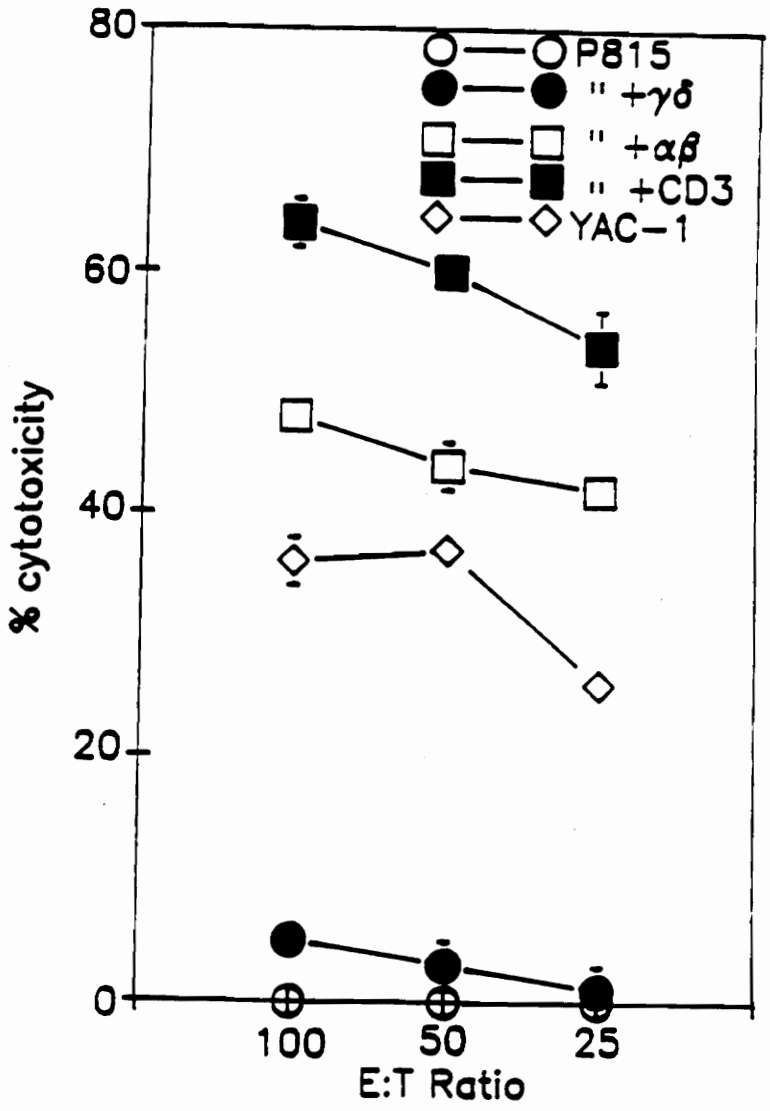

Fig 3.1 **Activation of DN T cells from *lpr* mice through the TCR leads to efficient induction of cytotoxicity.** Freshly isolated and purified DN T cells from *lpr* mice were tested for spontaneous cytotoxicity against YAC-1 or P815 tumor targets. The cytotoxicity against P815 targets was performed in the absence or presence of mAbs against CD3, α/ß or γδ TCR using ^{51}Cr-release assay. The data is expressed as mean percent cytotoxicity ± SEM at various effector:target (E:T) cell ratios.

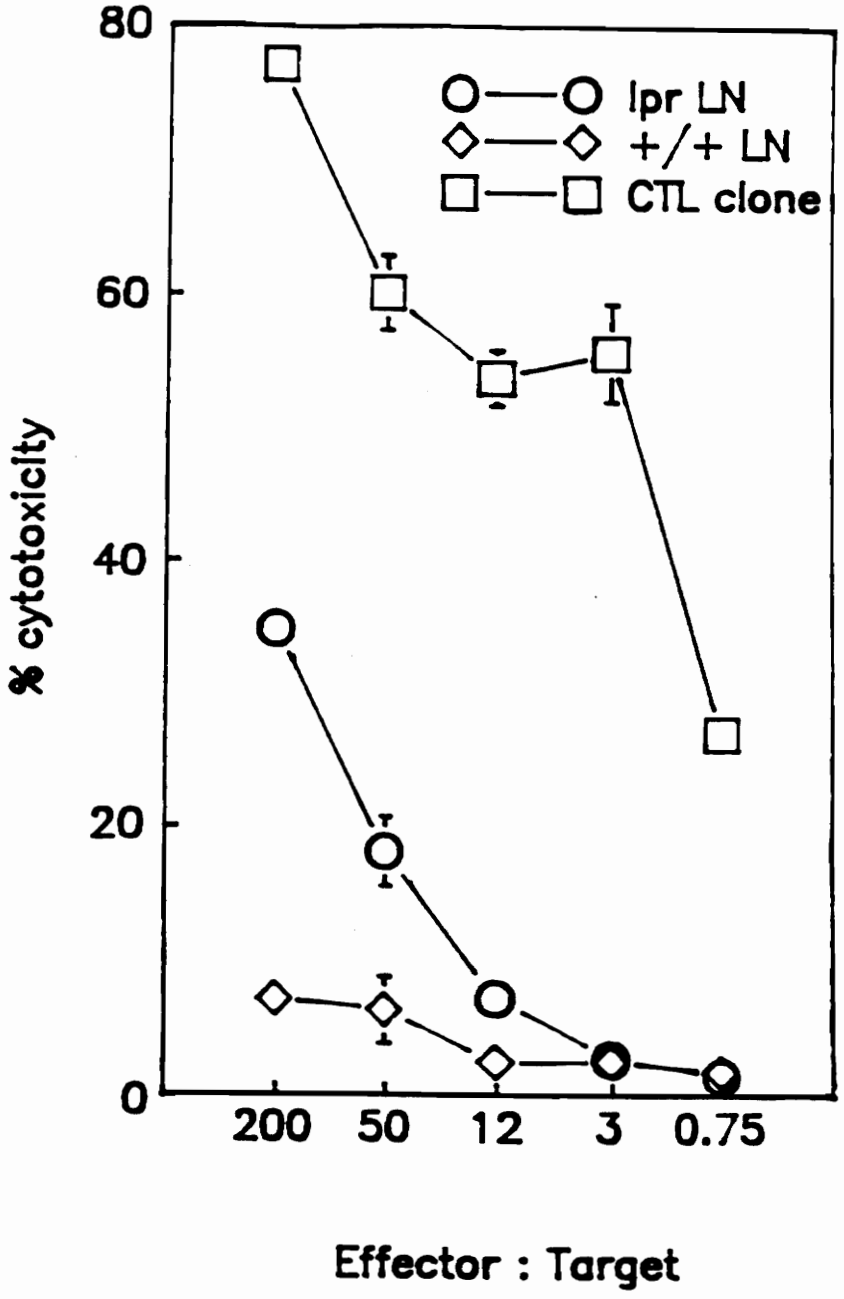

Fig 3.2 **Lytic activity of LN cells from MRL-+/+ or MRL-lpr/lpr mice.** Freshly isolated LN cells from 4-month-old MRL-+/+ or MRL-lpr/lpr mice were tested for spontaneous cytotoxicity against P815 tumor targets in the presence of anti-CD3 mAbs as described in Fig. 3.1. The CTL clone, PE-9, was used as a positive control.

involved in organ-specific homing of lymphocytes. Recently, we demonstrated that activated CTL that expressed CD44 and gp90^{MEL-14}, could mediate lysis of target cells when activated via these adhesion molecules, independent of the TCR (Seth *et al.*, 1991).

To study whether the DN T cells could also be activated to mediate lysis via the TCR-independent alternate pathway, DN T cells were incubated with a variety of FcγR$^+$ tumor targets in the presence of Abs against adhesion molecules (Figure 3.3 A). Interestingly, mAbs against CD44 and gp90^{MEL-14} induced strong lysis of P815 target cells, whereas mAbs against LFA-1, FcγR, and CD45R failed to trigger significant lysis. Similar observations were also made using other allogeneic FcγR$^+$ tumor cells such as LSA and EL-4 (Figures 3.3 B and C). This fact was further corroborated by demonstrating that DN T cells could also mediate lysis of hybridomas secreting mAbs against CD3, CD44, and gp90^{MEL-14} (Figure 3.3 D). In this experiment, DN T cells failed to mediate significant lysis of hybridomas secreting mAbs against CD45R and TCR-γ/δ, which also served as appropriate negative controls for other hybridomas. Together these studies demonstrated that *lpr* DN T cells can mediate efficient lysis of target cells when activated through the CD3-α/ß TCR complex, as well as other adhesion molecules such as CD44 and gp90^{MEL-14}.

The cytoplasmic granules of CTL and NK cells have been shown to contain a number of proteins, known as perforin, and a family of serine esterases (Gause *et al.*, 1988; Podack *et al.*, 1991). In this study, we addressed whether the DN T cells from *lpr* mice would exhibit perforin and serine esterases such as granzyme A either spontaneously or after activation via the CD3-TCR complex or through adhesion molecules. To this effect, we lysed purified DN T cells freshly isolated from *lpr* mice

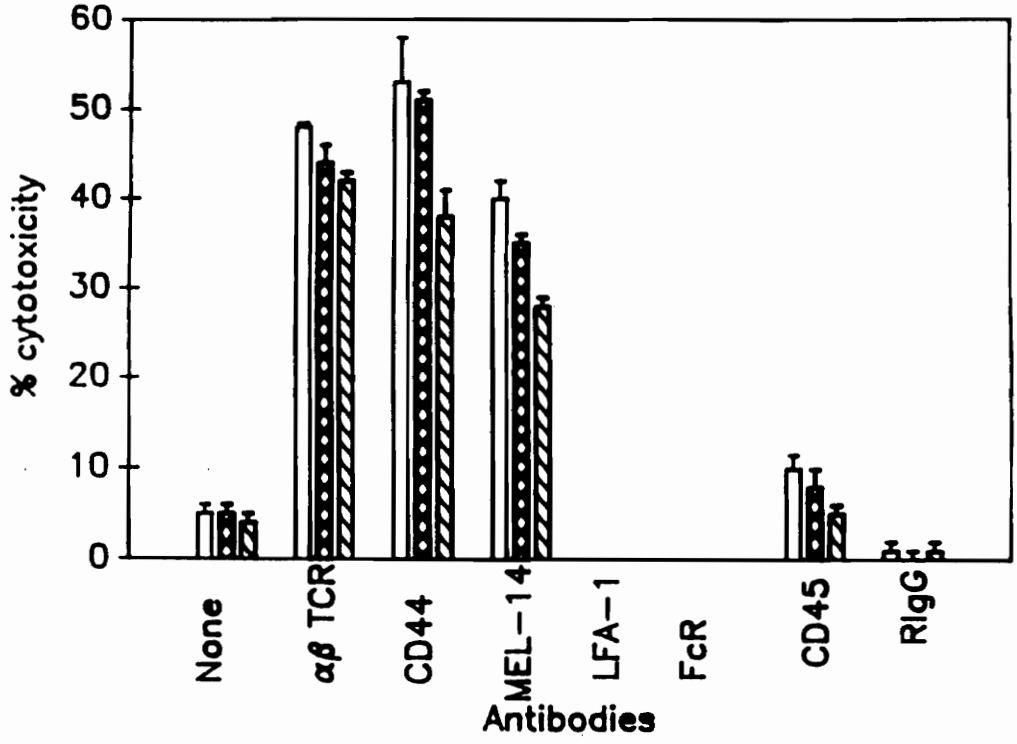

Fig 3.3A **Role of adhesion molecules in the activation of lytic activity by *lpr* DN T cells.** Freshly isolated and purified *lpr* DN T cells were tested for cytotoxicity against ^{51}Cr-labeled **P815** targets in the presence or absence of mAbs against a variety of adhesion molecules expressed by the DN T cells. The cytotoxicity was studied as described in Fig 3.1. *Open, hatched, and diagonal* bars represent cytotoxicity obtained at E:T ratios of 100, 50, and 25, respectively.

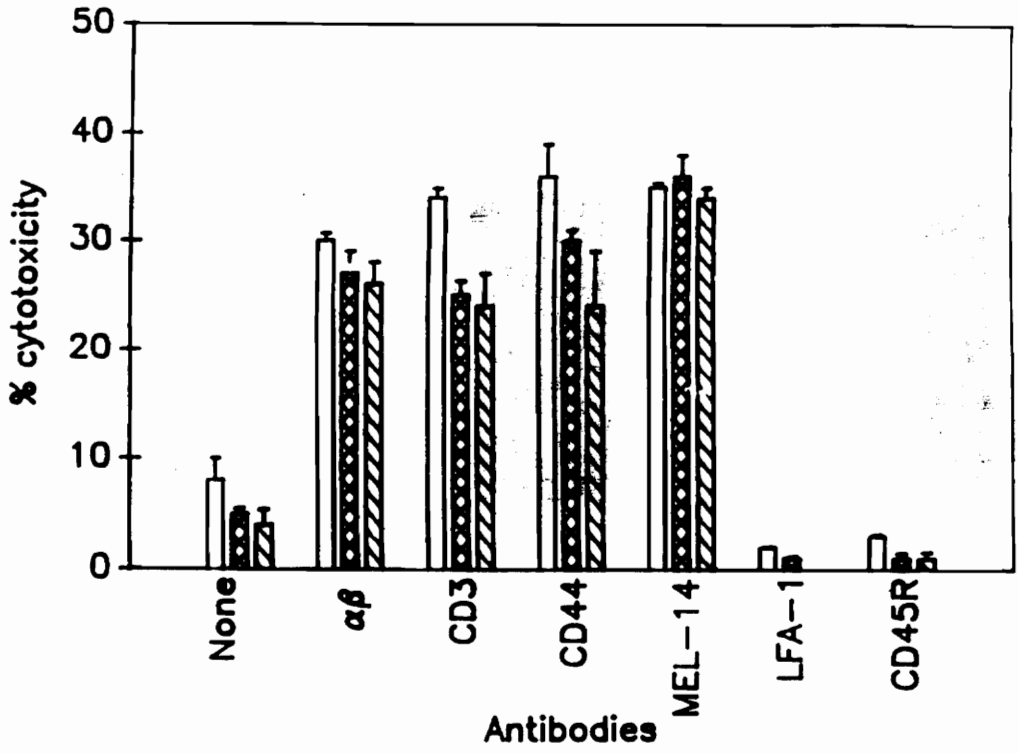

Fig 3.3B **Role of adhesion molecules in the activation of lytic activity by *lpr* DN T cells.** Freshly isolated and purified *lpr* DN T cells were tested for cytotoxicity against ^{51}Cr-labeled **LSA** targets in the presence or absence of mAbs against a variety of adhesion molecules expressed by the DN T cells. The cytotoxicity was studied as described in Fig 3.1. *Open, hatched, and diagonal* bars represent cytotoxicity obtained at E:T ratios of 100, 50, and 25, respectively.

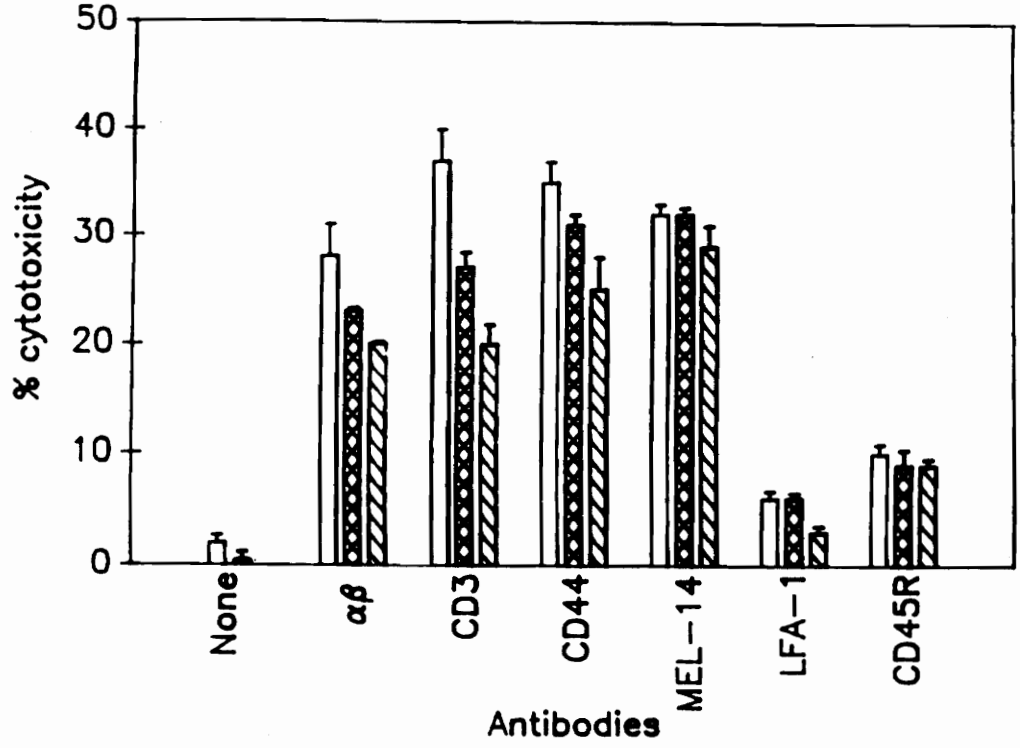

Fig 3.3C **Role of adhesion molecules in the activation of lytic activity by *lpr* DN T cells.** Freshly isolated and purified *lpr* DN T were tested for cytotoxicity against ^{51}Cr-labeled **EL-4** targets in the presence or absence of mAbs against a variety of adhesion molecules expressed by DN T cells. The cytotoxicity was studied as described in Fig. 3.1. *Open, hatched, and diagonal* bars represent cytotoxicity obtained at E:T ratios of 100, 50, and 25, respectively.

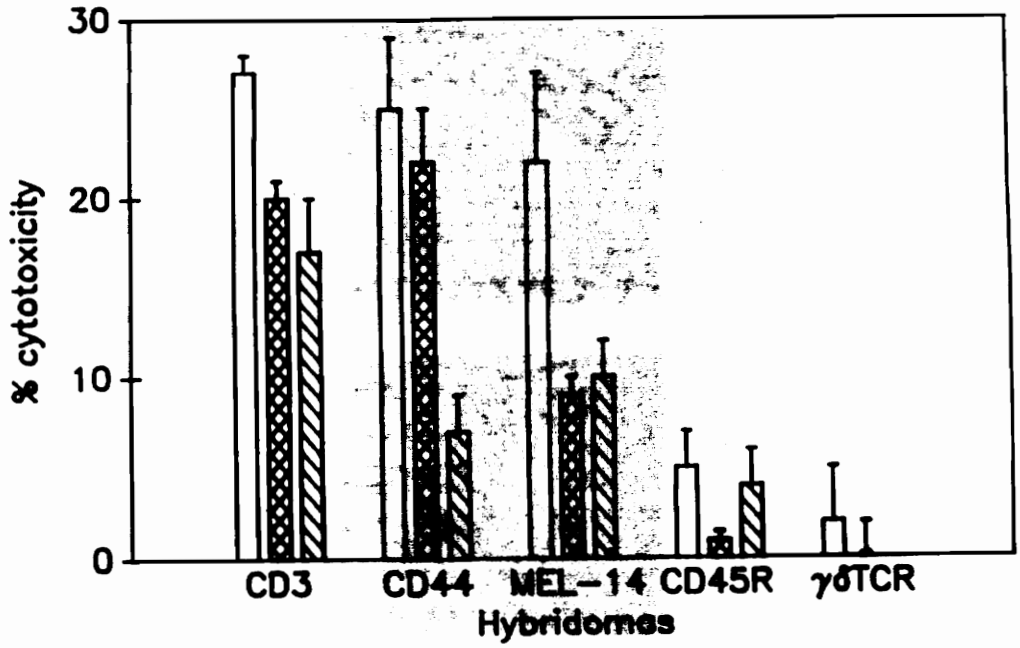

Fig 3.3D **Role of adhesion molecules in the activation of lytic activity by *lpr* DN T cells.** Freshly isolated and purified *lpr* DN T cells were tested for cytotoxicity against ^{51}Cr-labeled **hybridoma** targets secreting mAbs against a variety of adhesion molecules expressed by the DN T cells. The cytotoxicity was studied as described in Fig. 3.1. *Open, hatched, and diagonal* bars represent cytotoxicty obtained at E:T ratios of 100, 50, and 25, respectively.

and measured the level of BLTE. As a positive control, we used the *in vitro* activated CTL clone, PE-9 (Seth *et al.*, 1991). The data in Figure 3.4 suggested that whereas the CTL clone exhibited dose-dependent BLTE activity, the DN T cells failed to demonstrate spontaneous BLTE activity. Furthermore, activation of the DN T cells through the CD3-TCR complex or through adhesion molecules such as CD44, $gp90^{MEL-14}$, CD45R, etc., also failed to trigger BLTE activity (data not shown), even at a concentration of 10^6 cells. These data together demonstrated that DN T cells from *lpr* mice fail to demonstrate detectable levels of granzyme A.

Naive CTL do not express perforin. However, after activation, they synthesize perforin (Podack *et al.*, 1991). We therefore investigated whether *lpr* DN T cells transcribed perforin either constitutively or after activation. The presence of perforin in *lpr* DN T cells was detected by studying the expression of perforin gene in unstimulated cells using PCR. In addition, we also tested whether the *lpr* DN T cells would express IL-2 gene spontaneously. In this assay, we used primers for ß-actin as an internal standard to allow comparison of various samples. The data shown in Figure 3.5 indicated that unstimulated *lpr* DN T cells transcribed the perforin but not IL-2 gene. The DN T cells failed to express IL-2 gene despite stimulation through the TCR (data not shown), consistent with previous studies that *lpr* DN T cells fail to produce IL-2 when activated (Andrews *et al.*, 1978).

It is possible that the perforin band in the PCR using DN T cells may have been contributed by contaminating NK cells. To exclude this possibility, we isolated total RNA from MRL-*lpr*/*lpr* or MRL-+/+ LN cells and from the control CTL clone, PE-9. Next, 0.5 or 1 μg fo RNA was processed for PCR analysis, and the product was

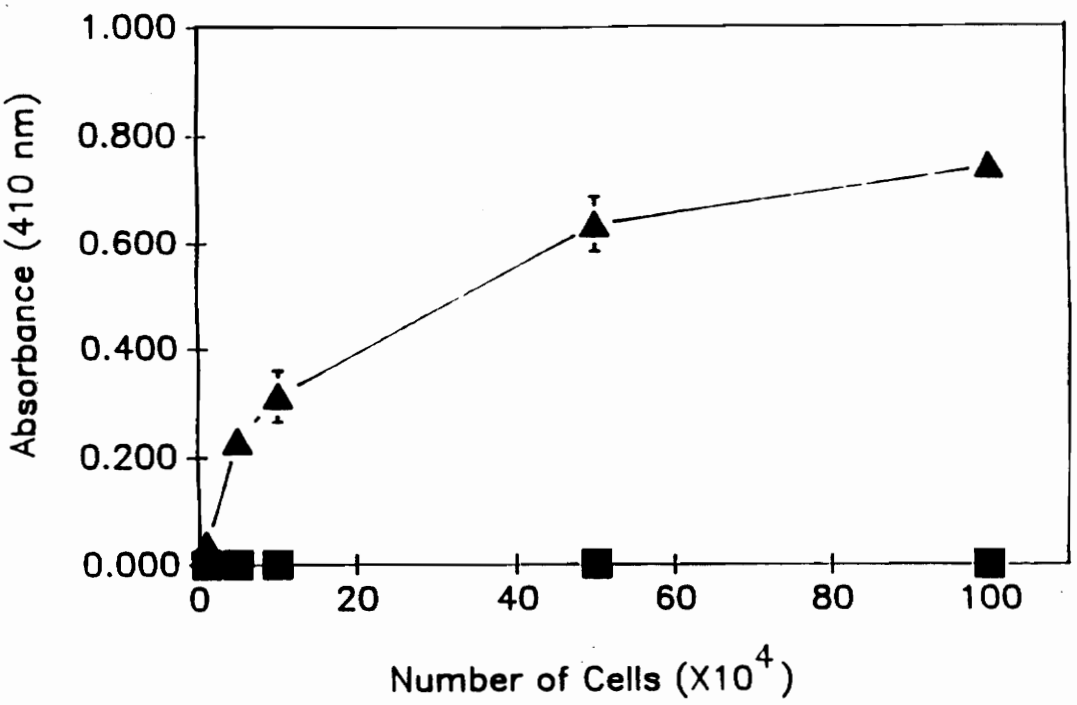

Fig 3.4 **BLTE (granzyme A) activity in *lpr* DN T cells.** Freshly isolated and purified *lpr* DN T cells (◆) or activated CTL clone, PE-9 (▲) used as a positive control, were lysed and BLTE activity was measured as described in Materials and Methods.

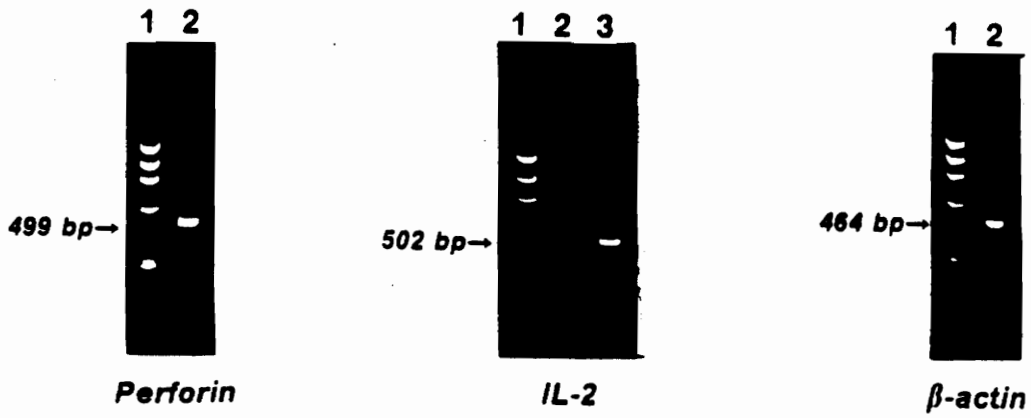

Fig 3.5 **Spontaneous expression of perforin gene by DN T cells from lpr mice.** Total RNA was extracted rom cells, reverse transcribed, and cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for perforin, IL-2 and ß-actin. The PCR products were electrophoresed through a 1.5% agarose gel containing ethidium bromide. *Lane 1* is a molecular standard, *lane 2* depicts DN T cells, and *lane 3* for IL-2 represents normal splenic T cells stimulated with anti-CD3 mAbs.

electrophoresed as described earlier. The data shown in Figure 3.6 suggested that when 0.5 μg of RNA was used in PCR analysis, no visible perforin band was seen using MRL-+/+ or PE-9 cells. However, a demonstrable perforin band was seen using MRL-*lpr/lpr* LN cells. In this experiment, the expression of ß-actin served as an internal control. Furthermore, when PCR was initiated with 1 μg of RNA for all samples, the expression of perforin was seen in all three cell types. However, the expression of perforin band was stronger using MRL-*lpr/lpr* cells when compared with MRL-+/+ cells. These data suggested that perforin expression seen using *lpr* LN can be attributed to *lpr* DN T cells.

This study has two important implications on the nature *lpr* DN T cells. First, *lpr* DN T cells although incapable of producing IL-2 and dividing when activated through the TCR, and therefore believed to be unresponsive, are fully functional as cytotoxic cells. The ligand for the DN TCR is not known. If the ligand is a self-antigen expressed on *lpr* cells, the DN T cells may kill such cells, thereby contributing to the autoimmune disease process. However, it should be noted that *lpr* DN T cells have been shown to have undergone negative selection and that their TCR is polyclonal in nature (for a review see Cohen and Eisenberg, 1991). Thus, it is less likely that they would cause damage to autologous cells after activation through the TCR. However, our findings that DN T cells can be activated via other adhesion molecules such as CD44 and gp90^{MEL-14}, to mediate cytotoxicity, further suggests that the DN T cells may be cytotoxic to autologous cells that bear ligands for CD44 and gp90^{MEL-14}. Such a mechanism may explain the observation that *lpr* LN contain cells capable of spontaneous cytotoxicity against autologous cells (Muraoka and Miller, 1988). Second, CD44 and gp90^{MEL-14} have been implicated in lymphocyte adhesion to

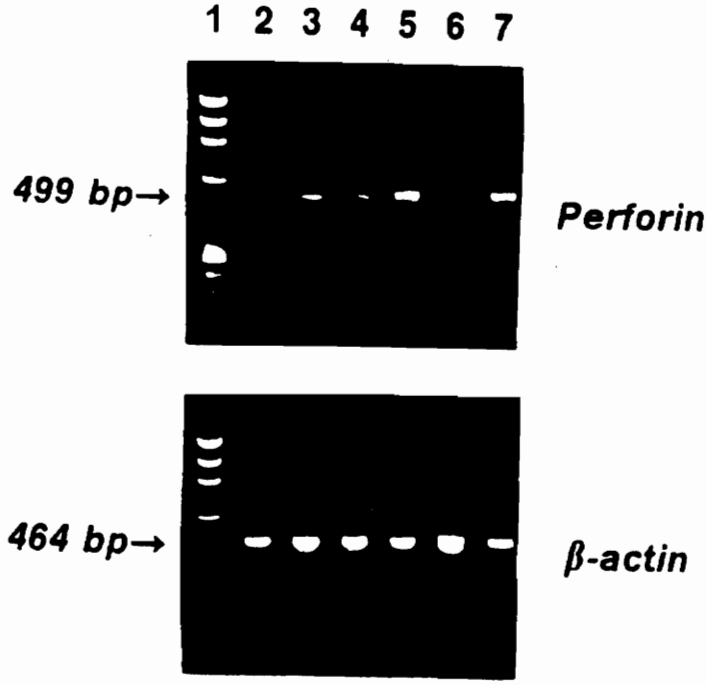

Fig 3.6 **Comparison of expression of perforin gene by LN cells from MRL-lpr/lpr and MRL-+/+ mice.** Perforin gene expression at the mRNA level was studied as described in Fig. 3.5, using varying concentrations of RNA in the PCR analysis as follows: *lane 1* is a molecular standard; *lane 2*, 0.5 μg of RNA from MRL-+/+ cells; *lane 3*, 1.0 μg of RNA from MRL-+/+ cells; *lane 4*, 0.5 μg of RNA from MRL-lpr/lpr cells; *lane 5*, 1 μg of RNA from MRL-lpr/lpr cells; *lane 6*, 0.5 μg of RNA from CTL clone, PE-9; and *lane 7*, 1 μg of RNA from CTL clone, PE-9.

endothelial cells (Haynes *et al.*, 1989). Thus, it is possible that the interaction between DN T cells and endothelial cells can lead to activation of the lytic properties of the DN T cells and consequently damage endothelial cells, resulting in vascular disease seen in *lpr* mice (Hewicker and Trautwein, 1987). Our data support earlier observations that depletion of DN T cells reduces the immunopathology (Mountz *et al.*, 1987) and that the DN T cells may mediate their effect by their capacity to spontaneously transcribe IFN-γ and TNF-α genes (Murray and Martens, 1989).

Based on this study, we suggest that DN T cells are not inert or anergic T cells but may represent activated cytotoxic cells constitutively expressing certain cytokines. This combined with the fact that DN T cells can be activated via a variety of adhesion molecules to mediate cytotoxicity suggests that they play an important role in the induction of autoimmune disease.

Chapter 4: Double-Negative T cells from Mice Homozygous for *lpr* but not *gld* Mutation Exhibit Cytotoxic activity: Role of Fas and Fas-Ligand Interactions.

Introduction

Mice homozygous for *lpr* gene develop an autoimmune disease similar to that seen in human systemic lupus erythematosus and develop massive infiltration of an unusual subset of $\alpha\beta TCR^+$ T cells which are $CD4^-CD8^-$ (double negative, DN) (reviewed in Cohen and Eisenberg, 1991). The mutant *lpr* gene encodes a defective form of Fas, a molecule involved in the signal transduction of apoptosis (Watanabe-Fukunaga *et al.*, 1992). Similarly, mice homozygous for the *gld* mutation also develop an identical disease and *gld* mutation has been characterized to result in a defective and nonfunctional expression of the ligand for the Fas molecule (Takahashi *et al.*, 1994). Despite extensive research, the nature and significance of the DN T cells that

accumulate in *lpr* and *gld* mice is not clear. The DN T cells are unique not only in their inability to express CD4 and CD8 molecules, but also exhibit an unusual phenotype not normally expressed by naive T cells, such as, the expression of J11d, Ly6C, CD45R, and CD44 (Morse *et al.*, 1982). Furthermore, the DN T cells are refractory to stimulation with mitogens or antibodies against the CD3-TCR complex as measured by their inability to proliferate, acquire IL-2 receptors and produce IL-2 (Davignon *et al.*, 1988; Kakkanaiah *et al.*, 1990; Wofsy *et al.*, 1981; Kakkanaiah *et al.*, 1991). Such studies have suggested that *lpr* DN T cells are inactive or anergic T cells whose role in autoimmune disease remains unknown.

Recent studies from our lab demonstrated that the DN T cells from *lpr* mice constitutively express perforin gene and exhibit spontaneous NK-like cytotoxicity (Kakkanaiah *et al.*, 1990; Hammond *et al.*, 1990). Furthermore, it was noted that mAbs against the CD3 or $\alpha\beta$TCR complex as well as against certain adhesion molecules such as CD44 and gp90$^{MEL\text{-}14}$ could activate the lytic potential of the *lpr* DN T cells (Hammond *et al.*, 1993; Wang *et al.*, 1993). These studies suggested that *lpr* DN T cells may represent activated cytotoxic T cells and that they may participate in the autoimmune disease process by being able to mediate lysis of autologous cells bearing the specific ligands for the adhesion molecules involved in the signalling of the cytotoxicity.

In the current study, we further investigated whether the DN T cells from *gld* mice would also exhibit spontaneous or "redirected" cytotoxicity when stimulated through the TCR or other adhesion molecules, to mediate lysis of non-specific FcR$^+$ targets. Surprisingly, we observed that unlike the *lpr* DN T cells *gld* DN T cells failed to exhibit any cytotoxicity in vitro. The inability of *gld* DN T cells to mediate

cytotoxicity was due to the fact that the *gld* DN T cells failed to express the Fas-ligand. Furthermore, we also investigated whether *lpr* DN T cells would mediate cytotoxicity of endothelial cells, inasmuch as, our previous studies had demonstrated that the DN T cells and $CD8^+$ $\alpha\beta TCR^+$ cytotoxic T lymphocytes (CTL) could be activated through homing receptors to mediate redirected cytotoxicity (Hammond *et al.*, 1993; Seth *et al.*, 1991). These experiments revealed that the *lpr* DN T cells could mediate efficient lysis of syngeneic endothelial cells but not syngeneic fibroblast cells. Furthermore, the cytotoxicity of endothelial cells by the *lpr* DN T cells was dependent on the recognition of hyaluronate expressed on the endothelial cells. Together, our data suggested that the interaction between Fas expressed on target cells and Fas-ligand expressed by DN T cells plays an important role in the induction of cytotoxicity. Secondly, because the DN T cells express high levels of CD44 they may be able to interact with the endothelial cells and this combined with the fact that the DN T cells spontaneously express several cytokines, suggests that they may also participate in the induction of vascular disease that is seen in these autoimmune mice.

Materials and Methods

Mice. MRL-*lpr/lpr* (*lpr*), MRL-+/+., C57BL/6-+/+, and C57BL/6-*gld/gld* (*gld*) mice were originally obtained from the Jackson Laboratories (Baar Harbor, Maine) were bred in our animal facilities as described (Seth *et al.*, 1988).

Antibodies. The monoclonal antibodies were in culture supernatants and were from the following hybridomas: KM201 directed against CD44 which recognizes hyaluronate binding site (Miyake *et al.*, 1990); MEL-14 against lymphocyte homing receptor ($gp90^{MEL\text{-}14}$); 24G2 against FcR; H57-597 against TCR-αß; 145.2C11 against CD3; 6B2 against CD45R; and J11d against heat stable antigen. All hybridomas were procured and mAbs were purified as described elsewhere (Hammond *et al.*, 1993). Anti-Fas mAbs were purchased from Pharmingen (San Diego, CA); anti-IA^k, anti-IA^b, and anti-$H\text{-}2^k$ polyclonal antibodies were kindly provided by NIH (Bethesda, MD).

Medium and reagents. Tissue culture medium, RPMI-1640 (GIBCO/BRL, Grand Island, NY) was used throughout these studies, supplemented with 2mM L-glutamine, 50 uM 2-mercaptoethanol, gentamicin (50 ug/ml), and 10% (vol/vol) fetal bovine serum (Atlanta Biologicals, Norcross, GA). Phorbol myristate acetate (PMA), calcium ionophore, hyaluronate (bovine trachea), and hyaluronidase (bovine testes) were purchased from Sigma Chemical Co. (St. Louis, MO).

DN T cells. The DN T cells were purified as described elsewhere (Kakkanaiah *et al.*, 1990; Kakkanaiah *et al.*, 1991; Hammond *et al.*, 1993) by treating LN cells from

4 month old *lpr* or *gld* mice twice with anti-CD4, anti-CD8, and either anti-IA^k (MRL-*lpr/lpr*) or anti-IA^b (C57BL/6-*gld/gld*) antibodies followed by complement. Next, the viable cells were isolated via density gradient centrifugation over histopaque (Sigma Chemical Co., St. Louis, MO). The purity of the DN T cells was >95% as determined by flow cytotometry as described (Hammond *et al.*, 1993).

Cell lines. P815, a natural killer (NK) cell-resistant mastocytoma and YAC-1, an NK-sensitive Moloney virus-induced lymphoma were maintained in vitro by serial passage as described elsewhere (Hammond *et al.*, 1993). A mouse endothelial cell line immortalized by SV40 (TME-3H3) bearing H-2^k developed by A. Hamann (Harder *et al.*, 1991) was kindly provided by J. Lesley, The Salk Institute, San Diego, CA and an SV40 transformed fibroblast cell line (SVC3H) also of H-2^k origin, kindly provided by L. Gooding, Emory University, Atlanta, GA were maintained in culture as described (Gooding, 1977).

Generation of alloreactive cytotoxic T lymphocytes (CTL). Alloreactive CTL were generated as described (Seth *et al.*, 1991). Briefly, $3x10^6$ splenic T cells from C57BL/6-+/+ or C57BL/6-*gld/gld* (H-2^b) mice were cultured in 24-well plates containing 2 ml of complete medium, with $3x10^6$ irradiated (2000 rads) DBA/2 (H-2^d) or C3H-HJ (H-2^k) spleen cells. After 5 days of culture at 37°C, live alloreactive CTL were harvested and isolated by density gradient centrifugation over histopaque (Sigma Chemical Co., St. Louis, MO) and tested for cytotoxicity against ^{51}Cr-labeled targets bearing the same MHC as the stimulator cells.

Cytotoxicity assay. The "redirected" cytotoxicity was performed by studying the ability of the DN T cells to lyse the FcγR$^+$ tumor target cells in the presence of mAbs against adhesion molecules expressed by the DN T cells (Seth *et al.*, 1991; Hammond *et al.*, 1993). The direct cytotoxicity was carried out by studying the ability of DN T cells to mediate cytotoxicity of NK-sensitive YAC-1 target cells. The cytotoxicity was studied by ^{51}Cr-release assay as described (Seth *et al.*, 1991; Hammond *et al.*, 1993). Tumor target cells were labeled with ^{51}Cr and seeded at $5x10^3$ cells/well along with varying numbers of effector cells and mAb supernatants in redirected cytotoxicity or the target cells alone, in direct cytotoxicity assays. The plates were incubated at 37°C for 4 hours. The amount of ^{51}Cr released by target cells was measured using a gamma counter (TM Analytic, Elk Grove, IL). Percent cytotoxicity was calculated from the ^{51}Cr-release as follows:

%cytotoxicity = 100X (experimental release-control release)/(total release-control release). In redirected cytotoxicity, the spontaneous release was measured both in the presence or absence of mAbs to ensure that the Abs alone did not trigger additional release of ^{51}Cr from the target cells. Also, in some cytotoxicity assays, PMA (10 ng/ml) and calcium ionophore (0.5 μM) were added to enhance the cytotoxicity of the DN T cells against the endothelial cells. To prepare Fas$^+$ and Fas$^-$ targets in redirected lysis, spleen cells from MRL-+/+ or MRL-*lpr/lpr* mice (less than 2 months of age) were stimulated with LPS (50 μg/ml) for 24 hours and the B cell blasts were labelled with ^{51}Cr and used as targets in redirected cytotoxicity assays. In assays involving hyaluronidase treatment, after labeling with ^{51}Cr, TME-3H3 cells were incubated in complete medium containing 60 U/ml hyaluronidase at 37°C for 30 min and rinsed three times in medium prior to mixing with effector cells.

PCR analysis of cytokine gene expression in DN T cells. PCR method was employed to study whether the *lpr* and *gld* DN T cells spontaneously transcribed various cytokine genes as described previously (Hammond *et al.*, 1993). Briefly, purified DN T cells were lysed in a buffer containing 0.14 M NaCl, 1.5 mM $MgCl_2$, 10mM Tris chloride, pH 8, 0.5% NP-40, and 1mM 2-ME. The total RNA was isolated by digesting the cell lysate at 37°C for 35 minutes with Proteinase K (50 μg/ml) in a digestion buffer containing 0.2 M Tris-chloride pH 7.6, 25 mM EDTA pH 8, 0.3 M NaCl, and 2% SDS. Next, proteins were extracted in a phenol:chloroform (1:1) mixture. Nucleic acids from aqueous phase were precipitated in ethanol at -80°C overnight. The concentration of recovered RNA was measured by UV absorption spectrophotometry and RNA was reverse transcribed to cDNA as describe in the Perkin-Elmer Cetus (Norwalk, CT) protocol. Reverse transcription was performed at 42°C for 45 min. followed by denaturation of the Reverse Transcriptase at 99°C for 5 min. The resulting cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers. The primers for ß-actin, used as a control, and perforin cDNA were selected using the Genetic Computing Group computer assisted search from GenBank sequences. TNF-α and IFN-γ cDNA primers were purchased from Clontech (Palo Alto, CA). The PCR was run according to the Clontech product protocol guide at 94°C for 2 min followed by 35 cycles of denaturing at 94°C for 45 sec., annealing at 60°C for 45 sec., and extension at 72°C for 2 minutes. The primer sequences were as follows for ß-actin 5'-TATCCTGACCCTGAACTACCCCATT and 3'-AGCACAGCTTCTCTTTGATGTCACG; for perforin 5'-GGTCAGAATGCAAGCAGAAGCACAAand3'-TTGAAGTCAAGGTGGAGTGGAGGTT; for TNF-α 5'-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC and 3'-

GTATGAGATAGCAAATCGGCTGACGGTGTGGG; and IFN-γ 5'-TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC and 3'-TGGACCTGTGGGTTGACCTCAAACTTGGC. Five μl of the PCR product was electrophoresed on 1.5% agarose gel stained with ethidium bromide. The demonstration of a single band of 464-, 499-, 354-, or 365-bp size was considered to be indicative of expression of ß-actin, perforin, TNF-α, and IFN-γ genes, respectively.

Results

***lpr* but not *gld* DN T cells exhibit cytotoxic activity:**

In a recent study we demonstrated that DN T cells could mediate spontaneous cytotoxicity of certain tumor targets and participate in redirected lysis of FcR^+ tumor targets in the presence of mAbs against the CD3/TCR complex as well as against homing receptors, such as, CD44 and $gp90^{MEL-14}$ (Hammond *et al.*, 1993). Recent studies have demonstrated that the interaction between Fas-ligand expressed on cytotoxic T cells and Fas receptor expressed on target cells may play a crucial role in the induction of cytotoxicity by the T cells. Inasmuch as, the *gld* DN T cells lack the expression of functional Fas-ligand and because previously it has not been investigated whether the *gld* DN T cells can exhibit cytotoxicity, we thought it would be interesting to test whether *gld* DN T cells can mediate spontaneous cytotoxicity of certain tumor targets as well as participate in redirected lysis in the presence of anti-CD3/TCR antibodies.

As shown in Fig. 4.1 (A), *lpr* DN T cells could mediate significant lysis of YAC-1 target cells and furthermore in the presence of PMA and calcium ionophore, the cytotoxicity was significantly enhanced. Interestingly, however, the *gld* DN T cells failed to mediate spontaneous lysis of YAC-1 target cells and furthermore, addition of PMA and calcium ionophore failed to activate the DN T cells to mediate lysis of YAC-1 target cells. It was also investigated whether the *gld* DN T cells would mediate redirected lysis in the presence of anti-CD3 mAbs (Fig 4.1B), or anti-$\alpha\beta$TCR mAbs (Fig. 4.1C). While *lpr* DN T cells could mediate efficient lysis of FcR^+ P815 target cells in the presence of anti-CD3 or anti-$\alpha\beta$TCR mAbs, *gld* DN T cells failed to mediate lysis in these assays. Also, unlike *lpr* DN T cells, *gld* DN T cells failed to mediate lysis of

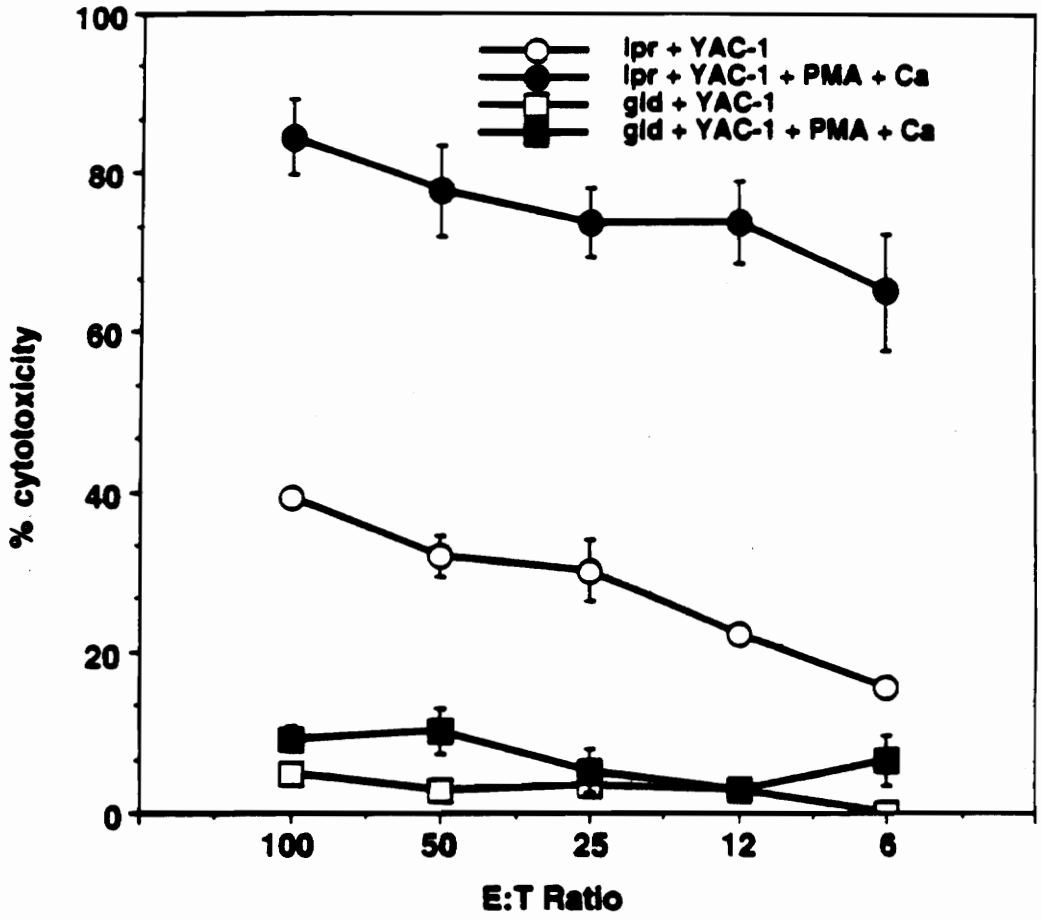

Fig 4.1A **DN T cells from *lpr* but not *gld* mice exhibit cytotoxic activity *in vitro*.** Freshly isolated DN T cells from lpr or gld mice were tested for spontaneous cytotoxicity against YAC-1 tumor targets in the presence or absence of PMA and calcium ionophore at various effector:target cell ratios.

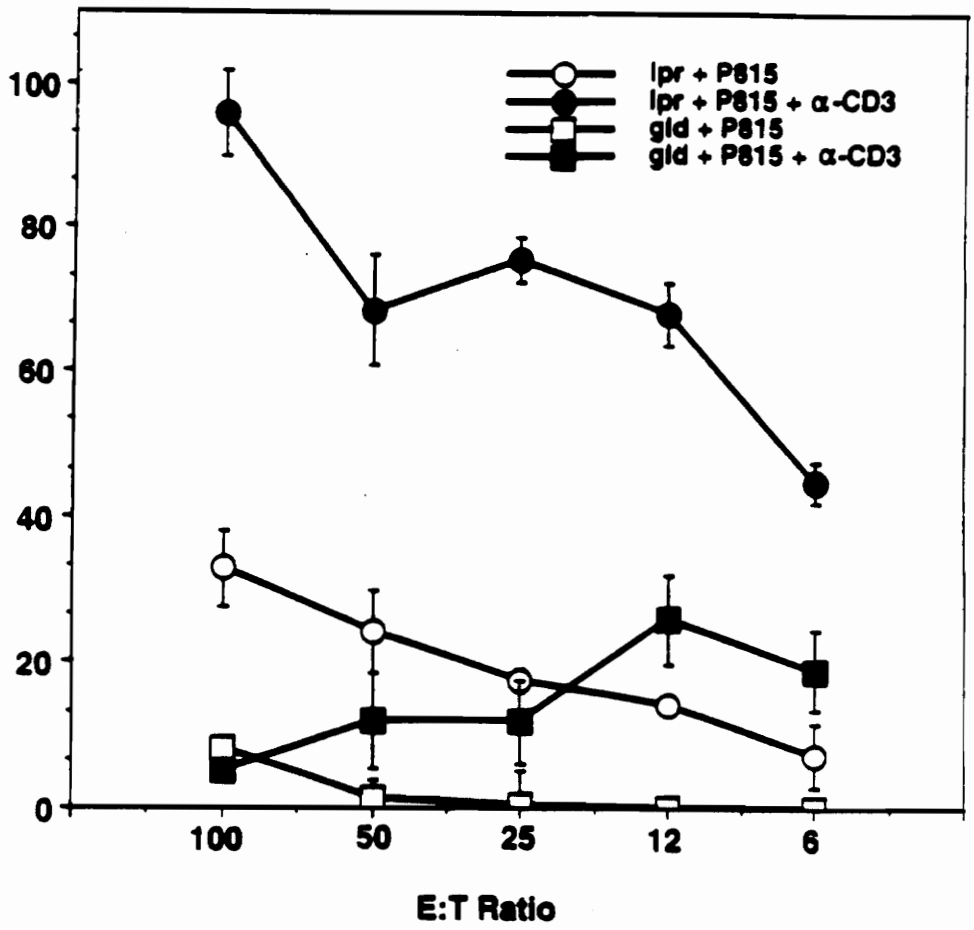

Fig 4.1B **DN T cells from *lpr* but not *gld* mice exhibit cytotoxic activity in vitro.** DN T cells from *lpr* or *gld* mice were tested for cytotoxicity against P815 tumor targets in the presence or absence of anti-CD3 antibodies (1:20 final dilution).

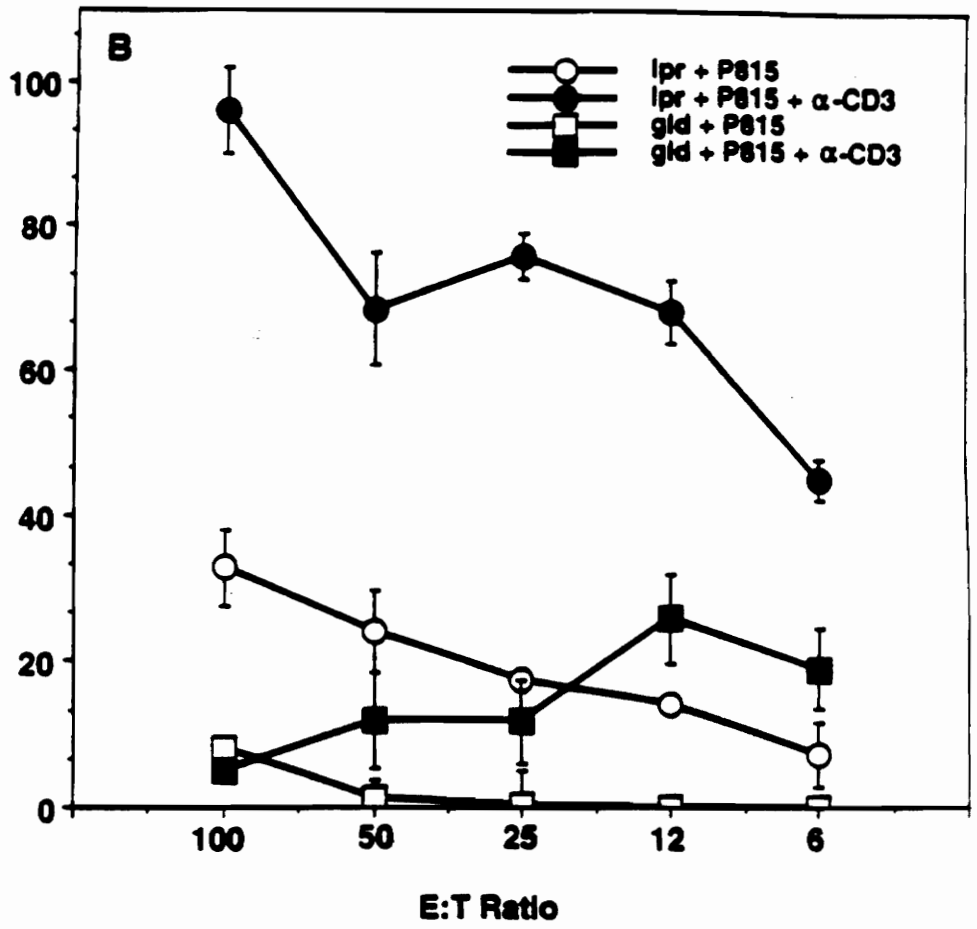

Fig 4.1C **DN T cells from *lpr* but not *gld* mice exhibit cytotoxic activity *in vitro*.** DN T cells from *lpr* or *gld* mice were tested redirected cytotoxicity against P815 tumor targets in the absence or presence of anti-αßTCR antibodies (1:10 final dilution).

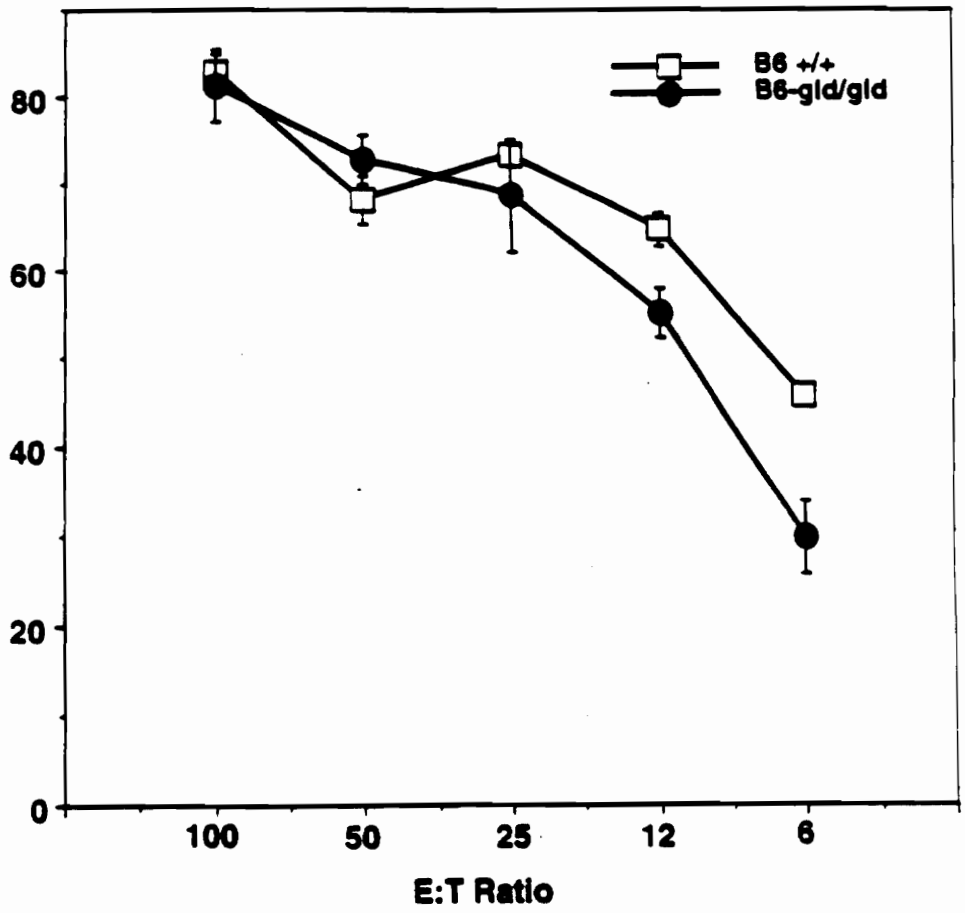

Fig 4.1D **DN T cells from *lpr* but not *gld* mice exhibit cytotoxic activity *in vitro*.** The ability of alloreactive CTL from *gld* and +/+ strains to mediate cytotoxicity of P815 tumor targets. T cells from *gld* or +/+ strain were stimulated in culture with irradiated spleen cells from DBA/2 (H-2^d) and following 5 days of culture the cells were harvested and tested for cytotoxic activity against P815 (H-2^d) tumor targets. The data is depicted as mean percent cytotoxicity ± standard errors from triplicate cultures.

target cells in the presence of anti-CD44 mAbs (data not shown). These data together demonstrated that unlike *lpr* DN T cells, *gld* DN T cells failed to mediate cytotoxicity of tumor targets when tested for both spontaneous cytotoxcity and redirected lysis.

We next investigated whether the inability of the *gld* DN T cells to mediate cytotoxicity was unique only to this population of T cells or whether a similar property was also demonstrated by other cytotoxic T cells from *gld* mice, such as the alloreactive CTL. To this end, splenic T cells from young *gld* or +/+ mice were cultured in vitro for 5 days in the presence of irradiated spleen cells from H-2^d strain. After 5 days, the cells were harvested and the alloreactive CTL were tested for their ability to mediate cytotoxicity against H-2^d (P815) tumor targets. The results depicted in Figure 4.1 (D) demonstrated that the alloreactive CTL from *gld* mice could mediate efficient lysis of P815 tumor targets comparable to the lysis exhibited by B6-+/+ mice. These data together demonstrated that while the *gld* DN T cells were exclusively dependent on the expression of functional Fas-ligand to mediate cytotoxicity, alloreactive CTL from *gld* strain could mediate efficient lysis of tumor targets even in the absence of functional Fas-ligand expression.

Recognition of Fas on target cells is critical for *lpr* DN T cell mediated cytotoxicity:

Although the above results demonstrated that *gld* DN T cells require the expression of a functional Fas-ligand to mediate cytotoxicity, it was not clear whether a similar requirement existed for the induction of cytotoxicity by *lpr* DN T cells. To address this we investigated whether YAC-1 and P815 tumor targets would express Fas. It was observed that >60% of these tumor cells expressed Fas molecule (Fig. 4.2). These

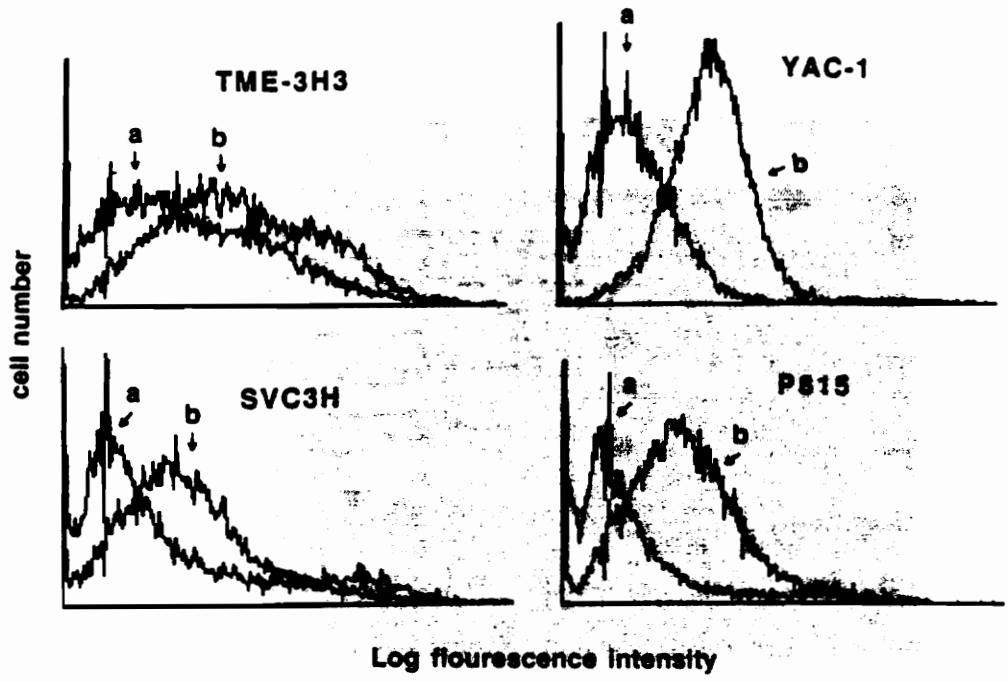

Fig 4.2 **Flow cytometric analysis of various tumor targets for the expression of Fas.** Tumor targets TME-3H3, YAC-1, SVC3H, and P815 were incubated with anti-Fas mAbs followed by staining with FITC-conjugated anti-hamster IgG and the cells were analyzed flow cytometrically. **(a)** Represents a negative control in which cells were stained with the secondary antibody alone and **(b)** cells stained with anti-Fas followed by the secondary antibody.

data suggesting that *lpr* DN T cells may mediate cytotoxicity of YAC-1 and P815 tumor targets following interaction between Fas-ligand and Fas. To further corroborate these findings, the ability of *lpr* DN T cells to mediate cytotoxicity of syngeneic Fas^+ and Fas^- target cells was investigated. To this effect, spleen cells from MRL-+/+ and MRL-*lpr/lpr* mice were stimulated with LPS to prepare FcR^+ blast cells which were used as target cells to study the ability of *lpr* DN T cells to mediate redirected lysis. The data depicted in Fig. 4.3 indicated that *lpr* DN T cells could mediate significant lysis of Fas^+ but not Fas^- target cells in the presence of anti-TCR mAbs thereby suggesting that *lpr* DN T cells were also dependent on the recognition of the Fas molecule on the target cells to mediate cytotoxicity when activated through the TCR.

Expression of adhesion molecules and cytokine genes by *lpr* and *gld* DN T cells:

It was also possible that the reason why *gld* DN T cells could not mediate cytotoxicity was due to the fact that the *gld* DN T cells may express differential levels of various adhesion molecules involved in the cytotoxicity when compared to the *lpr* DN T cells. To address this, 4 month old *gld* or *lpr* LN cells depleted of $CD4^+$ and $CD8^+$ T cells were stained with various mAbs directed against the TCR and other adhesion molecules. The data depicted in Fig. 4.4 indicated that *gld* LN cells exhibited similar percentages of DN T cells as *lpr* LN and furthermore, the density of expression of various adhesion molecules was similar and comparable in these strains of mice.

We had previously demonstrated that the *lpr* DN T cells constitutively transcribed perforin gene which is involved in cytotoxicity (Hammond *et al.*, 1993). Furthermore, other investigators have demonstrated expression of cytokines such as

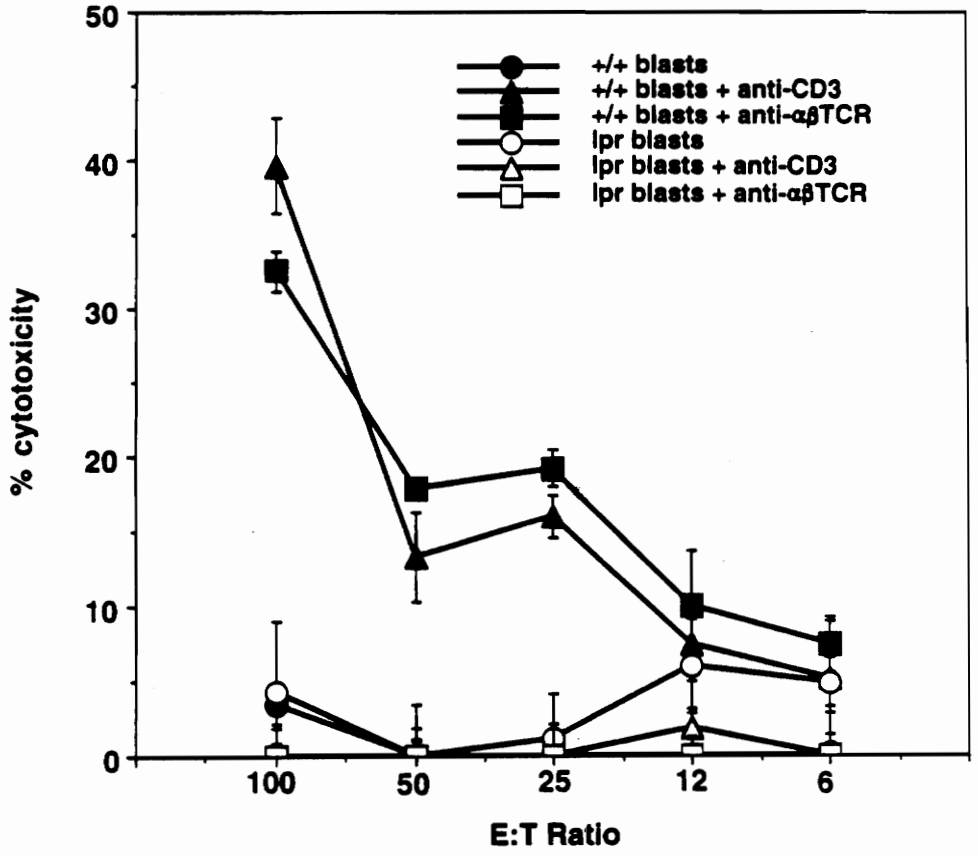

Fig 4.3 **DN T cells from *lpr* mice can mediate redirected cytotoxicity of Fas$^+$ (+/+) target cells but not Fas$^-$ (lpr) target cells.** DN T cells from *lpr* mice were tested for cytotoxicity against +/+ and *lpr* targets which were obtained by incubating spleen cells from +/+ or *lpr* mice with LPS for 24 hours and the LPS blast cells were used as targets in the presence or absence of anti-αßTCR mAbs in redirected cytotoxicity as described in Fig. 5.1B and C.

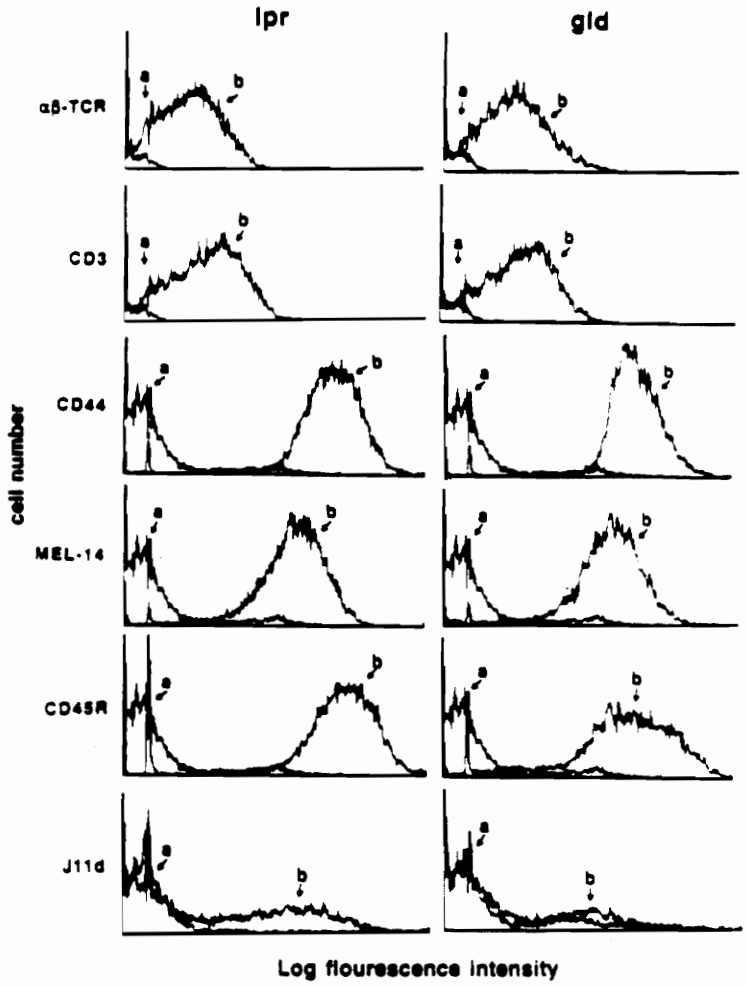

Fig 4.4

Flow cytometric analysis of the expression of various adhesion molecules on DN T cells from *lpr* and *gld* mice. DN T cells from *lpr* and *gld* mice (4 months of age) were incubated with various mAbs against adhesion molecules followed by staining with FITC-conjugated secondary antibodies as described in methods. These cells were analyzed flow cytometrically. **(a)** Depicts negative control in which cells were incubated with the secondary antibody alone and **(b)** represents cells stained with antibodies against adhesion molecules followed by the secondary antibodies. The percent positive cells in *lpr* mice were: *α*ßTCR (79%), CD3 (85%), CD44 (93%), gp90^{MEL-14} (89%), CD45R (86%), and J11d (37%). In *gld* mice the percentages were: *α*ßTCR (81%), CD3 (81%), CD44 (92%), gp90^{MEL-14} (86%), CD45R (84%), and J11d (18%). The mean intensity of fluorescence depicted as mean channel number fo *lpr* mice was: *α*ßTCR (57), CD3 (72), CD44 (178), gp90^{MEL-14} (145), CD45R (181), and J11d (130). Similarly for *gld* mice was : *α*ßTCR (65), CD3 (71), CD44 (163), gp90^{MEL-14} (143), CD45R (161), and J11d (102).

IFN-γ and TNF-α (Murray and Martens, 1989). In the current study therefore we investigated whether the failure of *gld* DN T cells to mediate cytotoxicity was related to the fact that these cells fail to spontaneously transcribe perforin or other cytokine genes. To this end, *gld* and *lpr* DN T cells were compared for the expression of various cytokine genes using PCR analysis. The data shown in Fig. 4.5 indicated that when similar quantities of RNA isolated from *lpr* or *gld* DN T cells were tested, comparable levels of perforin were demonstrable in *lpr* and *gld* DN T cells. However, the expression of TNF-α and IFN-γ was decreased in the *gld* cells when compared to the *lpr* cells. The fact that the *gld* DN T cells can constitutively express several cytokine genes involved in cytotoxicity suggested that the reason why these cells could not mediate cytotoxicity may not be due to the inability of these cells to produce these cytokines. It should be noted that *gld* DN T cells were not able to mediate cytotoxicity even following activation through the TCR or upon stimulation with PMA and calcium ionophore.

DN T cells can mediate cytotoxicity of endothelial cells:

lpr DN T cells as well as $CD8^+$ $\alpha\beta$-TCR^+ cytotoxic T cells were previously shown to mediate redirected cytotoxicity when activated through homing receptors such as CD44 and $gp90^{MEL-14}$ (Hammond *et al.*, 1993). Inasmuch as endothelial cells express the ligands for these homing receptors, it was suggested that such cytotoxic T cells may be able to mediate lysis of endothelial cells and this was recently demonstrated by us using the $\alpha\beta TCR^+$ $CD8^+$ cytotoxic T cells (Hammond-Mckibben *et al.*, in press). In the current study, we investigated whether *lpr* DN T cells would mediate spontaneous cytotoxicity of a syngeneic SV-40 transformed endothelial cell line designated TME-3H3. The data shown in Fig. 4.6(A) suggested that the *lpr* DN

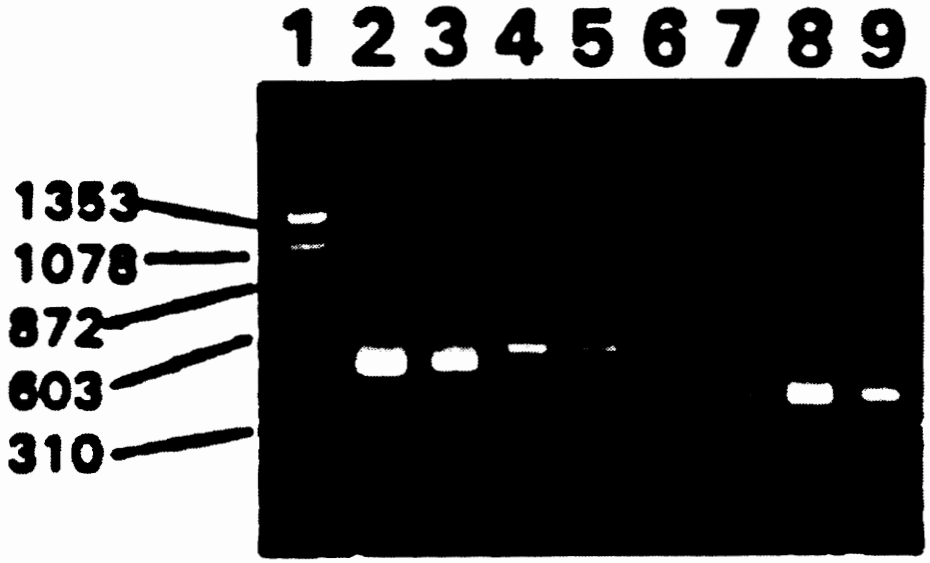

Fig 4.5 **PCR analysis of cytokine gene expression in lpr and gld DN T cells.** Total RNA was isolated from lpr and gld DN T cells and reverse transcribed as described in Materials and Methods. The resulting cDNA was then amplified using PCR with cytokine specific primers and 10 μl of PCR products were electrophoresed on a 1.5% agarose gel conataining ethidium bromide.

T cells could mediate moderate spontaneous lysis of the endothelial cells and when activated with PMA and calcium ionophore the cytotoxicity of endothelial cells was significantly enhanced. Using this approach, we next investigated the mechanism of lysis of endothelial cells by the *lpr* DN T cells. As shown in Fig. 4.6(B), while *lpr* DN T cells could mediate efficient lysis of TME-3H3 endothelial cells, they failed to lyse a syngeneic SV-40 transformed fibroblast cell line designated SVC3H. Also, the *gld* DN T cells failed to mediate cytotoxicity of endothelial (Fig. 4.6B) as well as the fibroblast cell line (data not shown). Together, these data suggested that *lpr* DN T cells can mediate efficient lysis of endothelial cells but not fibroblast cells. It should be noted that the inability of *lpr* DN T cells to mediate cytotoxicity of the fibroblast cell line (SVC3H) was not due to lack of expression of Fas because flow cytometric analysis revealed that SVC3H cells expressed significant levels of Fas, as did the endothelial cell line, TME-3H3 (Fig. 4.2).

Role of CD44-hyaluronate interaction in *lpr* DN T cell-mediated cytotoxicity of endothelial cells:

To further explore the mechanism of cytotoxicity of endothelial cells, anti-CD44 mAbs were added to the cytotoxicity assay to investigate whether the lysis of endothelial cells was mediated through CD44 expression by the DN T cells. In this assay we expected the anti-CD44 mAbs to act as a blocking factor rather than participate in redirected cytotoxicity because the TME-3H3 cells were found to be FcR^- (data not shown). As shown in Fig. 4.7A, mAbs against CD44 failed to inhibit and infact enhanced the lysis of the endothelial cells. It was possible that addition of anti-CD44 antibodies could lead to activation or other effects discussed later. Therefore

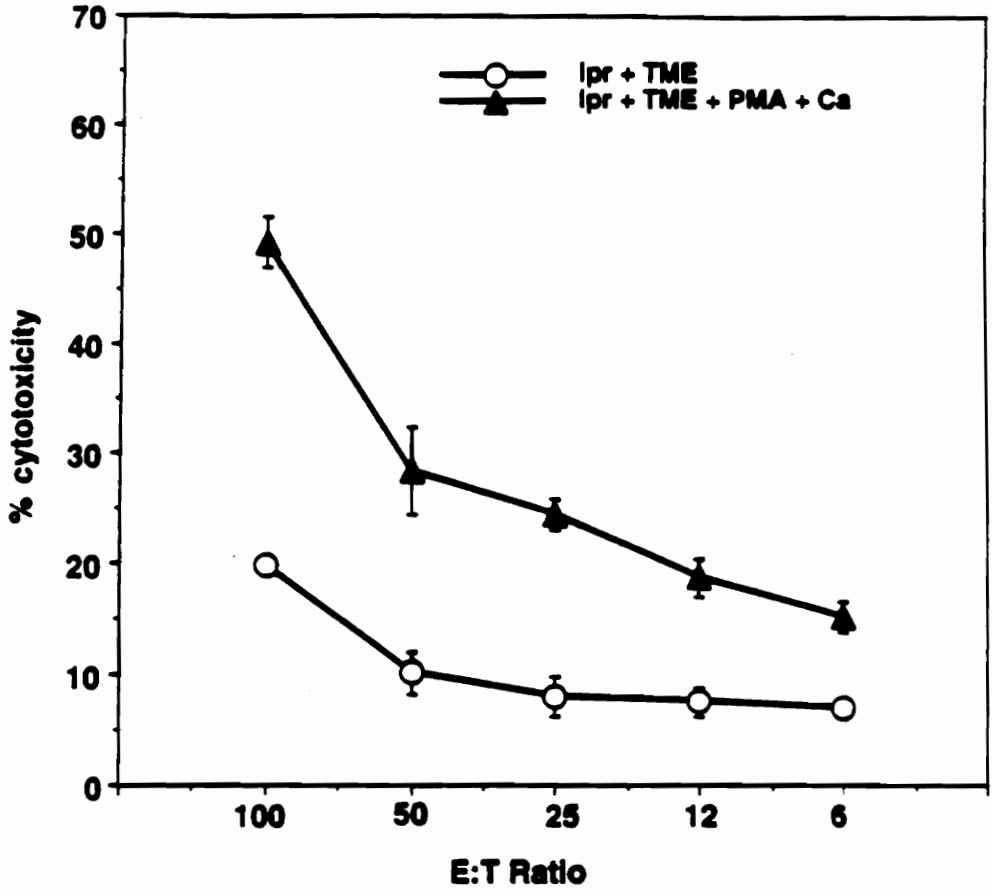

Fig 4.6A ***Lpr* but not *gld* DN T cells mediate cytotoxicity of endothelial cells.** *Lpr* DN T cells were tested for spontaneous cytotoxic activity against an SV40-transformed endothelial cell line, TME-3H3, in the absence or presence of PMA and calcium ionophore.

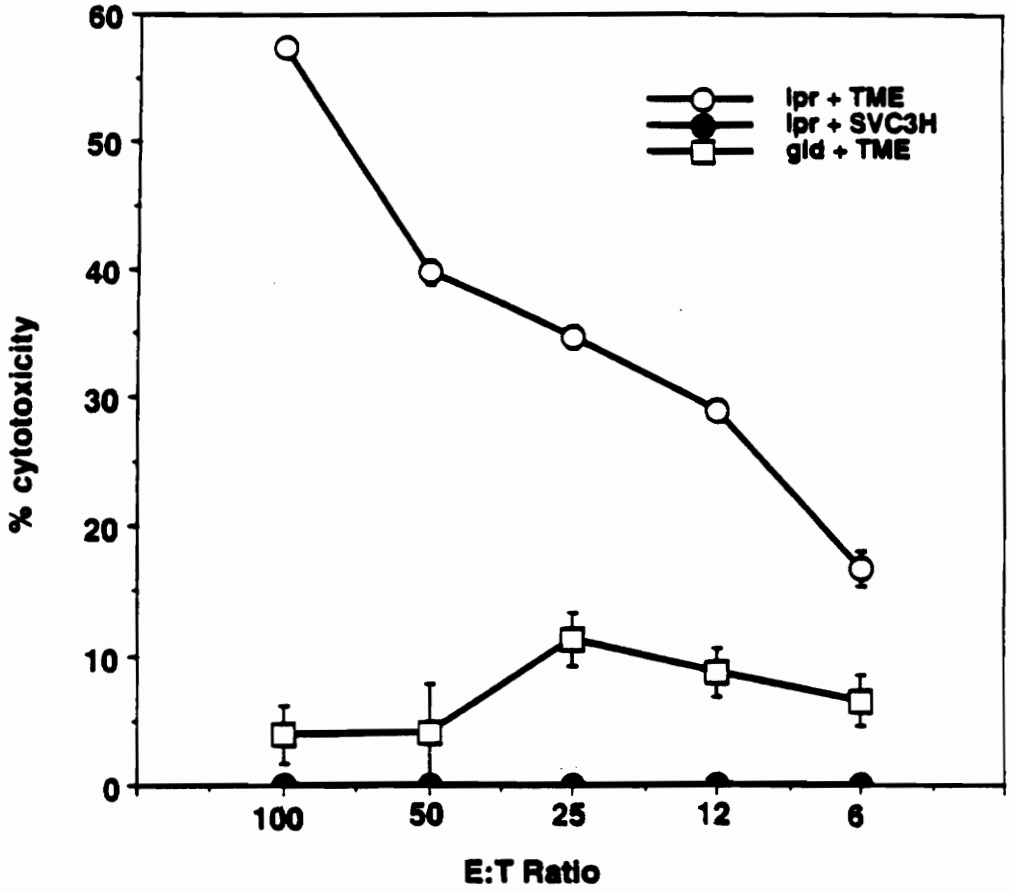

Fig 4.6B ***Lpr* but not *gld* DN T cells mediate spontaneous lysis of endothelial cells.** *Lpr* or *gld* DN T cells were tested for spontaneous cytotoxic activity against the endothelial cell line, TME-3H3, or a fibroblast cell line, SVC3H. The *gld* DN T cells were not able to mediate cytotoxicity of SVC3H cells or TME-3H3 cells in the presence of PMA and calcium ionophore (data not shown).

to further investigate the possible role of CD44, hyaluronate (HA) was added to the cytotoxicity assay because hyaluronate has been previously identified as one of the major ligands for CD44 (Miyake *et al.*, 1990). Addition of hyaluronate at 0.5 or 5 mg/ml to the cytotoxicity assay caused marked inhibition of the cytotoxicity of *lpr* DN T cells against endothelial cells (Fig. 4.7B). This inhibition of cytotoxicity was not due to nonspecific or toxic effect because similar addition of HA to the spontaneous cytotoxicity of YAC-1 targets did not cause any inhibition in the lysis of YAC-1 targets whereas in the same experiment, HA caused significant inhibition of the lysis of endothelial cells (Fig. 4.7C). Furthermore, treatment of endothelial cells with hyaluronidase caused marked decrease in the ability of *lpr* DN T cells to mediate cytotoxicity of the endothelial cells (Fig. 4.7D). Together, these data demonstrated that *lpr* DN T cells might recognize the hyaluronate on the endothelial cells and the interaction between CD44 and the hyaluronate may lead to activation of *lpr* DN T cells to mediate spontaneous lysis of endothelial cells as previously suggested (Hammond *et al.*, 1993).

The lysis of endothelial cells by DN T cells is MHC-unrestricted:

The above studies as well as the fact that *lpr* DN T cells lacking CD4 and CD8 expression can mediate cytotoxicity suggested that such cytotoxicity may be MHC-unrestricted and TCR-independent. To further corroborate this, the effect of the addition of anti-MHC or anti-TCR antibodies on the ability of *lpr* DN T cells to mediate cytotoxicity of endothelial cells was investigated. As shown in Fig. 4.8A, addition of anti-H-2^k antibodies failed to inhibit the cytotoxicity of the *lpr* DN T cells against the endothelial cells, whereas, addition of similar quantities of anti-H-2^k antibodies

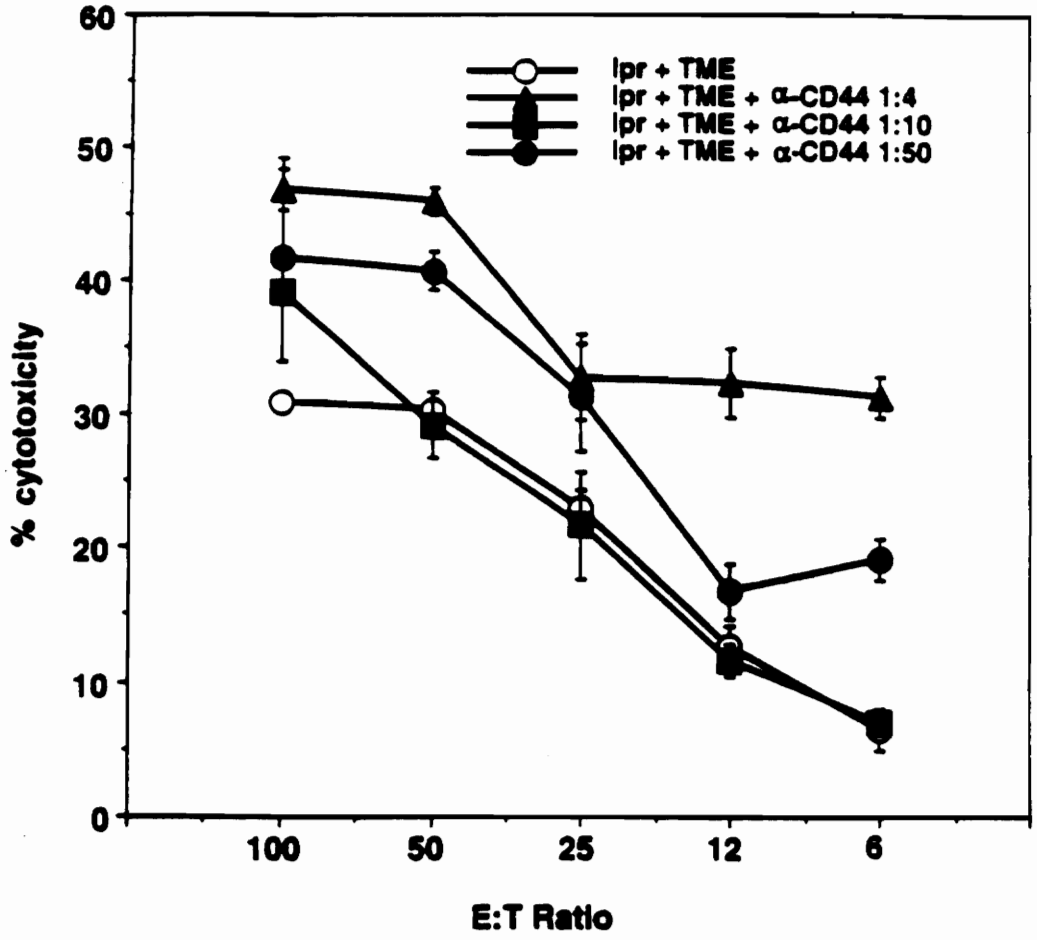

Fig 4.7A **Mechanism of *lpr* DN T cell mediated cytotoxicity of endothelial cells.** *Lpr* DN T cells were tested for cytotoxic activity against endothelial cell line, TME-3H3, in the presence or absence of anti-CD44 mAbs.

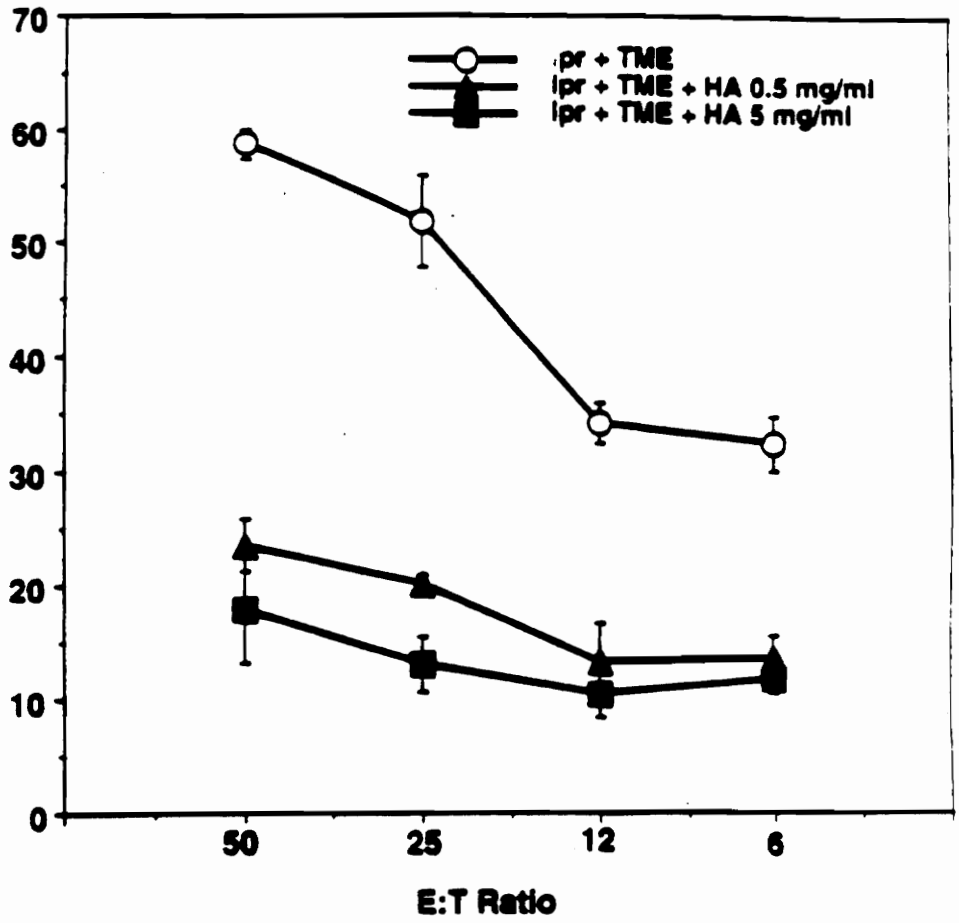

Fig 4.7B **Mechanism of *lpr* DN T cell mediated cytotoxicity of endothelial cells.** *Lpr* DN T cells were tested for cytotoxicity against TME-3H3 in the presence or absence of hyaluronate (HA).

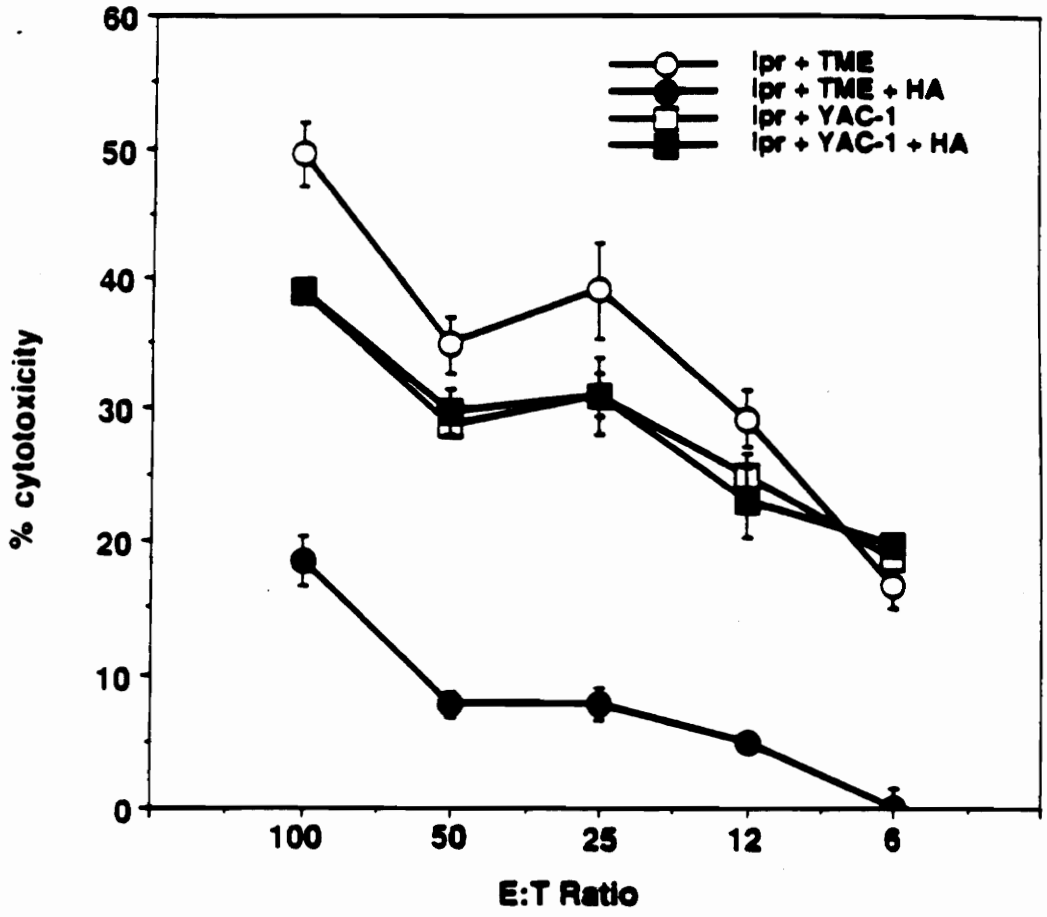

Fig 4.7C **Mechanism of *lpr* DN T cell mediated cytotoxicity of endothelial cells.** *Lpr* DN T cells were tested for spontaneous cytotoxic activity against endothelial cells (TME-3H3) or YAC-1 in the presence or absence of HA (0.5 mg/ml).

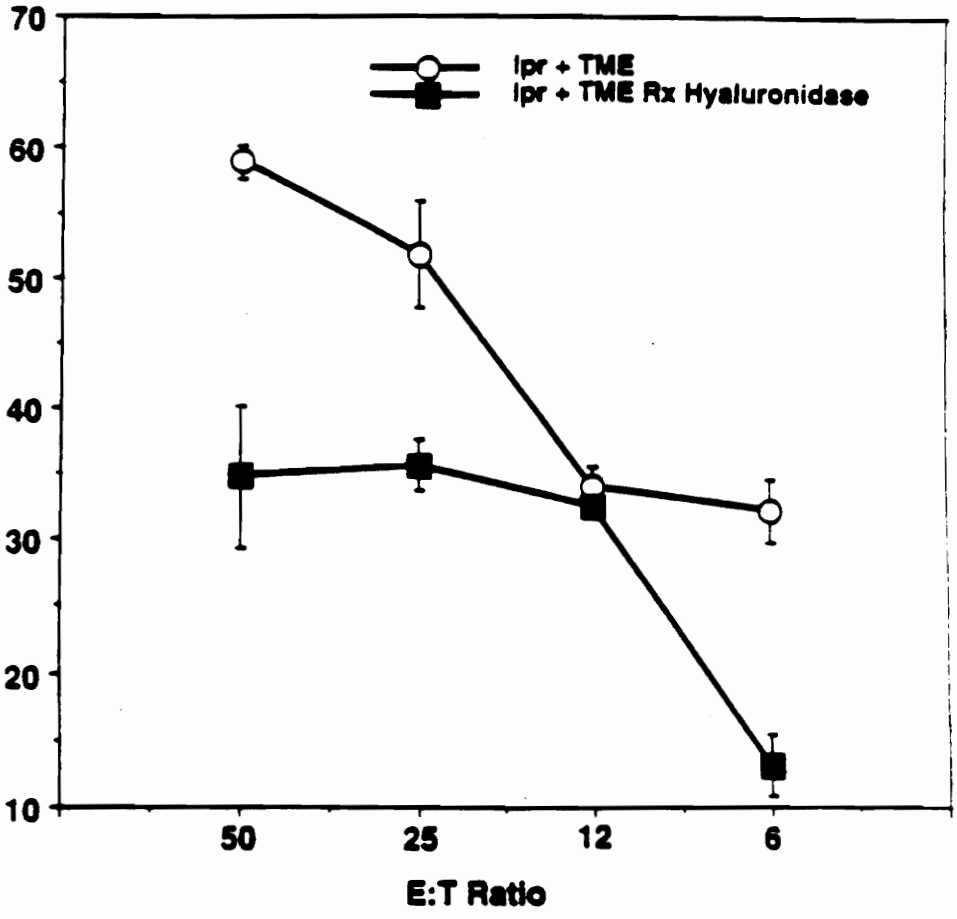

Fig 4.7D **Mechanism of *lpr* DN T cell mediated cytotoxicity of endothelial cells.** *Lpr* DN T cells were tested for spontaneous cytotoxic activity against endothelial cell line, TME-3H3, before or after treatment of targets with hyaluronidase (60 U/ml).

significantly inhibited the cytotoxicity of alloreactive T cells against the endothelial cells (Fig. 4.8B). In the above assay, the alloreactive CTL were generated by stimulating splenic T cells from H-2^b mice with irradiated H-2^k spleen cells and after 5 days of culture the effector T cells were tested against TME-3H3 endothelial bearing the H-2^k molecules. Next, addition of anti-CD3 or anti-$\alpha\beta$TCR antibodies failed to inhibit the cytotoxicity of *lpr* DN T cells against the endothelial cells and in fact caused significant increase in the cytotoxicity of endothelial cells possibly by further activating the DN T cells (Fig 4.8 C and D). These data together suggested that the cytotoxicity of *lpr* DN T cells against the endothelial cells was TCR-independent and MHC-unrestricted. These data also corroborated the findings that *lpr* DN T cells may use other adhesion molecules such as CD44 in the induction of cytotoxicity of endothelial cells.

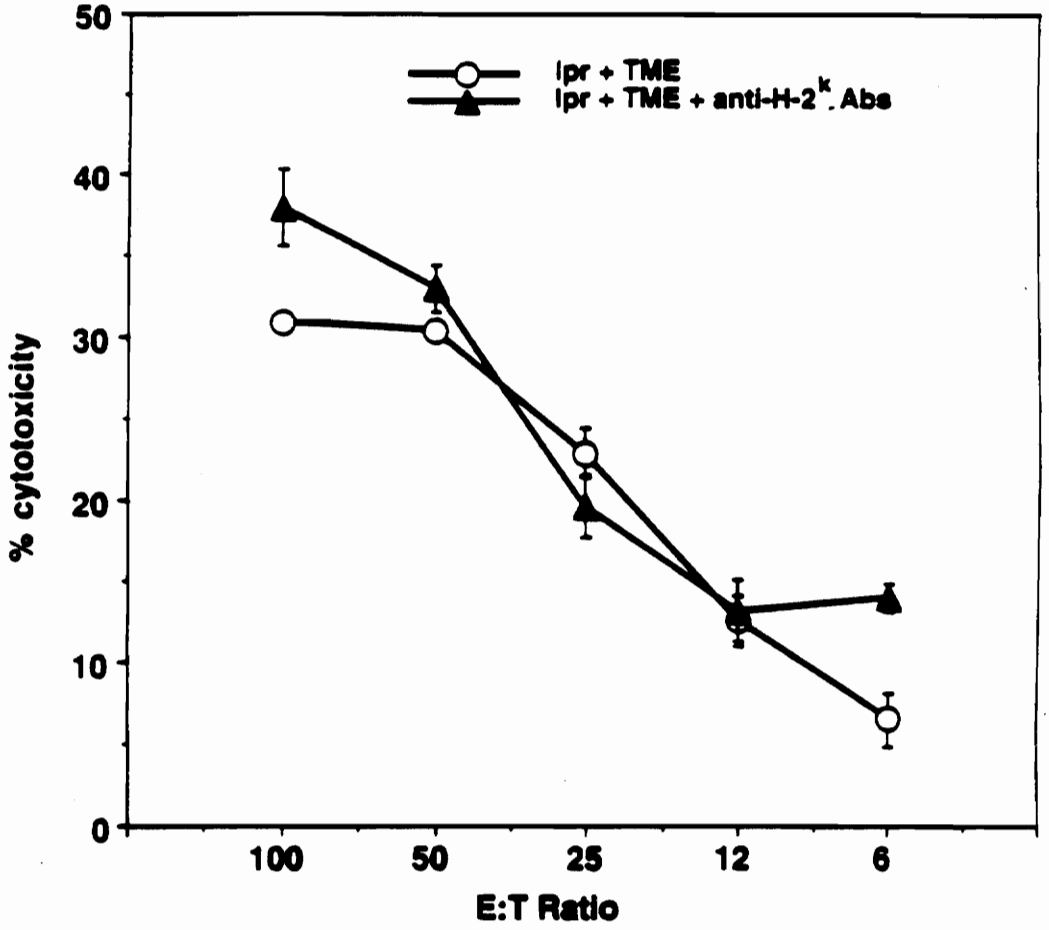

Fig 4.8A **The cytotoxicity of *lpr* DN T cells against endothelial cells is independent of the TCR and MHC molecules.** *Lpr* DN T cells were tested for cytotoxicity against endothelial cells (TME-3H3) as described in Fig. 5.7 in the presence or absence of anti-H-2^k antibodies (1:500 final dilution).

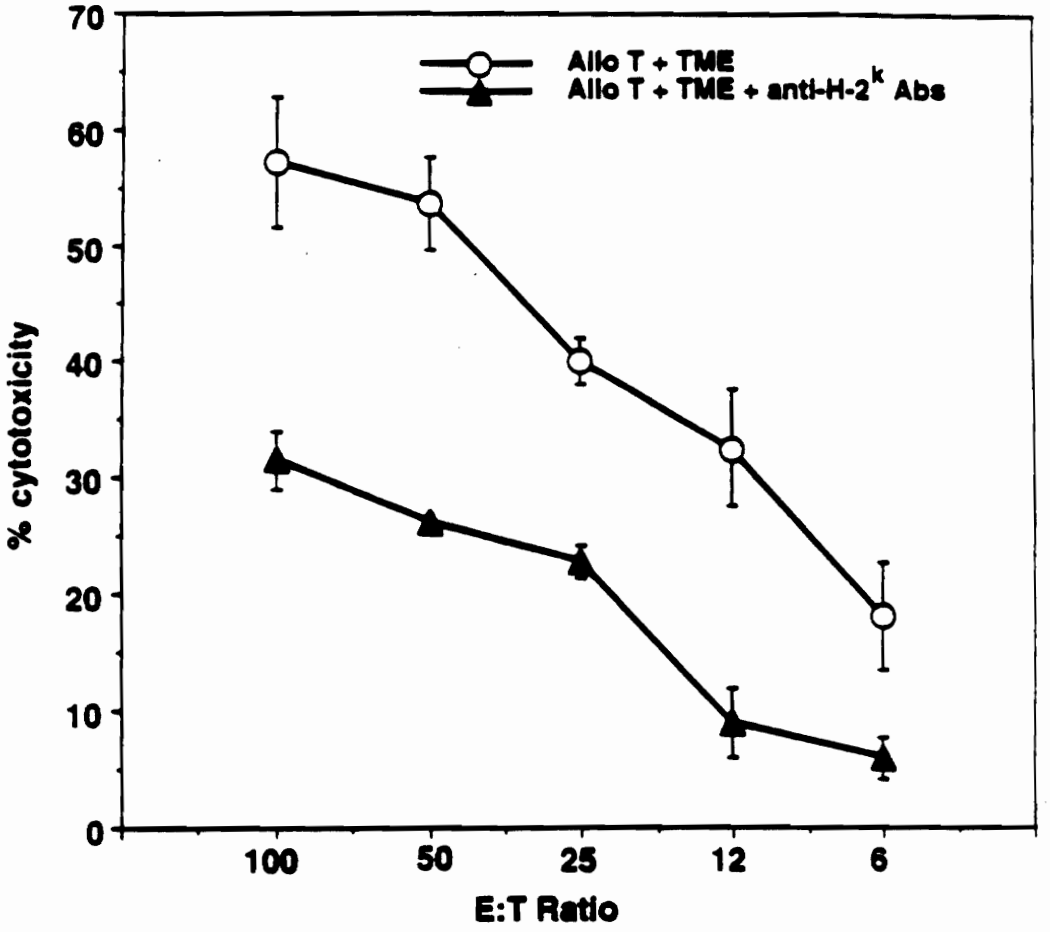

Fig 4.8B **The cytotoxicity of *lpr* DN T cells against endothelial cells is independent of the TCR and MHC molecules.** The ability of alloreactive T cells to mediate lysis of the endothelial cells, TME-3H3, is inhibited in the presence of anti-H-2^k antibodies (1:500 final dilution). The alloreactive CTL were obtained by culturing B6 T cells with spleen cells from H-2^k mice and after 5 days, the CTL were harvested and tested for cytotoxicity against H-2^k bearing endothelial cells.

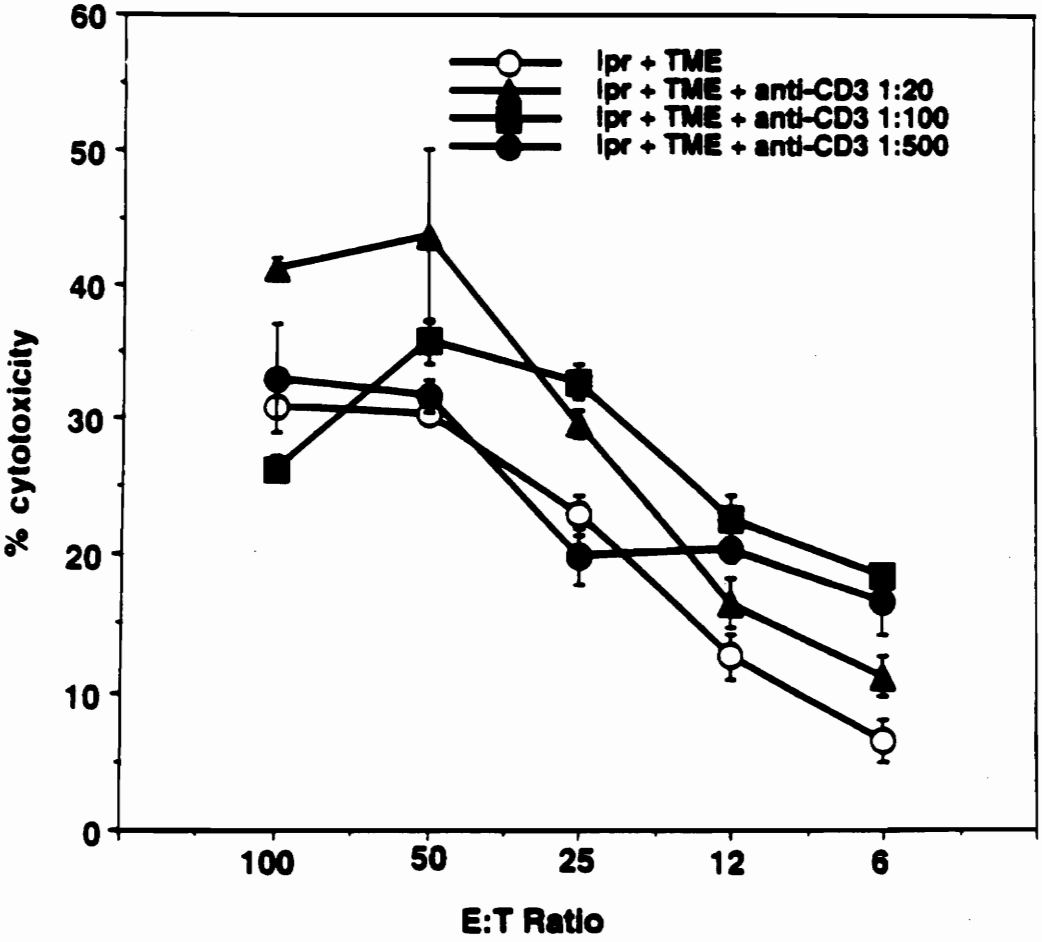

Fig 4.8C **The cytotoxicity of *lpr* DN T cells against endothelial cells is independent of the TCR and MHC molecules.** DN T cells from *lpr* mice were tested for cytotoxic activity against endothelial cell line, TME-3H3 in the presence or absence of various concentrations of anti-CD3 mAbs.

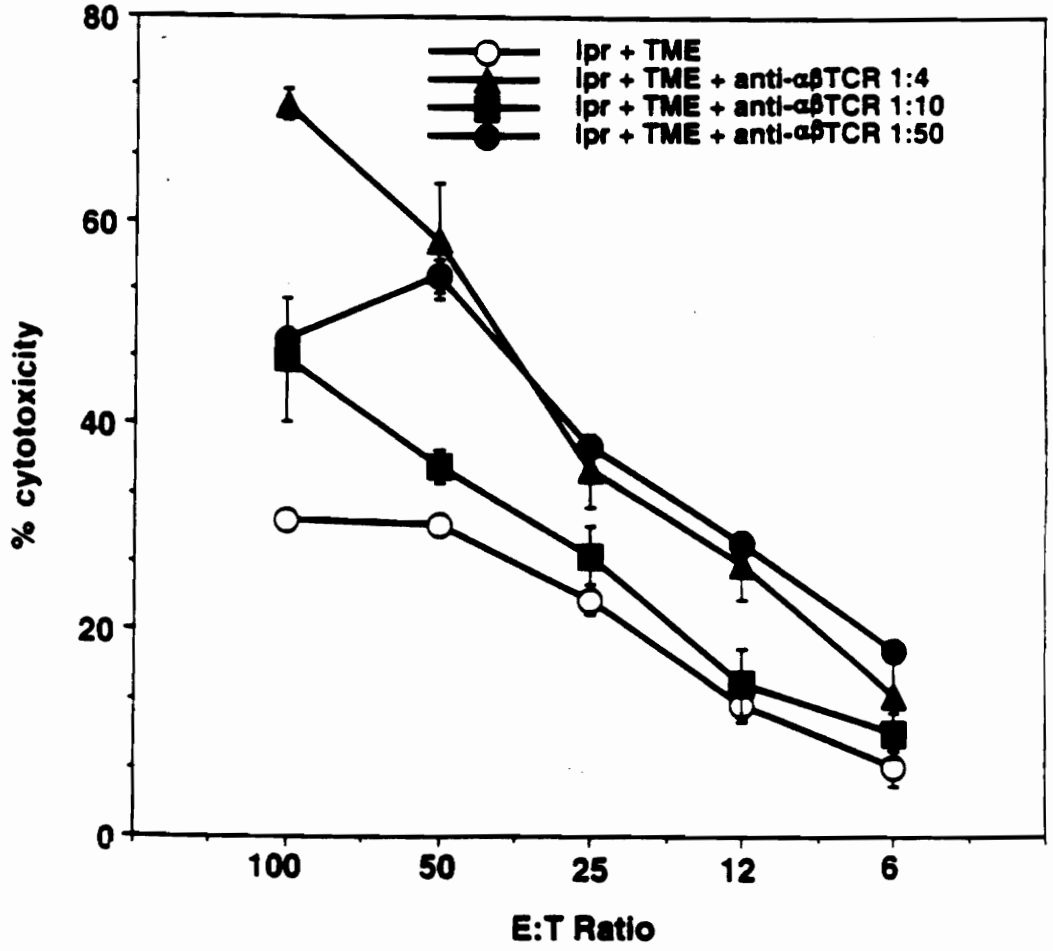

Fig 4.8D **The cytotoxicity of *lpr* DN T cells against endothelial cells is independent of the TCR and MHC molecules.** DN T cells from *lpr* mice were tested for cytotoxicity against TME-3H3 endothelial cells in the absence or presence of various dilutions of anti-αßTCR mAbs.

Discussion

Mice homozygous for *lpr* and *gld* gene mutations develop lymphoproliferative disease characterized by the accumulation of large numbers of DN T cells (Cohen and Eisenberg, 1991). In an earlier study it was demonstrated that DN T cells from *lpr* mice are cytotoxic T cells constitutively expressing perforin gene (Hammond *et al.*, 1993; Wang *et al.*, 1993). In the current study we demonstrate that similar DN T cells which accumulate in *gld* mice fail to exhibit cytotoxicity despite activation through the TCR or using PMA and calcium ionophore. The cytotoxicity exhibited by *lpr* and *gld* DN T cells was clearly dependent on the expression of Fas on the target cells and Fas-ligand on the DN T cells. It was possibly due to this reason that the *gld* DN T cells which lack a functional Fas-ligand were not able to mediate cytotoxicity of target cells bearing the Fas antigen. Interestingly, it was also noted that *lpr* DN T cells could mediate cytotoxicity of syngeneic endothelial cells, whereas, they failed to mediate lysis of syngeneic fibroblast cells. Also, the cytotoxicity of endothelial cells was independent of the TCR and was MHC-unrestricted but appeared to depend on the expression of hyaluronate by the endothelial cells. These data suggested that the interaction between CD44 expressed on the DN T cells and hyaluronate expressed on endothelial cells may play a critical role in the activation of DN T cells leading to the cytotoxicity of endothelial cells. Our studies demonstrate that the DN T cells may be able to interact closely with the endothelial cells and contribute to the vasculitis seen in the autoimmune mice.

There are several aspects of target cell cytotoxicity mediated by CTL which remain unresolved. Two important models have been described by which CTL lyse the

target cells (reviewed in ref. Henkart, 1985; Martz and Howell, 1989). The first is called necrosis in which the killer cells disrupt the target cell membranes leading to osmotic lysis of the cells. The second model is called apoptosis in which the target cells die from DNA fragmentation. The Fas (CD95) is a 45 kD cell surface protein belonging to the TNF-nerve growth factor receptor family which has been identified as a principal receptor for the induction of apoptosis in cells (reviewed in Nagata and Goldstein, 1995). Several recent studies suggested that the ability of cytotoxic cells to kill target cells may depend on the expression of Fas on the target cells and Fas-ligand on the CTL (Mosmann and Coffman, 1989; Cher and Mosmann, 1987; Boom *et al.*, 1988; Ransdell *et al.*, 1994). In the current study we observed that unlike the *lpr* DN T cells which could participate in spontaneous cytotoxicity of YAC-1 tumor targets and endothelial cells, as well as in redirected lysis when activated through antibodies against the TCR, *gld* DN T cells could not mediate cytotoxicity despite of activation through the TCR or using PMA and calcium ionophore. Several pieces of evidence suggested that lack of a functional Fas-ligand expression on *gld* DN T cells accounted for their inability to mediate cytotoxicity. Furthermore, the *gld* DN T cells were phenotypically very similar to the *lpr* DN T cells and expressed similar levels of perforin when compared to the *lpr* DN T cells. Also, the *gld* DN T cells constitutively transcribed TNF-α and IFN-γ. These data suggested that the lack of cytotoxicity demonstrated by *gld* DN T cells was not due to decreased expression of adhesion molecules involved in cytotoxicity or the inability to spontaneously transcribe the cytokines involved in lysis of target cells. Although we did observe that the *gld* DN T cells transcribed lower levels of TNF-α and IFN-γ, this may not have contributed to the total lack of expression of cytotoxicity because despite stimulation with PMA and

calcium ionophore or the TCR, which could possibly enhance the cytokine gene expression, the *gld* DN T cells failed to mediate cytotoxicity. The important role played by Fas-ligand expressed on DN T cells and Fas expressed on target cells in determining the cytotoxicity was also evident from the data obtained using *lpr* DN T cells. For example, *lpr* DN T cells which express Fas-ligand were able to kill Fas^+ target cells but failed to mediated cytotoxicity of Fas^- target cells.

It should be noted that interaction between Fas-ligand and Fas appeared to play an important role in the cytotoxicity exhibited by the *gld* DN T cells but not by the alloreactive CTL that were generated in primary cultures from *gld* mice. These data are consistent with a recent study which demonstrated that $CD4^+$ T cells or LN T cells activated with PMA and ionomycin from *gld* mice failed to lyse Fas^+ human targets (Mosmann and Coffman, 1989; Ransdell *et al.*, 1994). Interestingly, T cells from *gld* mice specifically generated against allogeneic targets could kill such cells (Mosmann and Coffman, 1989). Thus, together the current study demonstrates that while the DN T cells that accumulate in *lpr* and *gld* mice may exclusively use Fas-dependent pathway, the alloreactive CTL were capable of using Fas-independent pathway. Furthermore, the DN T cells appear to resemble functionally the $CD4^+$ T cells or T cells from LN cells activated with PMA and ionomycin which are strictly dependent on expression of Fas-ligand to mediate the cytotoxicity. We also noted that although DN T cells mediate NK-like cytotoxicity, they use a distinct pathway from NK cells because NK cells from *gld* mice are able to mediate cytotoxicity of YAC-1 target cells unlike the purified DN T cells from *gld* strain (unpublished data). It should also be noted that recent studies have characterized two distinct mechanisms of cytotoxicity, Fas-based and perforin-based, which appear to be independent, based on the fact that cytotoxic

cells from perforin knockout mice can lyse target cells using Fas-dependent pathway and *gld* cytotoxic cells can lyse by perforin pathway (reviewed in Nagata and Goldstein, 1995). In this context, our data suggest that DN T cells are unique inasmuch as, they are dependent on Fas pathway despite constitutively expressing perforin.

Despite extensive research, the nature and the role played by DN T cells in autoimmune disease remains elusive. Based on the ability of DN T cells to spontaneously transcribe several cytokine genes *in vivo* as well as mediate cytotoxicity when activated through a variety of adhesion molecules, it was suggested that DN T cells may play an important role in induction of autoimmune disease (Murray and Martens, 1989; Hammond *et al.*, 1993). Most *lpr* mice die by approximately 6 months of age from renal failure and vasculitis. They develop proliferative glomerulonephritis with mononuclear cell infiltration, endothelial and messangial cell proliferation and crescent formation (Cohen and Eisenberg, 1991; Theofilopoulous and Dixon, 1985). They also develop necrotizing medium sized arteries with involvement of kidneys, mesentery and occasionally coronary circulation (Theofilopoulous and Dixon, 1985). Although the vasculitis may involve neutrophils and immune complexes representing Type III hypersensitivity, there are also lesions characterized by lymphocyte and macrophage perivascular cuffs which initiate vascular wall destruction (Moyer and Reinisch, 1984) and such types of vasculitis have been described in human (Sokoloff and Banion, 1957; McCluskay and Feinberg, 1983) autoimmune diseases as well as in the *lpr* model (Hewicker and Trautwein, 1987; Moyer and Reinisch, 1984). Furthermore lymphocytes cultured with activated endothelium have been shown to induce autoimmune type of vasculitis (Hart *et al.*, 1983) and also lymphokine activated

T cells or LAK cells have been shown to kill endothelial cells (Damle *et al.*, 1987). Together such studies demonstrated that cell-mediated immune mechanisms may play an important role in the induction of vasculitis.

Earlier studies from our lab demonstrated that *lpr* DN T cells as well as $\alpha\beta TCR^+$ $CD8^+$ T cells can mediate cytotoxicity when activated through homing receptors such as CD44 and $gp90^{MEL\text{-}14}$ (Seth *et al.*, 1991; Hammond *et al.*, 1993; Hammond-McKibben *et al.*, in press). Inasmuch as, endothelial cells bear ligands for these homing receptors, our studies suggested that DN T cells which constitutively express abnormally high levels of CD44, may be able to interact directly with endothelial cells and such an interaction may mediate damage to the endothelial cells. To test this hypothesis, we used a well characterized SV-40 transformed syngeneic endothelial cell line to address whether DN T cells could mediate lysis of these cells. The data demonstrated that the *lpr* DN T cells could mediate significant lysis of endothelial cells and furthermore they were not able to mediate cytotoxicity of SV-40 transformed syngeneic fibroblast cells. This cytotoxicity was MHC-unrestricted and was not inhibited by mAbs against the TCR. However, the cytotoxicity was inhibited in the presence of hyaluronate and treatment of endothelial cells with hyaluronidase led to a significant decrease in the cytotoxicity of endothelial cells. Together these data demonstrated that the *lpr* DN T cells may recognize the endothelial cells via interaction between CD44 and hyaluronate which is considered as one of the important ligands involved in cytotoxicity (Galandrini *et al.*, 1994). Also, addition of hyaluronate inhibited the cytotoxicity of endothelial cells but not that of YAC-1 cells. These data suggested that the cytotoxicity of YAC-1 cells may depend on other adhesion receptor-ligand interactions as hypothesized for MHC-unrestricted cytotoxicity often exhibited

by the CTL (Thiele and Lipsky, 1989).

Recently, HA interaction with CD44 was shown to trigger intracellular Ca^{2+} mobilization in T cells (Bourguignon *et al.*, 1993) and trigger cytotoxic activity (Galandrini *et al.*, 1994). It should be noted that in the current study, our attempts to block the cytotoxicity with anti-CD44 mAbs failed, similar to other studies (Galandrine *et al.*, 1994). This may be because anti-CD44 mAbs can directly activate the T cells, cause aggregation or lead to further efficient binding to HA (Haynes *et al.*, 1989; Belitsos *et al.*, 1990; Lesley *et al.*, 1992).

Despite the demonstration that DN T cells are capable of mediating cytotoxicity of endothelial cells, the significance of these findings with respect to the induction of vasculitis or other autoimmune disease pathology remains questionable because our studies also demonstrated that to mediate cytotoxicity, *lpr* DN T cells are exclusively dependent on the interaction between the Fas-ligand expressed on the DN T cells and Fas expressed on the target cell. Thus, in *lpr* mice, because the endothelial cells or other target cells do not express Fas, the DN T cells although capable of mediating cytotoxicity in vitro may not be able to accomplish this *in vivo*. However, we would like to point out that the *lpr* DN T cells may be able to contribute towards vasculitis or other autoimmune disease pathology by virtue of being able to express abnormally high levels of CD44, bind to the endothelial cells and produce cytokines such as TNF-α, IFN-γ and possibly other cytokines which have not been characterized yet. Constant interaction with endothelial cells and constitutive expression of cytokines, could trigger chronic inflammatory response which may lead to significant damage to the endothelial cells or other autologous cells, giving rise to vasculitis or glomerulonephritis. Similarly the *gld* DN T cells despite being unable to mediate direct cytotoxicity, may still be able

to interact with the endothelial cells due to expression of high levels of CD44 and being able to constitutively express several cytokine genes, be able to trigger a similar inflammatory response thereby contributing to the autoimmune disease process. Moreover, IFN-γ and TNF-α can upregulate Ia expression (Lu and Unanue, 1982), which in turn can induce the proliferation of $CD4^+$ autoreactive T cells capable of activating B cells (Nagarkatti *et al.*, 1985a; Nagarkatti *et al.*, 1985b). These cytokines can directly or indirectly trigger IgG_{2a} secretion as well as enhance the proliferation of activated B cells (Snapper and Paul, 1987; Kehrl *et al.*, 1987). The potential role played by DN $\alpha\beta TCR^+$ T cells in autoimmunity was also highlighted by the findings that the numbers of these cells in the blood of SLE patients increases proportionally with the severity of the disease and that such cells can help B cells to produce anti-DNA antibodies (Shivakumar *et al.*, 1989).

Together our studies suggest that DN T cells are not inert or anergic T cells as commonly believed. The DN T cells may represent unique cytotoxic T cells which exclusively use the Fas-dependent pathway to mediate cytotoxicity of the target cells. Furthermore, by expressing abnormally high levels of a variety of adhesion molecules as well as constitutively expressing a variety of cytokines, they may be able to interact with autologous cells such as endothelial cells and thereby trigger an inflammatory response leading to the induction of pathogenesis of autoimmune disease.

Chapter 5: Characterization of Factors Regulating Successful Immunotherapy using a Tumor-specific Cytotoxic T lymphocyte Clone: Role of Interleukin-2, Cycling Pattern of Lytic Activity and Adhesion Molecules

Introduction:

In the previous chapter, our finding that *lpr* DN T cells are constitutively activated *in vivo* and are capable of mediating redirected lysis when stimulated through the CD3/TCR complex as well as through the adhesion molecules CD44 and $gp90^{MEL-14}$ was discussed. This chapter discusses experiments designed to characterize the importance of adhesion molecule expression by a CTL clone, designated PE-9, in mediating both *in vitro* cytotoxicity and *in vivo* adoptive immunotherapy in tumor-bearing mice. In addition, the ability of the CTL clone to mediate damage to endothelial cells in a TCR-independent mechanism when activated with IL-2 both *in vitro* and *in vivo* will be examined. The PE-9 clone expressed high levels of CD44 and

gp90^{MEL-14} when activated and could mediate redirected lysis when activated through these molecules (Seth *et al.*, 1991), similar to *lpr* DN T cells. Because DN T cells do not grow *in vitro*, it is difficult to assess the lytic ability of these cells at different stages of activation. Thus, the PE-9 clone, which readily grows *in vitro*, served as an excellent model for investigating the correlation between density of adhesion molecule expression with cytolytic capabilities.

Tumor infiltrating lymphocytes (TIL) have been the subject of extensive research, recently, following the demonstration that such cells exert anti-tumor effect *in vivo*, both in murine models of advanced metastatic disease and in human melanomas (Rosenberg *et al.*, 1986; Topalian *et al.*, 1987). The TIL have been shown to demonstrate similar properties as lymphokine-activated killer (LAK) cells exhibiting nonspecific cytolytic activity or have been identified as $CD8^+$ T cells demonstrating MHC-restricted tumor-specific cytotoxicity (Topalian *et al.*, 1987; Itoh *et al.*, 1986; Muul *et al.*, 1987). Adoptive immunotherapy against cancer has met with encouraging but sometimes limited success (Parmiani, 1990) the reasons for which are not well understood.

Studies from our laboratory and elsewhere have demonstrated that treatment of C57BL/6 mice bearing a syngeneic T cell lymphoma, designated LSA, with a nitrosourea such as 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), can "cure" over 90% of the mice and interestingly 100% of the BCNU-cured mice can reject subsequent inoculation with homologous but not heterologous syngeneic tumor (Nagarkatti and Kaplan, 1985; Nagarkatti *et al.*, 1988; Nagarkatti *et al.*, 1989; Selvan *et al.*, 1990; Nagarkatti *et al.*, 1990). BCNU-treatment was effective only in immunocompetent mice but not in irradiated or nude mice (Nagarkatti *et al.*, 1988). These studies

demonstrated that in addition to the tumoricidal activity of BCNU, the host's immune system was essential for inducing successful cures. Recently, we demonstrated that after BCNU-treatment, there was an active infiltration of $CD4^+$ and $CD8^+$ T cells in the peritoneal cavity, the predominant site of LSA tumor-growth and to a lesser degree in the spleen where the tumor cells metastasize at advanced stages (Nagarkatti et al, 1990). Such tumor-associated lymphocytes (TAL) isolated from spleen and peritoneal exudate cells (PEC) bearing the CD4 phenotype were shown to be Th1 cells, inasmuch as, they secreted IL-2 and gamma-IFN, activated macrophages, elicited tumor-specific delayed type hypersensitivity (DTH) reaction and could independently mediate tumor-rejection in nude mice (Nagarkatti *et al.*, 1988; Nagarkatti *et al.*, 1990). The $CD8^+$ TAL exhibited tumor-specific cytolytic activity and could also cause tumor-rejection *in vivo* (Nagarkatti *et al.*, 1985; Nagarkatti *et al.*, 1988; Selvan *et al.*, 1991). These studies together demonstrated that BCNU-treated LSA tumor-bearing mice may serve as an ideal host to isolate and establish long term clones of TAL and to study their properties.

In the present study we established several long term cell lines and clones from the TAL isolated from the PEC of BCNU-cured mice rechallanged with LSA tumor. All the T cell clones isolated were found to be $\alpha\beta TCR^+$, $CD8^+$ cytotoxic T lymphocytes (CTL). Using one of these clones we tried to delineate various factors that influence the successful tumor immunotherapy. We demonstrate in the current study that the success of immunotherapy against the LSA tumor using the CTL clone depends on the cycling pattern of cytolytic activity exhibited by the CTL clone, the regulation of adhesion molecule expression and cytokine production by the cells, and the MHC-unrestricted lysis exhibited by the CTL clone upon administration of high

concentrations of IL-2.

Materials and Methods

Mice. Adult, female C57BL/6 normal and nude mice of 8-12 weeks of age were obtained from the National Cancer Institute (Bethesda, MD) and maintained in our animal facility in a laminar flow cabinet that provided sterile environment.

Cell lines. LSA, a thymic lymphoma; EL-4, a chemically induced leukemia; P815, a natural killer (NK) cell-resistant mastocytoma and YAC-1, an NK-sensitive Moloney virus-induced lymphoma were maintained *in vitro* and *in vivo* by serial passages as described elsewhere (Nagarkatti *et al.*, 1985). A mouse endothelial cell line immortalized by SV40 (SVEC4-10) bearing H-2^k, kindly provided by K. A. O'Connel and M. Edidin, Johns Hopkins Univ., Baltimore, MD and an SV40 transformed fibroblast cell line also of H-2^k origin (SVC3H), kindly provided by L. Gooding, Emory University, Atlanta, GA, were maintained in culture as described (O'Connell and Ediden, 1990). Another SV40 transformed endothelial cell line designated TME-3H3 bearing H-2^k developed by A. Hamann (Harder *et al.*, 1991) was kindly provided by J. Lesley, The Salk Institute, San Diego, CA.

Antibodies. Monoclonal anti-CD4 (GK1.5; rat IgG), anti-CD8 (53-6.72; rat IgG), MEL-14 (lymphocyte homing receptor, rat IgG), anti-CD45R (6B2, rat IgG), CD44 (9F3, rat IgG), anti-LFA-1 (M17/4, rat IgG), 7D4 (anti-IL-2R; rat IgM) and J11d2 (anti-immature T cells and B cells; rat IgM) were grown *in vitro* or *in vivo* as ascites

as described elsewhere (Seth *et al.*, 1991; Nagarkatti *et al.*, 1990). The anti-CD3 mAb (145.2C11; hamster IgG) was kindly provided by J.A. Bluestone, University of Chicago, Chicago, IL, the pan $\alpha\beta$-TCR reactive mAb (H57-597; hamster IgG) by R.T. Kubo, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO, and 9F3 hybridoma was a generous gift from F. J. Dumont, Merck Sharpe & Dohme Research Lab., Rahway, NJ. All other hybridomas were obtained from American Type Culture Collection (ATCC, Rockville, MD). For flow cytometric analysis, affinity purified fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and PE-conjugated anti-CD8 antibodies were purchased from Becton-Dickinson (Mountain View, CA) and used (Selvan *et al.*, 1990). FITC-conjugated anti-hamster IgG $F(ab')_2$ was obtained from Cappel Laboratories (Malvern, PA). Affinity purified normal rat gammaglobulin was obtained from Jackson Immune Laboratories (West Grove, PA). $F(ab')_2$ fragments of mAb against $\alpha\beta$-TCR was obtained by treatment with pepsin (Sigma Chemical Co., St. Louis, MO) as described elsewhere (Seth *et al.*, 1991). A polyclonal mouse serum designated Ebk carrying antibodies against $H\text{-}2^k$ antigens was obtained from National Institutes of Health Repository, Rockville, MD.

Interleukins. Recombinant human interleukin-2 (rIL-2) was kindly provided by Hoffman La Roche Inc., Nutley, NJ. It was tested for activity against IL-2 dependent HT-2 cells as described elsewhere (Nagarkatti *et al.*, 1990).

Drug treatment. BCNU was kindly provided by the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD). BCNU solution was

prepared freshly by dissolving in absolute ethanol and diluting further with PBS pH 7.2 (Nagarkatti *et al.*, 1985). Single i.p. injection of BCNU was administered into tumor-bearing mice at a concentration of 20-40 mg/kg body weight 5 days after $1x10^6$ LSA tumor cell inoculation (Nagarkatti *et al.*, 1985; Nagarkatti *et al.*, 1988). Mice that survived for more than one month following this treatment were referred to as BCNU-cured LSA mice in the text.

Establishment of LSA specific T-Cell Lines. LSA tumor-specific CTL lines were established as described earlier (Seth *et al.*, 1991; Selvan *et al.*, 1991). C57BL/6 mice were injected with $1x10^6$ live LSA tumor cells. Five days later, these mice were injected i.p. with 20 mg/kg bodyweight of BCNU. After five days, the PEC were harvested and suspended in culture medium consisting of RPM1-1640 supplemented with 10% fetal calf serum (Gibco Laboratories, Grand Island, NY), 10 mM HEPES, 1 mM glutamine, 50 μM 2-mercaptoethanol, and 40 μg/ml gentamicin sulfate referred to as complete medium. T-cells were enriched by passing the splenic cells through nylon wool columns. The cells were treated with J11d + C′ to deplete any contaminating LSA tumor cells (Nagarkatti *et al.*, 1989). The LSA tumor cells to be used as stimulator cells, were irradiated at 5000R. Primary culture was carried out in 24-well Costar plates (Costar, Cambridge, MA) by incubation in 2 ml complete medium, $3x10^6$ responder T cells along with $1x10^5$ LSA and recombinant IL-2 (50 units/ml). After establishing long-term cultures, the cell line was cloned by limiting dilution (Seth *et al.*, 1991; Selvan *et al.*, 1991). The clones were maintained in the presence of 50 units/ml of IL-2 by repeated subculture twice a week. To maintain their specificity the clones cultured with IL-2

were occasionally stimulated with irradiated LSA tumor cells.

Phenotypic analysis of T cell clones. The expression and density of various adhesion receptors on the T cell clone was determined by immunofluorescence technique (Seth *et al.*, 1991; Selvan *et al.*, 1990). Briefly, $1x10^6$ cloned cells were washed in PBS containing 0.1% sodium azide and incubated at 4°C for 30 min in a test tube with antibodies directed against CD3, $\alpha\beta$-TCR, CD8, CD44, CD45R and LFA-1. After washing the cells twice, FITC-conjugated secondary Ab was added. To detect the presence of CD3 and $\alpha\beta$-TCR, the secondary Ab consisted of FITC-conjugated anti-hamster IgG $F(ab')_2$ and to detect CD45R, LFA-1, CD44 and CD8, the secondary antibody consisted of FITC-conjugated anti-rat IgG $F(ab')_2$ (Cappel Lab., Malvern, PA). The negative controls consisted of fluorescence obtained by staining cells with normal rat IgG or hamster IgG depending on the type of antibody used, followed by FITC-conjugated secondary antibody. The tubes were incubated on ice for 30 min and washed thrice. The cells were analyzed for fluorescence intensity by flow cytometry (Epics V, Model 752, Coulter Electronics, Hilleah, FL) (Seth *et al.*, 1991). The fluorescence profiles obtained with negative controls were subtracted from the positive staining to obtain the actual intensity of fluorescence. The density of adhesion receptors which is proportional to intensity of immunofluorescence was expressed as mean channel number.

Alloreactive CTL. Alloreactive CTL were generated as described in detail elsewhere (Seth *et al.*, 1991). Briefly, $3x10^6$ splenic T cells from C57BL/6 mice ($H\text{-}2^b$) were cultured in 24-well plates containing 2 ml of complete medium, with

$3x10^6$ irradiated spleen cells from MRL-+/+ mice (H-2^k). After 5 days, the cells were harvested, viable cells were purified and tested for cytotoxicity against ^{51}Cr-labelled TME-3H3 (H-2^k) targets.

Assay for Cytotoxicity. The cell mediated lysis elicited by the clones was determined using ^{51}Cr-release assay (Nagarkatti *et al.*, 1985). Briefly, 4-$6x10^6$ targets cells (LSA, EL-4, P-815, YAC-1, or Con A and LPS blasts) were labelled with 200 μCi of $^{51}Na_2CrO_4$ by incubating at 37°C for 60 min. To obtain Con A and LPS blasts, syngeneic splenic cells were stimulated with Con A (2 μg/ml) or LPS (5 μg/ml) and cultured for 48 hr at 37°C. The targets were washed four times and labelled with ^{51}Cr. Varying ratios of effector : target cells in triplicates were and mixed in 96-well round-bottom plates (Falcon 3910, Becton Dickinson and Company, Lincoln Park, NJ) and incubated for 4 hr at 37°C. Spontaneous release was measured by incubating ^{51}Cr-labelled targets alone and maximum release was determined by incubating the target cells with 0.1 M sodium dodecyl sulfate. After 4 hr incubation, the supernatants were harvested with the Titertech collecting system (Skatron Inc., Sterling, VA) and radioactivity was measured with a gamma counter (TmAnalytic, Elk Grove Village, IL). The mean percentage of specific cytotoxicity ± S.E.M. was calculated from triplicate cultures using the standard formula: %specific cytotoxicity = 100 X [(experimental release - spontaneous release)/(Maximum release - spontaneous release)]. Spontaneous release from targets was always less than 15% of maximum releasable counts.

Adoptive immunotherapy using TAL. In all experiments, the CTL clone cultured for

48 hours with IL-2 was used for adoptive immunotherapy unless stated otherwise. The PE-9 cells were injected i.p. into normal or nude syngeneic C57BL/6 mice at a concentration of $\sim 8x10^6$ cells suspended in 0.2ml PBS. The control mice received PBS alone. These mice were challenged with $1x10^6$ live LSA tumor cells by the i.p. route and the mean survival time (MST) and percent survival of mice was noted. The number of mice used in each group has been given under each figure legend. Each experiment was repeated at least twice with consistent results.

Model for quantitation of vascular leak syndrome (VLS). The toxicity resulting from administration of high concentrations of rIL-2 was studied by measuring the extravasation of ^{125}I-albumin into various organs, designated as VLS, as described in detail by Rosenstein *et a*l. (Rosenstein *et al.*, 1986). Groups of 3-4 mice were irradiated at 500 rads and injected intraperitoneally with $\sim 13x10^6$ PE-9 cells followed by $\sim$130,000 units of rIL-2 three times daily for 5 days. On the 5th day, mice received 0.5 μCi of ^{125}I-BSA (specific activity = 1-2 μCi/μg kindly provided by New England Nuclear, Boston, MA) i.v. in 0.5ml PBS. Two hours later mice were bled to death, under anaesthesia, and lungs, liver and spleen were harvested, placed in vials and counted in a gamma counter. Negative controls consisted of normal mice or irradiated mice injected with PBS alone instead of IL-2 and as a positive control of VLS, normal mice injected with IL-2 alone were included. The experiment was repeated twice with consistent results.

Studies on cycling pattern of lytic activity. To study how culture conditions would influence the cytotoxicity, expression of adhesion molecules and granzyme A

expression, the following method was employed. PE-9 cells cultured with rIL-2 + irradiated LSA tumor cells were harvested 48 hours after the initial culture and viable cells were isolated by centrifugation on ficoll-hypaque (Nagarkatti *et al.*, 1990). Next, the cells were subcultured in 24 well tissue culture plates at $4x10^5$ cells/well in 2ml medium containing 50 units/ml of rIL-2 continuously for 1-5 days and during this culture period, various properties such as expression of adhesion molecules, cytotoxicity against LSA, cytokine gene expression and granzyme A activity were studied. These experiments were repeated several times with different CTL lines and obtained consistent results.

Detection of cytokine production by PE-9 cells. PE-9 cells were grown *in vitro* as described above for studying the cycling pattern of lytic activity. Cells were harvested either on day 1 or day 4 after the initial day of culture and cytoplasmic RNA was extracted (Hammond *et al.*, 1993). One μg of RNA was reverse transcribed for each PCR sample using reverse transcriptase-PCR kit (Perkin-Elmer, Norwalk, CT). The primers for ß-actin and perforin cDNA were selected using the Genetic Computing Group program assisted search from GenBank sequences. TNF-α and IFN-γ primers were purchased from Clontech (Palo Alto, CA). The PCR run consisted of 30 cycles of denaturing at 94°C for 45 sec. followed by annealing at 60°C for 45 sec. and extension at 72°C for 2 min. The resulting cDNA was amplified with primers for ß-actin, perforin, TNF-α , and IFN-γ and 10 μl of PCR product was electrophoresed on a 1.5% agarose gel with ethidium bromide. The primer sequences were as follows: For ß-actin 5'-TATCCTGACCCTGAACTACCCCATT and 3'-AGCACAGCTTCTCTTTGATGTCACG;

for perforin 5'-GGTCAGAATGCAAGCAGAAGCACAA and 3'-TTGAAGTCAAGGTGGAGTGGAGGTT; for TNF-α 5'-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC and 3'-GTATGAGATAGCAAATCGGCTGACGGTGTGGG; for IFN-γ 5'-TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC and 3'-TGGACCTGTGGGTTGTTGACCTCAAACTTGGC. The demonstration of a single 464, 499, 354, or 365 bp band was considered to be indicative of the expression of ß-actin, perforin, TNF-α, or IFN-γ, respectively.

Detection of granzyme A. The total cellular content of granzyme A was determined by measuring BLT esterase as described elsewhere (Hammond *et al.*, 1993). Briefly, PE-9 cells were lysed with 1% Triton X-100. Of this lysate, 20 μl were added to microtiter wells containing 180 μl of assay solution consisting of PBS, pH 7.2 with 2.2×10^{-4}M 5,5'dithio-bis (2-nitro)-benzoic acid (Calbiochem Corp., LaJolla, CA) and 2×10^{-4}M BLT (Sigma Chemical, St. Louis, MO). After 30min. incubation at room temperature, the absorbance was read in an ELISA reader at 410nm.

Statistical Analysis. The survival rate of various experimental groups of mice was compared to the controls and the statistical significance was calculated using chi-squared test.

Results

Surface phenotype of T cell clones isolated from TAL:

Attempts were made to isolate and grow TAL from LSA tumor-bearing mice or BCNU-treated LSA bearing mice. We observed that TAL from tumor-bearing mice failed to divide in culture and eventually died. In contrast, TAL from BCNU-treated mice grew rapidly and we were able to establish several cell lines. Within few weeks of culture, the cell lines lost alloreactivity and all cell lines isolated lysed LSA tumor targets and were found to be $CD8^+$. These cell lines were further cloned by limiting dilution and several T cell clones isolated were phenotypically characterized. All clones tested were found to be $CD3^+$, $\alpha\beta$-TCR^+, $CD8^+$, $CD4^-$, LFA-1^+, MEL-14^+, $CD44^+$, and $CD45R^+$, as shown recently elsewhere (Seth *et al.*, 1991). Interestingly, none of the cell lines or clones isolated were $CD4^+$. This may be because the tumor-specific antigens on LSA were more likely to be processed and presented through endogenous (MHC class I) pathway thereby activating the $CD8^+$ T cells.

Specificity of TIL:

To examine the specificity of the T cell clones, several clones isolated from the PEC (PE-2, PE-3, PE-9) were screened for their lytic activity against a variety of specific and nonspecific tumor targets at different effector:target (E:T) ratios. Representative data at 25:1 ratio depicted in Figure 5.1 suggested that all clones tested exhibited specific lytic activity against LSA tumor but not against a heterologous syngeneic tumor (EL-4) or allogeneic tumor (P815). Similar results were obtained at other E:T ratios. The clones also failed to lyse syngeneic T and B

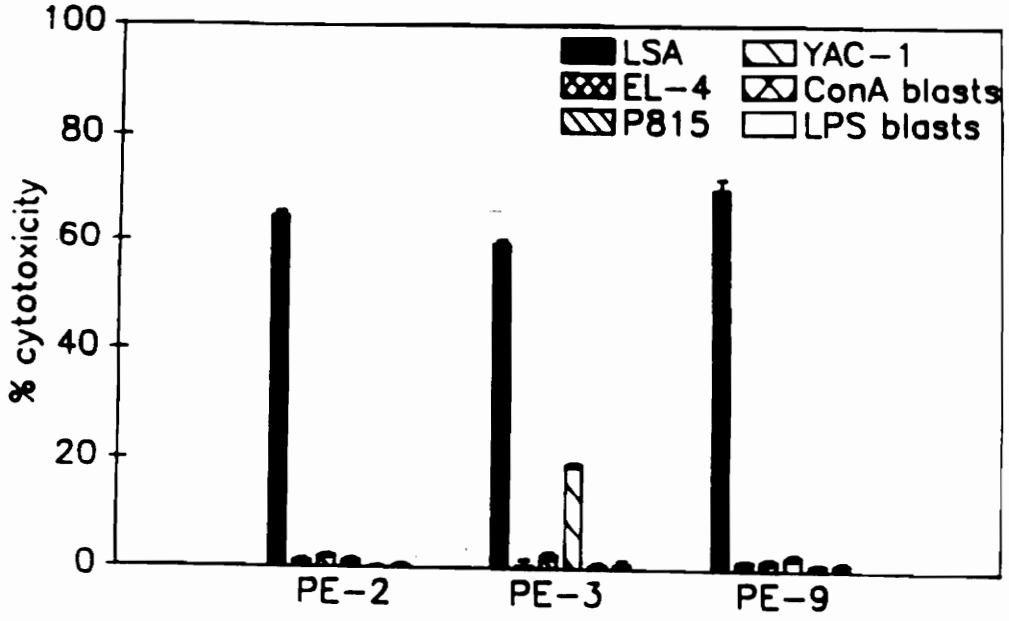

Fig 5.1 **Specificity of the lytic activity of CD8⁺ TAL clones.** The cytotoxicity mediated by various CTL clones was tested against a variety of ^{51}Cr-labeled targets, at an effector:target ratio of 25:1, in a 4 hour assay, as described in Materials and Methods. The vertical bars represent mean percent cytotoxicity ± S.E.M.

cell blasts. When tested against NK-sensitive tumor target, only 1 of the 3 clones tested exhibited low levels of cytotoxicity whereas the other clones failed to lyse YAC-1 targets.

Adoptive immunotherapy of LSA tumor-bearing normal or nude mice using CTL clones:

Since all T cell clones tested exhibited similar properties, further adoptive transfer studies were carried out using clone PE-9. Initially attempts were made to investigate whether PE-9 would cause LSA tumor-rejection in normal or nude syngeneic C57BL/6 mice, because in an earlier study we had observed that the immunotherapeutic efficacy using LSA sensitized T cells varied in normal and nude mice (Nagarkatti *et al.*, 1988). The data shown in Figure 5.2 suggested that normal mice injected with LSA tumor cells all died by day 18. In contrast, transfer of $8x10^6$ PE-9 cells into similar mice caused ~50% survival of mice for > 50 days ($p<0.001$). Furthermore, transfer of PE-9 cells into EL-4 bearing mice failed to provide any protection thereby confirming the specificity of the CTL clone. Interestingly, similar experiments in nude mice yielded different results (Figure 5.3). Nude mice injected with LSA alone died by 17 days. Injection of PE-9 prolonged the MST slightly, although, all mice died by day 25 ($p>0.05$). Injection of large quantities of rIL-2 (75,000 units/mouse) twice a day for one week along with PE-9, also failed to induce LSA tumor-rejection ($p>0.05$). It should be noted that IL-2 when injected alone into non-tumor-bearing normal/nude mice did not cause any deaths (data not shown). Furthermore, injection of PE-9 + IL-2 + LSA appeared to shorten the survival of mice when compared to those injected with PE-9 + LSA.

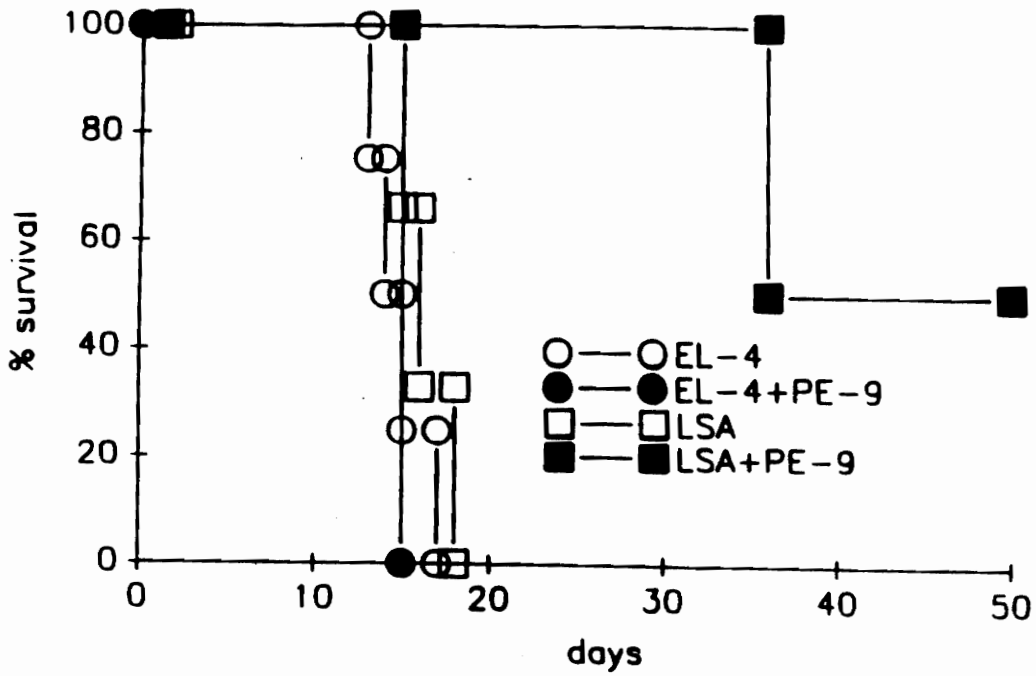

Fig 5.2 **CTL clone PE-9 can induce specific tumor-rejection in normal syngeneic C57BL/6 mice.** Normal mice were injected i.p. with $1x10^6$ live syngeneic LSA (specific) or EL-4 tumor cells (nonspecific), followed by PE-9 cells ($8x10^6$) i.p. The mice were studied for tumor-growth and survival. Number of mice in each group shown in parenthesis were: LSA (6), EL-4 (4), EL-4 + PE-9 (4), LSA + PE-9 (6). The statistical significance in different groups was as follows: EL-4 + PE-9 when compared to EL-4 ($p > 0.99$), and LSA + PE-9 when compared to LSA ($p < 0.001$).

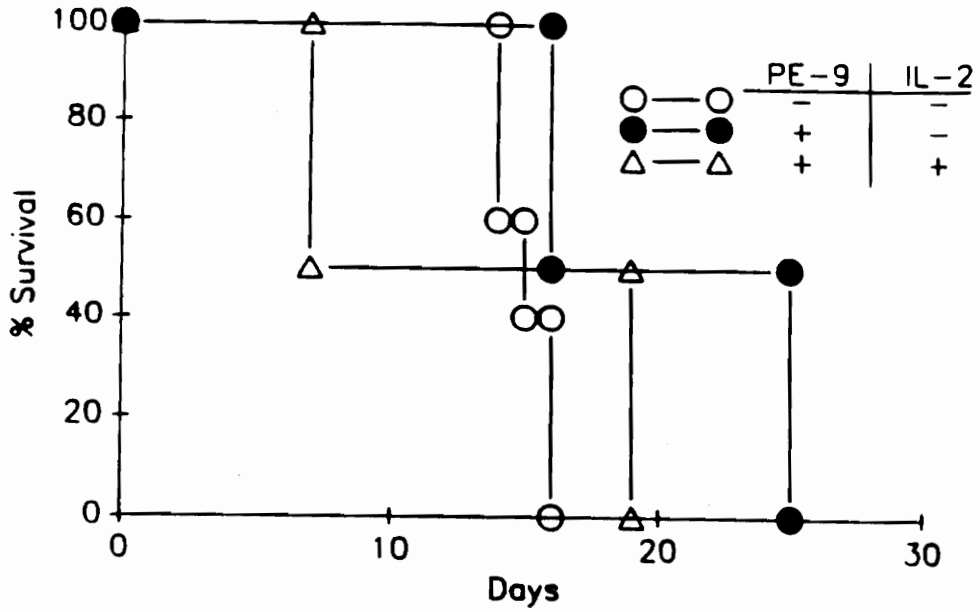

Fig 5.3 **CTL clone PE-9 fails to cure nude mice bearing LSA tumor.** Nude mice were injected with LSA tumor cells as described in Fig. 4.2 followed by PE-9 cells alone or a combination of PE-9 + IL-2. rIL-2 was injected i.p. (75,000 U/mouse) twice a day for one week and the mice were observed for tumor growth and survival. Nude mice injected with IL-2 alone did not die from any toxicity. The number of mice in each group shown in parenthesis were: LSA (5), LSA + PE-9 (4), LSA + PE-9 + IL-2 (4). The statistical significance in different groups was as follows: LSA + PE-9 when compared to LSA ($p > 0.05$), LSA + PE-9 + IL-2 when compared to LSA ($p > 0.05$).

This difference in the survival was, however, statistically not significant ($p > 0.05$).

Because the nude mice lack $CD4^+$ T helper cells, the fact that PE-9 clone was effective in normal but not in nude mice suggested the possible beneficial role played by $CD4^+$ Th in normal mice. It was also possible that PE-9 cells may require interleukins other than IL-2 for growth and activation *in vivo*, which can possibly be provided by $CD4^+$ T cells. To address this, nude mice bearing LSA tumor were injected with PE-9 clone and $CD4^+$ T cells purified from the spleens of normal syngeneic C57BL/6 mice. Each nude mouse was reconstituted with $\sim 14 \times 10^6$ $CD4^+$ T cells which approximates the number found in one spleen. Interestingly, injection of $CD4^+$ T cells along with clone PE-9 led to ~40% survival of nude mice bearing the LSA tumor ($p < 0.05$), whereas, similar mice injected with normal $CD4^+$ T cells alone, died ($p > 0.05$)(Figure 5.4A), similar to our earlier observation (Nagarkatti *et al.*, 1988).

Role of IL-2 in the induction of toxicity and VLS associated with immunotherapy:

We next addressed whether IL-2 was beneficial in the rejection of LSA tumor by normal C57BL/6 mice when injected along with PE-9 cells. To test this, groups of mice were injected with LSA alone or LSA + PE-9 or LSA + PE-9 + IL-2. The data shown in Figure 5.4B demonstrated that injection of PE-9 cells alone could cure ~50% of the LSA-bearing mice ($p < 0.05$) as seen before (Figure 5.2). Interestingly, administration of IL-2 did not facilitate, but on the contrary, decreased the efficacy of LSA tumor-rejection mediated by PE-9. These data were similar to

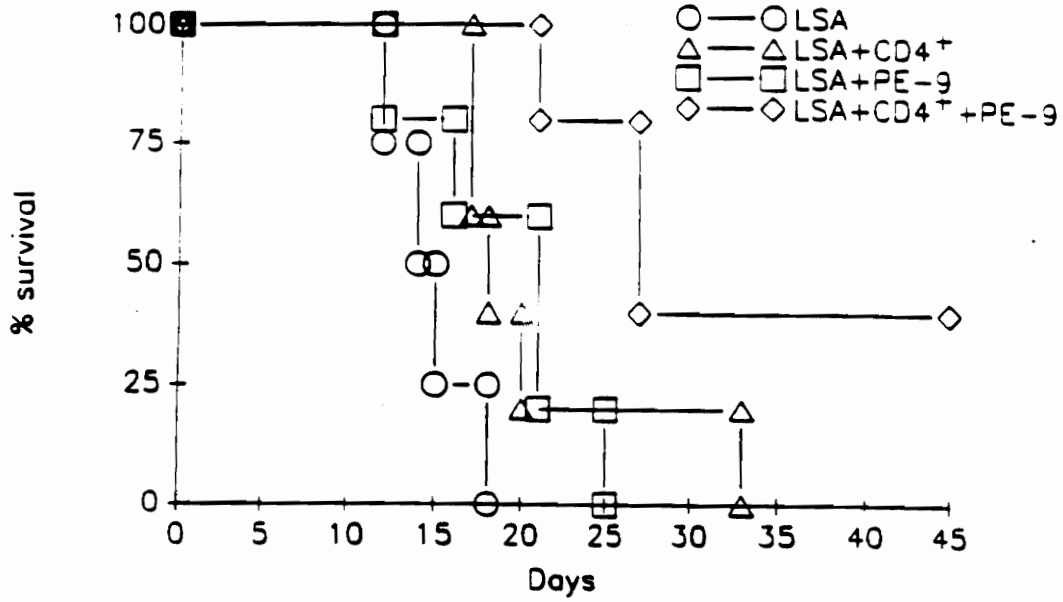

Fig 5.4A **Immunotherapy of LSA bearing normal or nude mice.** Adoptive transfer of normal $CD4^+$ T cells along with CTL clone PE-9, helps in the immunotherapy of nude mice bearing LSA tumors. Nude mice were injected with LSA tumors and CTL clone PE-9, as described in Fig. 4.3. Some mice were also injected with 14×10^6 $CD4^+$ T cells isolated from normal C57BL/6 mice. The number of mice in each group as shown in parenthesis were: LSA (4), LSA + $CD4^+$ T cells (5), LSA + PE-9 (5), LSA + $CD4^+$ T cells + PE-9 (5). Statistical significance was as follows: LSA + $CD4^+$ T cells when compared to LSA ($p > 0.05$), LSA + PE-9 when compared to LSA ($p > 0.05$) and LSA + $CD4^+$ T cells + PE-9 when compared to LSA ($p < 0.05$).

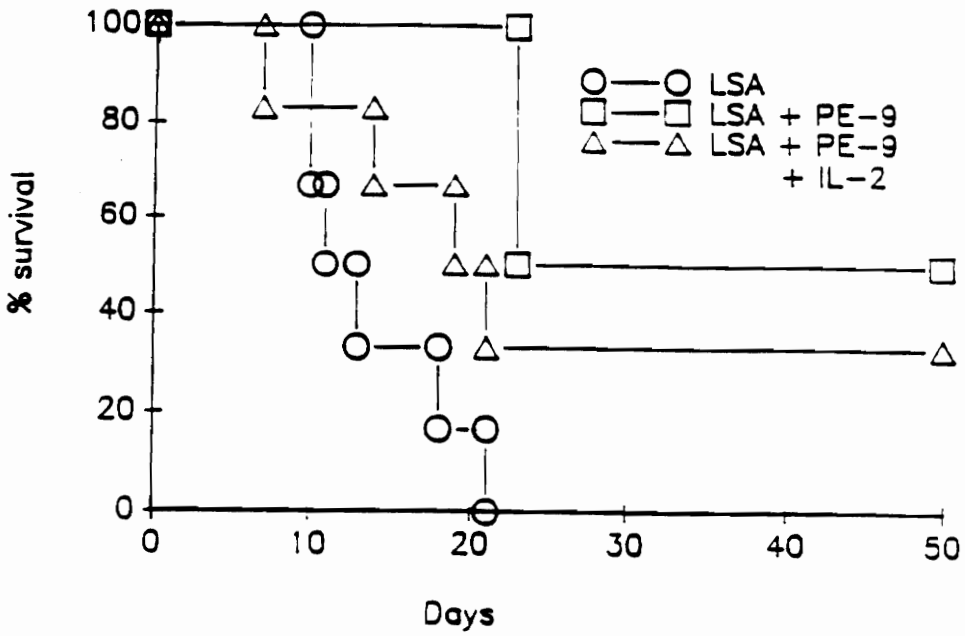

Fig 5.4B **Role of IL-2 in the immunotherapy of LSA tumors in normal mice using CTL clone, PE-9.** Normal mice were injected with LSA tumor cells and PE-9 cells as described in Fig. 4.2. These mice also received rIL-2 (75,000 U/mouse) twice a day for one week as described in Fig. 4.3. The number of mice in each group shown in parenthesis were: LSA (6), LSA + PE-9 (4), and LSA + PE-9 + IL-2 (6). The statistical significance in different groups was as follows: LSA + PE-9 versus LSA ($p < 0.05$) and LSA + PE-9 + IL-2 versus LSA ($p > 0.1$).

the observations made using nude mice (Figure 5.3).

Inasmuch as, these data suggested that IL-2 was not beneficial, we investigated the possible reason for this. Because, LSA is a thymic lymphoma, we first investigated whether LSA tumor cells may express IL-2 receptors (IL-2R). Flow cytometric analysis of LSA revealed that ~54% of the cells expressed IL-2R (data not shown). This suggested that IL-2 may facilitate the growth of LSA *in vivo*. However, incubation of LSA with rIL-2 (50units/ml or 500units/ml) *in vitro* had no significant effect on LSA tumor growth, and furthermore, *in vivo* injection of IL-2 into nude mice bearing LSA did not significantly alter survival of mice when compared to control nude mice bearing LSA alone (data not shown). Together these data suggested that IL-2 did not alter the growth characteristics of LSA. The second possibility was that IL-2 in combination with PE-9 was toxic to LSA-bearing mice. It had been previously demonstrated that injection of high concentrations of IL-2 into normal but not into irradiated mice induced vascular leak syndrome (VLS) and toxicity, thereby suggesting that IL-2 may activate the immune cells to induce VLS (Rosenstein, 1986). In the current study, therefore, we investigated whether administration of IL-2 along with CTL clone, into irradiated mice would induce VLS. To this effect, we injected ~130,000 units of IL-2 three times daily by i.p. route for 5 days. On the fifth day, mice were sacrificed and VLS was studied by measuring extravasation of ^{125}I-BSA in various organs. The data shown in Table 1 suggested that IL-2 induced significant VLS in normal mice when compared to the controls that received PBS alone. In contrast, IL-2 failed to trigger VLS in irradiated mice, consistent with an earlier study (Rosenstein *et al.*, 1986). PE-9 cells when injected alone into irradiated mice failed to induce VLS. However,

Table 5.1: **Induction of extravasation of ^{125}I-BSA into tissues following transfer of CTL clone + IL-2**

	Mean cpm[a]					
	Normal Mice		Irradiated Mice			
	PBS	IL-2[b]	PBS	IL-2	PE-9	PE-9 + IL-2
Lung	25,556	48,804(91)[c]	34,130	23,814(-31)	27,110(-21)	58,208(70)
Spleen	7,364	13,076(78)	6,728	6,418(-4)	6,498(-4)	15,980(137)
Liver	89,788	127,178(42)	95,414	82,776(-14)	86,072(-10)	126,337(32)

[a] ^{125}I-BSA (0.5 μCi) was injected i.v. in 0.5 ml PBS, 2 hours before mice were sacrificed. Mean injected counts of ^{125}I-BSA were 1.09×10^6, calculated from triplicate counts.

[b] IL-2 was injected i.p. (~ 130,000 units/mouse) thrice daily, for 5 days.

[c] Number in parenthesis represents percent increase in cpm when compared to the PBS control.

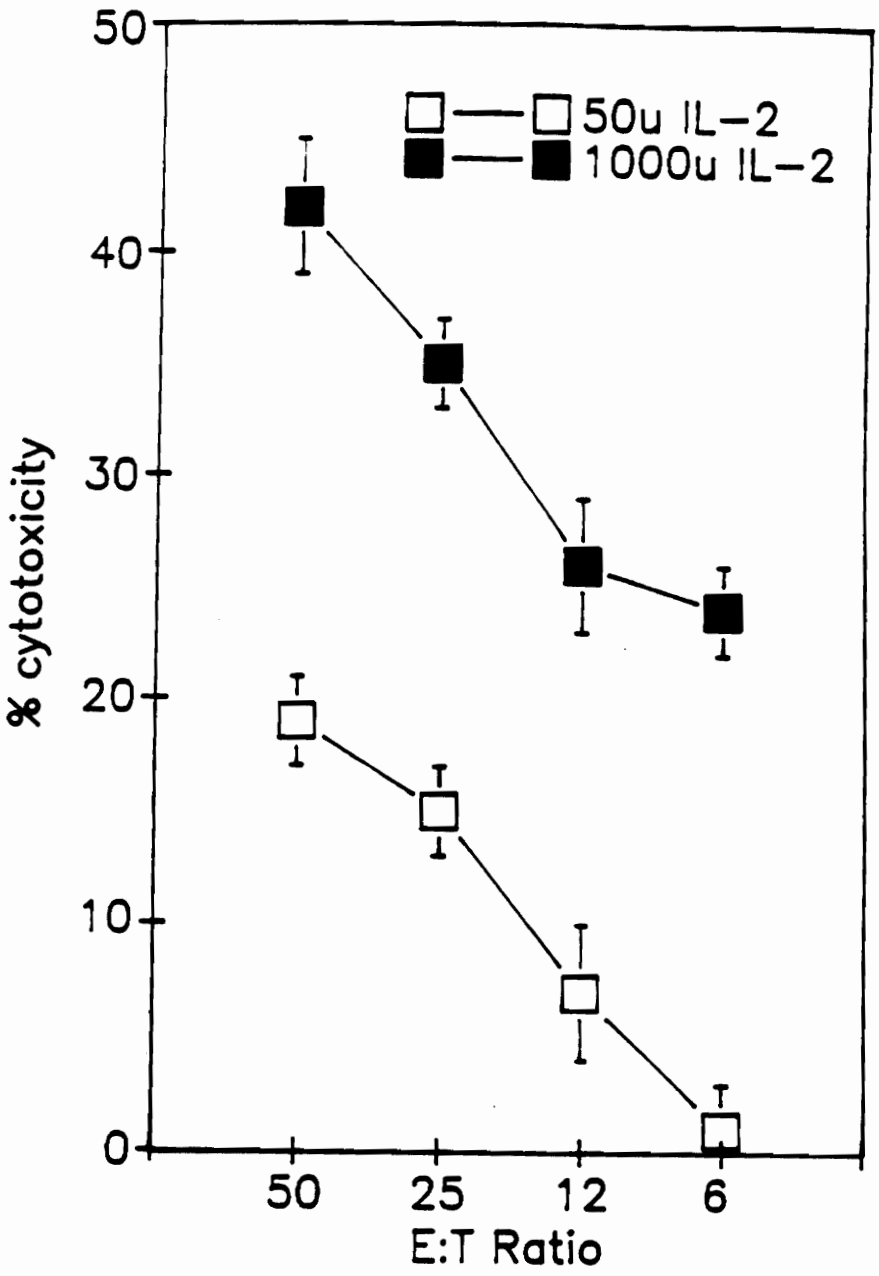

Fig 5.5A **Cytotoxicity of endothelial cell lines by the CTL clone, PE-9.** The PE-9 cells were cultured with either low doses of IL-2 (50 units/ml) or high doses of IL-2 (1000 units/ml) for one week, with one subculture using fresh IL-2 at the middle of the week. The PE-9 cells were tested for cytotoxicity against ^{51}Cr-labeled SVEC4-10 endothelial cells in a 4 hour assay.

combination of PE-9 + IL-2 into irradiated mice induced significant extravasation of ^{125}I-BSA particularly in the lungs and spleen. The experiment was repeated with consistent results.

Cytotoxicity of CTL clone on endothelial cells:

We tested whether the VLS associated with the administration of PE-9 + IL-2 resulted from nonspecific damage to the endothelial cells caused by PE-9 cells. To test this, we used a well-characterized endothelial cell line designated SVEC4-10 (O'Connell and Ediden, 1990). As shown in Figure 5.5A, the CTL clone PE-9 mediated moderate lysis of ^{51}Cr-labelled SVEC4-10 cells. However, culture of PE-9 cells with higher concentrations of IL-2 for one week induced increased lysis of endothelial cells. Furthermore, addition of F(ab')$_2$ fragments of $\alpha\beta$TCR-specific mAbs failed to inhibit the lysis of SVEC4-10 endothelial cells, while inhibiting the antigen-specific lysis of LSA (Figure 5.5B). These data suggested that the lysis of endothelial cells by PE-9 was TCR-independent. Inasmuch as, the endothelial cell line was derived from C3H mice, we tested whether PE-9 cells would lyse target cells of C3H origin and found that PE-9 cells failed to lyse T cell blasts from C3H mice (data not shown). In addition, using another SV-40 transformed endothelial cell line, designated TME-3H3, we also observed that the PE-9 cells could kill the endothelial cells but not an SV-40 transformed fibroblast cell line expressing the same MHC phenotype (Figure 5.5C). Interestingly, the TME-3H3 cell line was efficiently lysed by PE-9 cells even when the CTL was cultured with low concentrations of IL-2 (50-100 U/ml). Furthermore, the lysis of H-2^k bearing TME-3H3 endothelial cells was not inhibited by addition of polyclonal anti-H-2^k antibodies

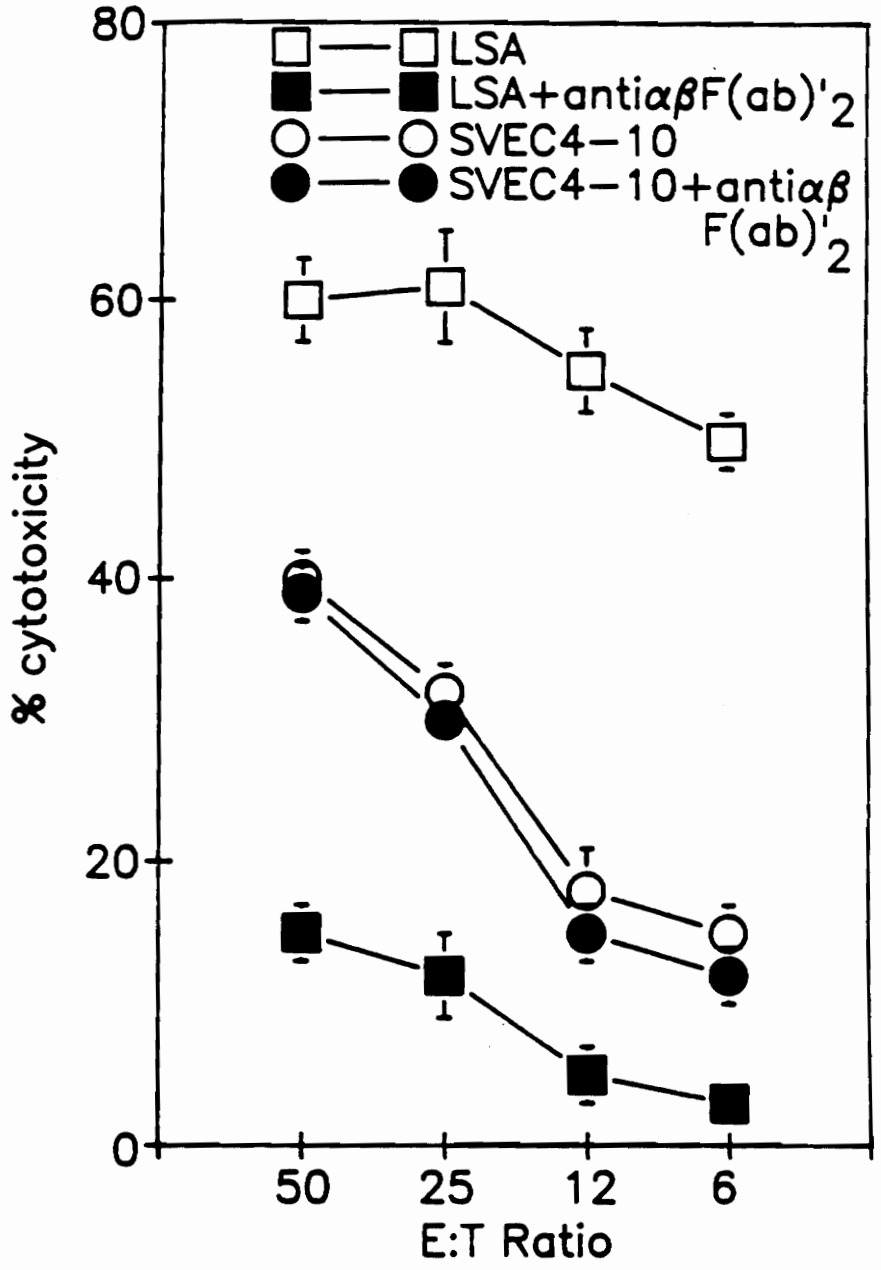

Fig 5.5B **Cytotoxicity of endothelial cell lines by the CTL clone, PE-9.** The cytotoxicity of SVEC4-10 cells by PE-9 was studied in the presence of $F(ab')_2$ fragments of anti-$\alpha\beta$TCR mAbs (1:10 dilution). As a control, the cytotoxicity of PE-9 on the specific LSA tumor was similarly tested in the presence of $F(ab')_2$ fragments of anti-$\alpha\beta$TCR mAbs.

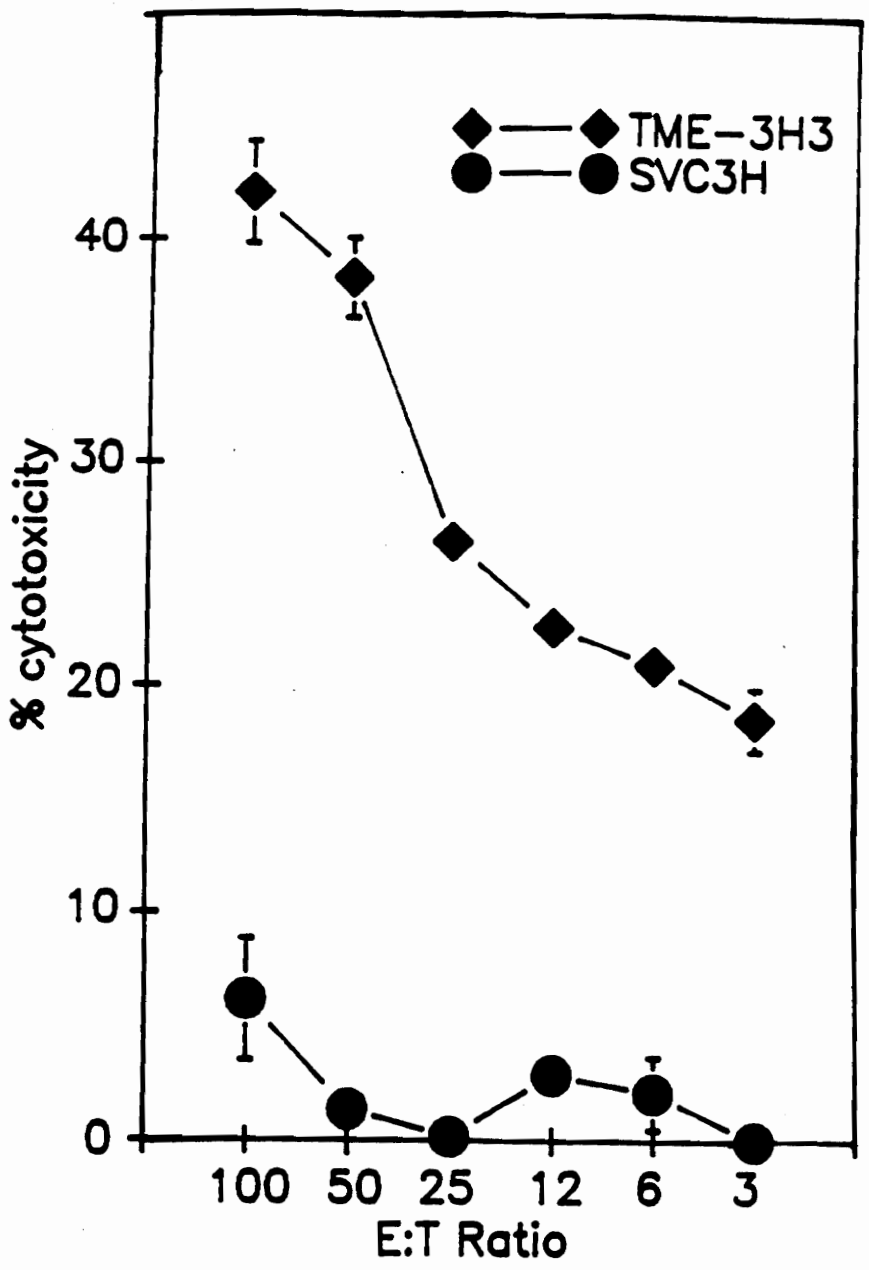

Fig 5.5C **Cytotoxicity of endothelial cell lines by the CTL clone, PE-9.** PE-9 cells cultured with 50 units/ml IL-2 in complete medium were tested for cytotoxicity against an endothelial (TME-3H3) or fibroblast cell line (SVC3H).

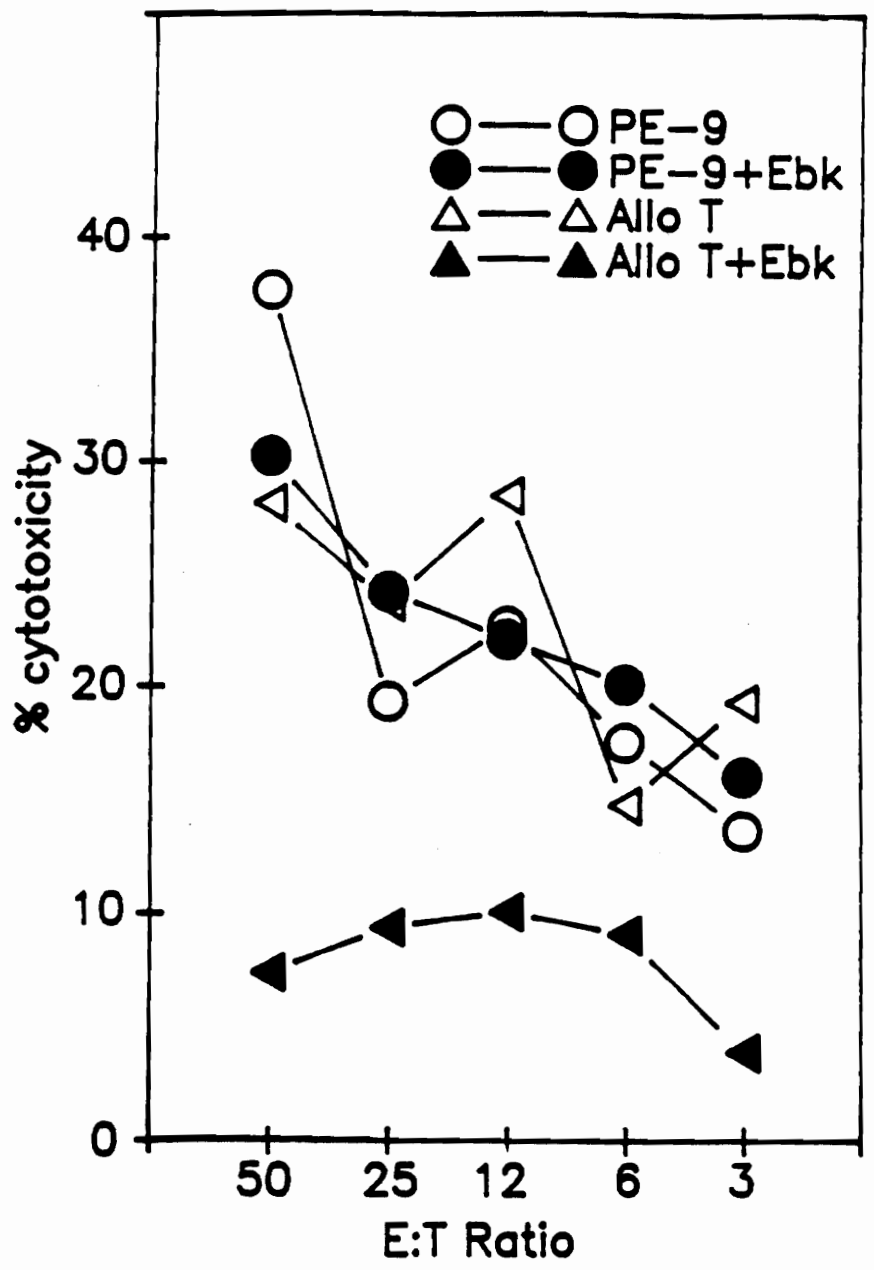

Fig 5.5D **Cytotoxicity of endothelial cell lines by the CTL clone, PE-9.** The ability of PE-9 cells and alloreactive CTL (H-2^b anti-H-2^k) to kill H-2^k bearing endothelial cells (TME-3H3) was tested in the presence of anti-H-2^k antibodies (Ebk) at 1:1000 final dilution in the ^{51}Cr-release assay.

whereas similar concentrations of antibodies could inhibit the lysis of the same target cells by specific alloreactive (H-2^b anti-H-2^k) CTL (Figure 5.5D). Together, these data suggested that PE-9 cells were mediating MHC-unrestricted and TCR-independent killing of the endothelial cells.

Cycling pattern of cytolytic activity of tumor-specific CTL clone and its effect on adoptive immunotherapy:

While performing *in vitro* cytotoxicity assays using PE-9 cells, we observed that the clone exhibited varying degrees of cytotoxic activity depending on the time of harvest after the initiation of culture. To study this effect systematically, PE-9 cells were subcultured for 1-4 days after addition of fresh IL-2 on day 0. The cells were harvested 1-4 days later and tested for cytotoxic activity at the same time against LSA targets. The data shown in Figure 5.6 demonstrated that PE-9 cells exhibited significant cytotoxicity at 24 hours after the initiation of culture. The cytotoxicity peaked at 48hr and gradually declined thereafter and virtually no cytotoxicity was demonstrable at 96hr or thereafter. This pattern of cytolytic activity was repeated following the harvest of cells after 4-5 days of culture and upon subsequent culture of cells with fresh IL-2. It should be noted that the cycling pattern of cytotoxicity exhibited by the CTL clone did not depend on the lack of IL-2 during later stages of culture because this phenomenon was observed despite of supplementing the cultures with fresh IL-2 everyday (data not shown). Also, the cycling pattern of lytic activity was exhibited by other CTL clones.

To study the effect of CTL cytotoxicity on immunotherapy, we next used PE-9 cells cultured for 48 or 96 hours in the immunotherapy of normal mice bearing LSA

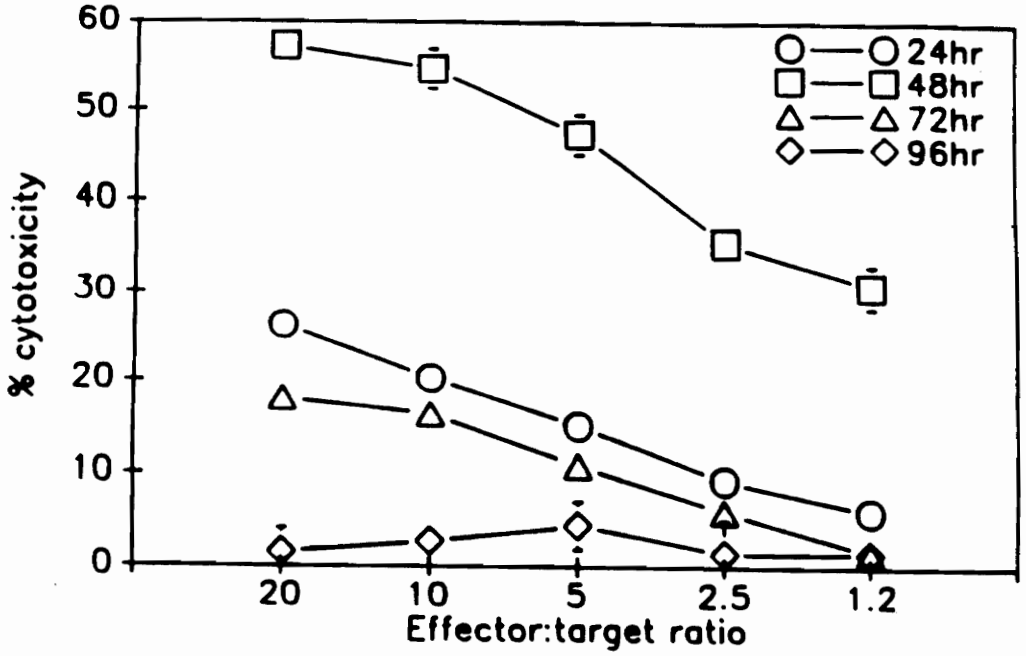

Fig 5.6 **Cycling pattern of cytolytic activity exhibited by CTL clone, PE-9.** The CTL clone PE-9 cultured with rIL-2 (50 units/ml) and irradiated (5000 rads) LSA ($5x10^4$ cells) were harvested, viable PE-9 cells were isolated and subcultured with fresh IL-2 (50 units/ml) added at 0 hours. At 24-96 hours post-incubation, the cells were harvested and tested for lytic activity against ^{51}Cr-labeled LSA tumor targets as described in Fig. 4.1.

tumors and found that PE-9 cells cultured for 48hr could cure ~50% the mice ($p<0.005$) whereas PE-9 cells cultured for 96hr failed to cure the tumor-bearing mice ($p>0.05$) (Figure 5.7). These data together suggested that CTL clones may exhibit cycling pattern of cytolytic activity *in vitro* and that the stage of CTL activation at the time of adoptive transfer may play a significant role in mediating effective cures.

Role of adhesion receptors in the regulation of cytolytic activity of PE-9 cells:

We investigated whether the cycling pattern of cytolytic activity expressed by the CTL clone depended on the density of expression of adhesion molecules. To address this, PE-9 cells cultured for 1-4 days as described above, were analyzed for various adhesion receptors, using flow cytometer. The density of adhesion molecules was studied using mean channel number of fluorescence as an index. The staining pattern of clone PE-9 at various days has been depicted in Figure 5.8 along with the data on the percentage of cells positively stained and the intensity of staining, depicted as mean channel number, shown in parenthesis. The data demonstrated that almost 90 or greater percent of cells exhibited adhesion receptors such as CD3, CD8, CD45R, LFA-1 and CD44, when tested on days 1-4. Interestingly, however, the percent of cells demonstrating positive fluorescence for $\alpha\beta$-TCR was ~75% on day 1 and this dramatically decreased to 28% on day 4. The density of most adhesion molecules also decreased significantly with increasing time in culture, particularly that of CD3, $\alpha\beta$-TCR and CD44.

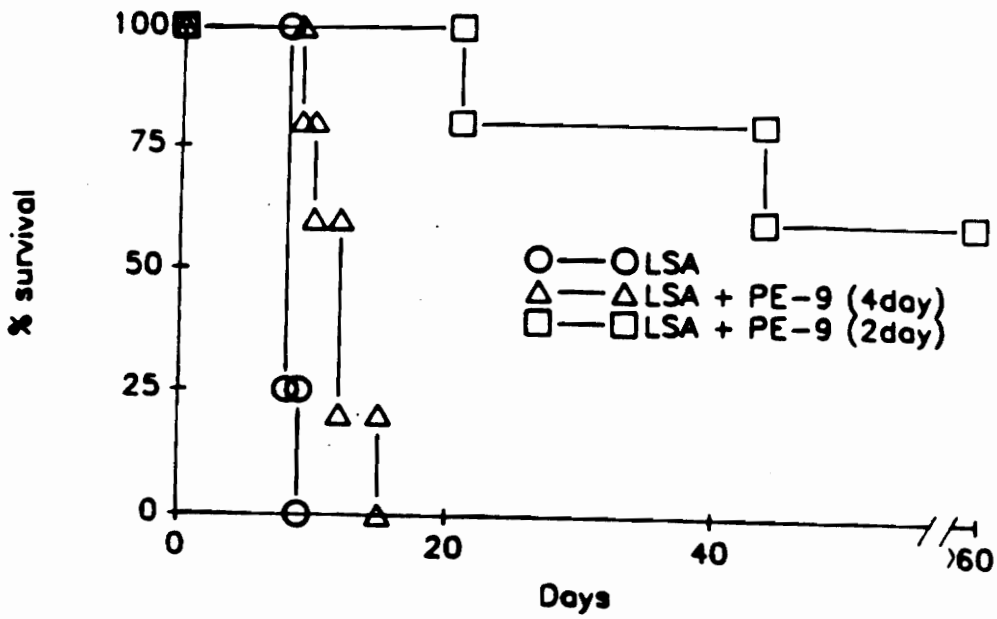

Fig 5.7 **The outcome of immunotherapy using the CTL clone depends on the post-stimulation time in culture.** The CTL clone PE-9 was subcultured with rIL-2 for 2 or 4 days as described in Fig. 4.6. The cells were harvested and viable cells ($8x10^6$) were injected i.p. into normal syngeneic mice which were challenged with $1x10^6$ live LSA cells. The mice were observed for tumor growth and survival. The number of mice in each group shown in parenthesis were: LSA (4), LSA + PE-9 cultured for 4 days (5), LSA + PE-9 cultured for 2 days (5). The statistical significance was as follows: LSA + PE-9 cultured for 4 days when compared to LSA alone ($p > 0.05$), LSA + PE-9 cultured for 2 days when compared to LSA alone ($p < 0.005$).

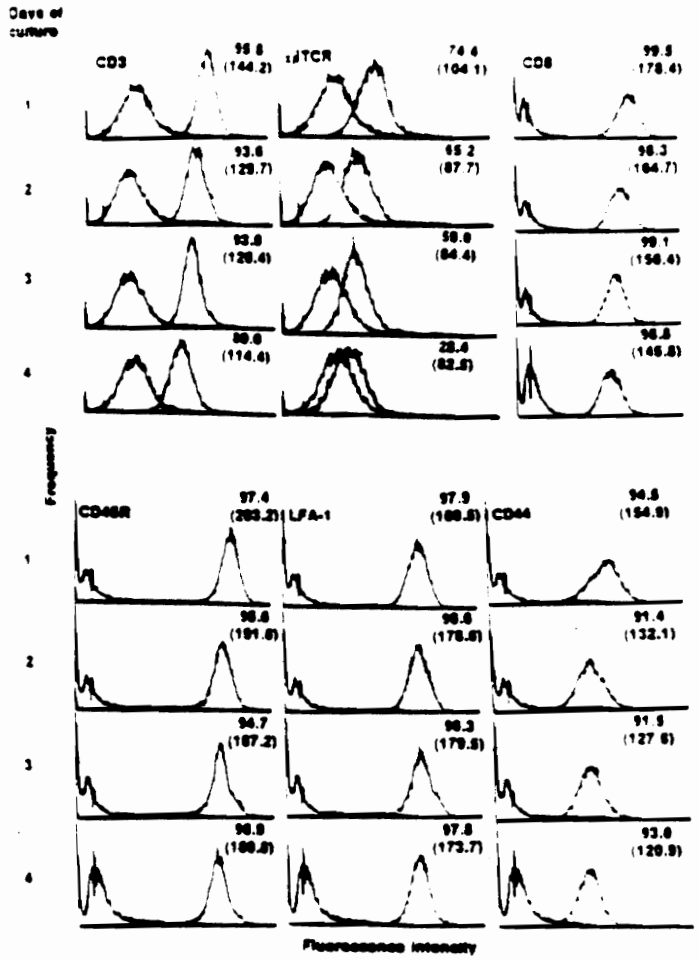

Fig 5.8 **Expression of adhesion receptors by the CTL clone during differentdays in culture.** The CTL clone PE-9 was cultured for 1-4 days as described in Fig. 4.6 and the cells were stained with mAbs against various adhesion receptors and analyzed using a flow cytometer. The figure depicts log fluorescent intensity versus frequency of cells. The first fluorescence profile represents negative control in which cells were incubated with rat or hamster IgG followed by FITC-conjugated secondary Ab as described in Materials and Methods. The second fluorescence profile represents positive staining in which cells were incubated with mAbs against various adhesion receptors + FITC conjugated secondary Abs. The numbers in each fluorescence profile represents percentage of cells depicting positive fluorescence. The numbers in parenthesis depict mean channel number used as an index of density of adhesion receptor expression.

Correlation between cycling pattern of lytic activity and cytokine activity exhibited by the CTL clone:

Inasmuch as, PE-9 cells exhibit perforins, TNF-α, IFN-γ and granzyme A activity which have been shown to participate in cytotoxicity exhibited by CTL, we investigated whether the cycling pattern of cytolytic activity exhibited by PE-9 resulted from altered levels of these cytokines produced during different days of culture. To this end, PE-9 cells cultured for 1 or 4 days were tested for cytokine gene expression for perforin, TNF-α and IFN-γ using PCR analysis (Hammond *et al.*, 1993). In this assay, we used 1 μg of RNA for all PCR analysis and studied the expression of ß-actin mRNA as an internal standard to allow comparison of various samples. The data shown in Figure 5.9A suggested that on day 4 of culture, the mRNA expression in PE-9 cells for all the cytokines tested decreased significantly when compared to day 1 of culture based on the intensity of the band seen in agarose gel electrophoresis. In fact, the expression of mRNA for TNF-α was not demonstrable at 4 days of culture of PE-9 cells. Interestingly, when granzyme A levels were measured, no significant alteration was seen on day 1 versus day 4 (Figure 5.9B).

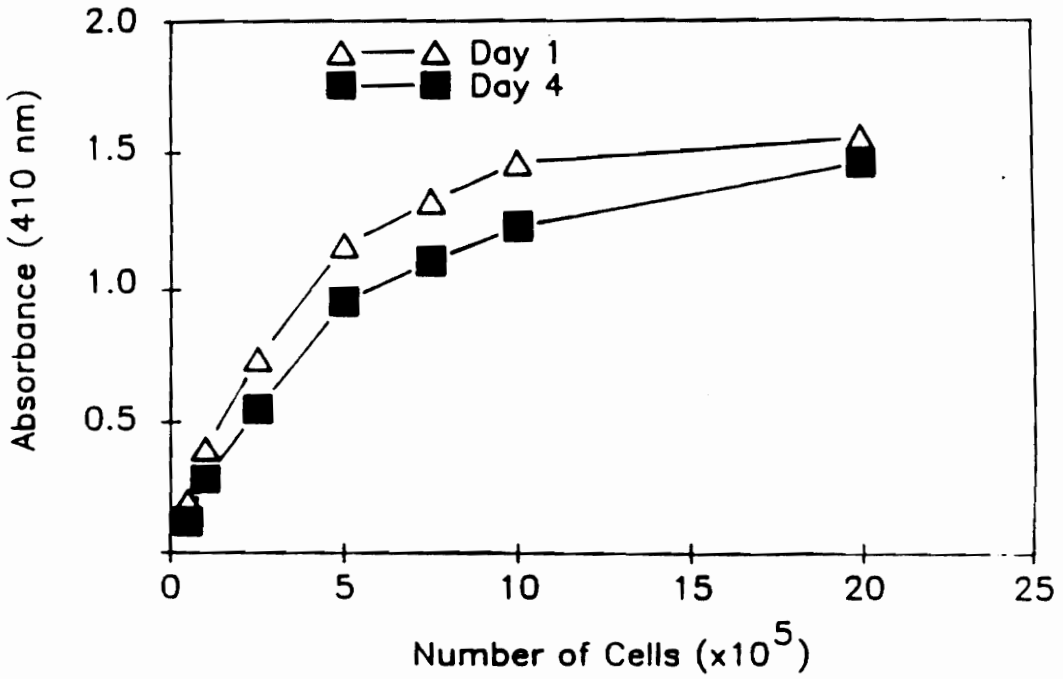

Fig 5.9A **Granzyme A expression in the CTL clone, PE-9.** PE-9 cells were cultured with IL-2 for 1-4 days as described in Fig. 4.6. Next, the cells were harvested and tested for granzy;me A activity by measuring the BLTE as described in Materials and Methods.

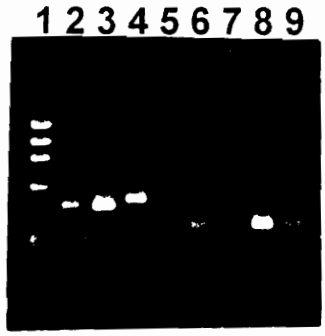

Fig 5.9B **Cytokine gene expression in the CTL clone, PE-9.** PE-9 cells were cultured with IL-2 for 1-4 days as described in Fig. 4.6. The expression of various cytokine genes in PE-9 cells was measured using PCR on day 1 (lanes 2, 4, 6, and 8) or day 4 (lanes 3, 5, 7, and 9) of culture. Lane 1 is a molecular standard (ΦX174 Hae III digest), lanes 2 and 3 depict 464 bp band for ß-actin, lanes 4 and 5 for perforin (499 bp), lanes 6 and 7 for TNF-α (354 bp), and lanes 8 and 9 for IFN-γ (365 bp).

Discussion

Adoptive immunotherapy using LAK cells or TIL and rIL-2 has been shown to be effective in treating metastatic cancer in murine models as well as in human cancer (Rosenberg *et al.*, 1986;Topalian *et al.*, 1987; Itoh *et al.*, 1986; Muul *et al.*, 1987; Lafreniere and Rosenberg, 1985; Rosenberg *et al.*, 1987). However, adoptive immunotherapy against cancer has met with limited but, for some, encouraging success (Parmiani, 1990), the reasons for which are not clear. The current study was initiated with the aim of characterizing various factors which may influence successful immunotherapy. Our data suggested that a) tumor-specific CTL clones are easier to culture and clone following BCNU-treatment when compared to untreated tumor-bearing mice, b) the CTL clone can cure LSA-bearing normal but not immunodeficient (nude) mice, c) transfer of $CD4^+$ T cells into nude mice increased the efficacy of the CTL clone to mediate tumor-rejection, d) administration of IL-2 did not facilitate tumor-rejection mediated by the CTL clone, e) administration of high concentrations of IL-2 into irradiated mice transferred with the CTL clone caused severe toxicity leading to VLS, f) culture of PE-9 cells with higher concentrations of IL-2 led to nonspecific killing of endothelial cells thereby suggesting that a similar mechanism may account for VLS seen *in vivo*, g) the CTL clone exhibited cycling pattern of lytic activity with high levels of lytic activity at 48 hours of subculture and virtually no cytotoxicity at 96 hours of *in vitro* culture, h) interestingly, cells cultured for 48 hours afforded significant protection in

tumor-bearing hosts whereas cells cultured for 96 hours failed to provide protection, i) the cycling pattern of lytic activity exhibited by the CTL clone correlated with density of the expression of a variety of adhesion molecules, particularly the $\alpha\beta$-TCR which was dramatically downregulated at 96 hours of culture and j) the mRNA expression for various cytokines was also decreased on day 4 of culture whereas the expression of granzyme A was not altered during the *in vitro* culture. Together our data suggested that *in vitro* culture conditions particularly the density of adhesion molecule expression and the IL-2 used, can influence the efficacy of immunotherapy as well as the toxicity associated with this method of treatment.

In the LSA tumor model, we have earlier demonstrated that the tumor-bearing mice fail to demonstrate tumor-specific CTL activity or delayed-type hypersensitivity (Nagarkatti and Kaplan, 1985; Nagarkatti *et al.*, 1989). However, following BCNU-treatment, enhanced anti-tumor immunity mediated by $CD4^+$ and $CD8^+$ T cells was demonstrable (Nagarkatti *et al.*, 1985; Nagarkatti *et al.*, 1988; Nagarkatti *et al.*, 1989; Nagarkatti *et al.*, 1990). This may explain why our attempts to grow TAL from the peritoneal cavity of tumor-bearing mice met with limited success, whereas following BCNU-treatment it was much easier to establish long term TAL cell lines. In this context it is interesting to note that cancer chemotherapy in humans was recently shown to lead to the appearance of $CD8^+$ effector cells having very strong lytic activity similar to that of LAK cells (Kivohara *et al.*, 1988). Also, under certain conditions, anti-cancer drugs have been shown to enhance the anti-tumor immunity (Mokyr, 1987). These data therefore suggested that in some tumor models, it may be possible to isolate and grow TAL, with

relative ease, after chemotherapy and such cells can be used effectively following discontinuation of chemotherapy.

The fact that PE-9 cells could protect normal but not nude tumor-bearing mice suggested the possibility that the CTL clone was unable to propagate in nude mice and required additional T helper cells, inasmuch as, such long term cultured $CD8^+$ T cells have limited ability to produce endogenous growth factors (Selvan *et al.*, 1990). The $CD4^+$ T cells exhibiting the properties of Th1 subset may also participate in LSA tumor rejection as shown in an earlier study (Nagarkatti *et al.*, 1990). To address whether the CTL clone would be effective in LSA-bearing nude mice in the presence of exogenous IL-2, we injected high concentrations of rIL-2. However, it was observed that the CTL clone failed to cause tumor rejection inspite of treatment with high doses of rIL-2. Also, treatment of normal mice bearing LSA with CTL clone + IL-2 failed to provide additional protection compared to the transfer of CTL clone alone. The reason why IL-2 failed to cure nude mice or provide extra protection in normal mice when administered along with the CTL clone, is not clear. One possibility is the short biological half life of IL-2. The second possibility was that IL-2 when administered along with the CTL clone was inducing toxicity.

Adoptive immunotherapy with IL-2 has been shown to lead to toxicity (Lotze *et al.*, 1986; Peace and Cheever, 1989). In humans, IL-2 therapy increases vascular permeability in multiple organs as well as interstitial pulmonary edema and major respiratory compromise (Seth *et al.*, 1991). Administration of high concentrations of IL-2 also triggers VLS in normal but not in immunosuppressed mice (Rosenstein *et al.*, 1986). These data suggested that IL-2 may mediate toxicity indirectly via its

action on immune cells, although the exact mechanism is not known.

In the current study we tested the mechanism by which IL-2 mediates toxicity during immunotherapy. It was observed that injection of IL-2 alone into irradiated but not into normal mice failed to trigger VLS, consistent with the earlier study (Seth *et al.*, 1991). Interestingly, injection of IL-2 along with PE-9 cells into irradiated mice could induce marked VLS. Furthermore, we found that PE-9 cells mediated significant lysis of endothelial cell lines *in vitro*. This lysis was TCR-independent and MHC-unrestricted thereby suggesting that molecules other than the TCR were involved in the killing of the endothelial cells. Our studies are consistent with earlier reports that IL-2 activated human lymphocytes can efficiently kill vascular endothelial cells (Damle *et al.*, 1987). Although the exact mechanism of such killing is not known, we and others have hypothesized based on earlier studies that activated CTL can lyse targets when triggered through certain adhesion/homing receptors (Thiele and Lipsky, 1989) particularly CD44 and $gp90^{MEL\text{-}14}$ (Seth *et al.*, 1991; Hammond *et al.*, 1993). Thus, it is possible that IL-2 may facilitate the binding of CTL to the endothelial cells leading to activation via such adhesion molecules leading to damage to the endothelial cells. It should be noted that PE-9 cells could kill another endothelial cell line (TME-3H3) efficiently, even when cultured with normal concentrations of IL-2. This may be because the cells may express the appropriate ligands necessary to trigger the CTL to mediate cytotoxicity. The fact that the CTL clone could kill the endothelial cells but not the fibroblast cells bearing the same MHC phenotype supports the hypothesis that CTL may use homing receptors to mediate endothelial cell lysis. However, more research needs to be carried out to test this hypothesis and such studies may

provide useful clues to prevent VLS during IL-2 mediated immunotherapy.

High dose IL-2 therapy has been reported to be toxic and the biological half-life of IL-2 is short (Lotze *et al.*, 1986). Also, CTL may require interleukins other than IL-2 to propagate *in vivo*. Thus, we reasoned that if the TIL clone required exogenous growth factors to proliferate *in vivo*, then, injection of $CD4^+$ Th cells may be able to provide this source of growth factors as seen in our earlier *in vitro* studies (Selvan *et al.*, 1990). Interestingly, when $CD4^+$ Th cells purified from normal mice were injected together with the $CD8^+$ TIL clone, a significant proportion of the nude mice bearing LSA tumors survived. These data suggested that the CTL clone may not be able to proliferate *in vivo* for prolonged period of time in the absence of exogenous help and that $CD4^+$ Th cells may represent a better source of help than the direct use of IL-2 in the nude mouse model. These data also suggested that adoptive immunotherapy, using $CD8^+$ CTL may not be effective in immunodeficient individuals, such as those infected with human immunodeficiency virus, who demonstrate marked decrease in $CD4^+$ T cells.

Another factor of interest which influenced the outcome of adoptive immunotherapy was the cycling pattern of cytolytic activity exhibited by the CTL clone *in vitro*. It was observed that after the initiation of culture, maximum CTL activity was demonstrable 48hr after *in vitro* culture, whereas, culture of cells for 96hr or more completely failed to exhibit lytic activity. Interestingly, the cells cultured for 48hr exhibited effective tumor-rejection when injected *in vivo* whereas those cultured for 96hr failed to cure LSA-bearing mice. These data together suggested that administration of cells at a time when they exhibit heightened lytic activity *in vitro* may play an important role in the outcome of immunotherapy

against the tumor. In the current study, attempts were also made to study the role played by adhesion molecules in regulating the cytotoxicity of TAL clone as well as its capacity to induce tumor-rejection *in vivo*. We investigated the density of expression of a variety of these adhesion receptors such as CD3, $\alpha\beta$-TCR, CD8, CD45R, CD44 and LFA-1 expressed by the CTL clone. The data demonstrated that almost 90% or greater number of cells exhibited adhesion receptors when tested on days 1-4. Interestingly, however, the percent of cells demonstrating positive fluorescence for $\alpha\beta$-TCR dramatically decreased from ~75% on day 1 to ~28% on day 4 of culture. Furthermore, the density of most adhesion molecules studied decreased gradually with increasing time in culture. There were two possible mechanisms to explain why PE-9 cells fail to lyse LSA tumor cells with increasing time in culture. First, on day 4 of culture, PE-9 cells exhibited decreased levels of lytic granules. To address this we looked for cytokine gene expression in PE-9 cells and found that the mRNA level for perforin, TNF-α, and IFN-γ was decreased on day 4 of culture when compared to day 1. Interestingly however, the levels of granzyme A were not significantly altered. This may be due to differential regulation of these cytotoxic molecules as reported by other investigators (85). However, if the cytotoxicity of LSA was mediated by perforin, TNF-α or IFN-γ, the decrease in their production may explain why the PE-9 cells were unable to lyse LSA with increasing time in culture. The second possibility was that PE-9 cells were unable to lyse LSA *in vitro* and cure LSA-bearing mice when cultured for 4 days *in vitro*, because of downregulation of $\alpha\beta$-TCR expression. Thus, downregulation of $\alpha\beta$-TCR expression and other adhesion molecules may prevent the binding, signaling and killing of LSA because the adhesion molecules play an

important role in effector:target cell interaction (Thiele and Lipsky, 1989).

The downregulation of adhesion molecule expression may not only affect the interaction of CTL with its target cell but also its homing capabilities when adoptively transferred *in vivo*. Since molecules such as CD44 not only participate in cell-cell adhesion and T cell activation but also in lymphocyte homing (Haynes *et al.*, 1989), the decreased expression of CD44 and possibly other homing receptors, may hinder the effective homing of the TIL clone *in vivo* leading to decreased tumor cures. In summary, the current study suggests that the immunotherapeutic efficacy of cultured TIL against specific syngeneic tumors depends on several culture conditions that regulate the cytotoxic activity of the CTL.

Chapter 6: Demonstration of antibodies against gp120 of Human Immunodeficiency Virus (HIV) following auto-sensitization in mice.

Introduction

Although human immunodeficiency virus (HIV) has been shown to be the causative agent of acquired immunodeficiency syndrome (AIDS), there is no clear consensus on how HIV infection actually leads to complete depletion of $CD4^+$ T cells. Several investigators in recent years have suggested that there could be other cofactors in addition to HIV infection which may lead to the collapse of the entire immune functions (Hoffman and Kion, 1991; Shearer, 1993; Andrieu *et al.*, 1986; Habeshaw *et al.*, 1992). One such hypothesis was proposed by Hoffman and colleagues (Hoffman and Kion, 1991) which states that AIDS may be a consequence of destabilization of the idiotypic network. Furthermore, Shearer (Shearer, 1993) suggested that there my be a link between alloimmunity and AIDS, based on similarities in the pathogenesis of AIDS and graft-versus-host disease.

Because individuals contracting AIDS are exposed to foreign lymphocytes that are present in blood or ejaculates, there may be two types of immune responses generated--one against HIV and the other, against allogeneic cells. Inasmuch as, gp120 of HIV is complementary to CD4 (Lasky, 1987) which in turn is complementary to class II MHC (Gay *et al.*, 1987), gp120 could resemble class II MHC (designated MHC-image, or MI) (Hoffamn *et al.*, 1991; Andrieu, 1986; Ziegler and Stites, 1986). Thus, part of anti-HIV response can be considered to be anti-self MHC image (anti-MI). Secondly, immune response of the host against foreign lymphocytes also includes that against the receptors on foreign lymphocytes which recognize the host. Inasmuch as, foreign lymphocytes express anti-self MHC receptors (Ramseier, 1972; Hoffman *et al.*, 1986), immune response against these receptors can be considered as "anti-anti-self MHC" or self MHC-image (MI) response. Based on the above, it was hypothesized that such immune reactions in the host can synergize and not only attack HIV and foreign anti-self idiotypes, but also the endogenous MHC-image and anti-class II helper cell idiotypes, thereby, destabilizing the Th cell network (Hoffman *et al.*, 1991). A combination of exposure to HIV and allogeneic stimuli could therefore initiate an autoimmune disease and AIDS may represent the final outcome of this.

In support of this, Kion and Hoffmann (Kion and Hoffman, 1991) made a surprising finding that normal mice exposed to allogeneic cells as well as autoimmune strains of mice, such as , MRL-*lpr/lpr* make antibodies against gp120 of HIV despite the fact that these mice had never been exposed to HIV. In these mice, it was possible to demonstrate the MHC-image (MI) antibodies. It was concluded that the production of MI and anti-MI may be responsible for the

pathogenesis of autoimmune disease and furthermore these findings supported the idea of synergy between immune response to allogeneic cells and HIV, leading to AIDS. However, it was not clear from these studies whether demonstration of anti-gp120 antibodies correlated with the onset of autoimmunity. To further investigate this as well as to address whether allogeneic immunizations in mice which differ only at class II MHC would lead to production of antibodies against gp120, we undertook the current studies. In addition, previously in our lab we have isolated autoreactive T cells capable of responding to self class II MHC molecules (Kakkanaiah *et al.*, 1990; Nagarkatti *et al.* 1985). In the current study, therefore, we investigated whether immunization using such autoreactive T cells in syngeneic mice would also lead to the production of antibodies against gp120. Our studies demonstrate that with the onset of autoimmune disease as well as following class II MHC related autoimmunization or alloimmunization, there is significant production of antibodies to gp120 of HIV.

Materials and Methods

Mice: MRL-+/+ and MRL-*lpr/lpr* mice were bred in our animal facilities as described (Hammond et al, 1993; Kakkanaiah *et al.*, 1990). Other strains, such as, C57BL/6, DBA/2, FVB, NZB were purchased from Jackson Labs (Bar Harbor, ME). All normal mice were female and were 4-8 months of age while the autoimmune strains of mice, MRL-*lpr/lpr* and NZB, were either young or aged. Young *lpr* and NZB mice were one month of age, and used prior to the onset of autoimmune disease, whereas, the aged mice were either 6 months (*lpr*) or 16 months of age (NZB) and therefore had developed the autoimmune disease. Mice were bled and serum was collected and tested at various dilutions for the presence of antibodies against gp120. Serum samples were obtained from groups of 3-5 mice and were pooled and tested for antibodies to gp120.

Detection of anti-gp120 antibodies by ELISA: Varying dilutions of serum samples were tested for anti-gp120 activity using MGScan HIV-1 ELISA kit (MicroGeneSys, Inc., Meriden, CT). Serum samples were diluted in sample diluent, provided with the kit and 100 μl of each dilution was incubated in triplicate wells of a gp120 coated ELISA plate at 37°C for one hour. At the end of the primary incubation with serum dilutions, the wells were rinsed three times with 400 μl of wash buffer. One hundred μl of 1:1000 dilution of horseradish peroxidase conjugated goat anti-mouse IgG was added to each well and incubated for one hour at 37°C followed by rinsing as described above. Substrate buffer was then added to the wells and the

reaction was allowed to proceed for 30 minutes at 37°C prior to reading the absorbance at a wavelength of 410 nm using an ELISA reader.

Alloantiserum: Several reference alloantisera of mouse origin were obtained from National Institutes of Health. These sera were produced following alloimmunizations to produce antibodies directed against class II MHC molecules. The sera that we tested included: 1) Fla.1,2,3,7 which was obtained by immunizing (A.SW x A.TH)F_1 with A.TL cells [($K^sI^sD^s$ x $K^sI^sD^d$) anti-$K^sI^kD^d$]. This antiserum is therefore directed against I region of H-2^k but it also cross reacts with I region of almost all other haplotypes. 2) F Ia.20, obtained by immunizing (B10.MBR x D2.GD)F_1 with B10 cells [($K^bI^kD^q$ x $K^dI^{d/b}D^b$)F_1 anti-$K^bI^bD^b$]; this antiserum was directed against I^b. 3) Y1-9-04-26-01 (B10.LG x A.TFR4)F_1 anti-B10.D2 ($K^dI^fD^s$ x $K^fI^fD^d$) anti-$K^dI^dD^d$. This antiserum was directed against I^d molecules and reacted with I region of several other haplotypes.

Immunization of MRL-+/+ mice with autoreactive T cell clone: The autoreactive T cell clone designated auto K1.4 was isolated from MRL-*lpr/lpr* mice as described earlier (Kakkanaiah *et al.*, 1990). Briefly, $CD4^+$ T cells from MRL-*lpr/lpr* mice were stimulated with irradiated syngeneic splenic adherent (SAC) cells. After secondary cultures, the cells were grown with SAC and IL-2. The cell line established was cloned. Genetic mapping studies revealed that the auto K1.4 cells were responding to syngeneic I-A^k molecules (Kakkanaiah *et al.*, 1990).

Results

Demonstration of antibodies to gp120 of HIV in the sera of mice following the onset of autoimmunity:

Initially we screened several normal strains of mice of different ages, from 4-8 months, for the presence of antibodies to gp120 of HIV using the ELISA assay. The data shown in Fig. 6.1 demonstrated that none of the normal strains of mice (C57BL/6, DBA/2, and FVB) demonstrated any significant level of antibodies to gp120 at 1:40 and 1:80 dilutions of the serum, when compared to the positive controls which consisted of known antibodies to gp120 and negative control which consisted of normal PBS. Also MRL-+/+ mice which develop autoimmune disease much later (over 12 months), did not exhibit significant levels of antibodies against gp120. We next investigated whether autoimmune susceptible strains of mice, such as, MRL-*lpr/lpr* and NZB would demonstrate the presence of antibodies to gp120 before and after the onset of autoimmune disease. The data depicted in Fig. 6.2 demonstrated that MRL-*lpr/lpr* mice before the onset of autoimmunity i.e. at one month of age, failed to demonstrate significant antibody response to gp120. Interestingly, however, after the onset of autoimmunity (at 6 months of age), MRL-*lpr/lpr* mice demonstrated high titers of anti-gp120 activity. Furthermore, NZB mice also demonstrated high levels of anti-gp120 antibodies at 16 months of age whereas they failed to demonstrate anti-gp120 antibodies at one month of age. Together these studies demonstrated that autoimmune-susceptible mice, prior to the onset of autoimmunity, do not exhibit significant levels of anti-gp120 activity, however, following the onset of autoimmunity, exhibit strong anti-gp120 response.

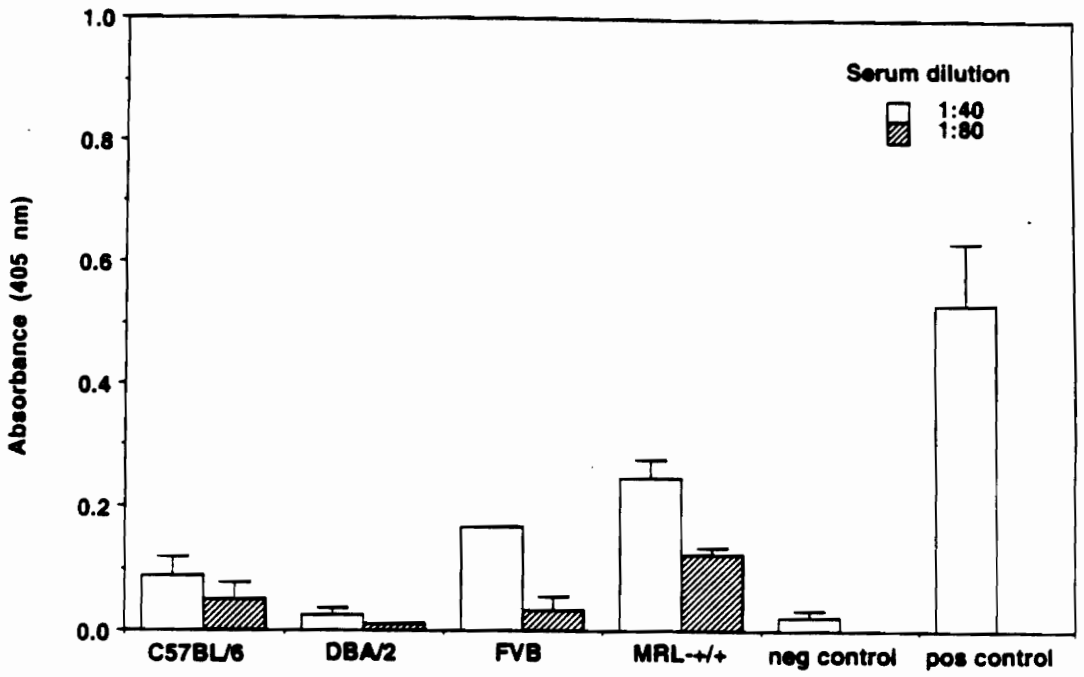

Fig 6.1 **Normal strains of mice do not exhibit antibodies against gp120 of HIV.** Serum samples from various strains of mice were tested for the presence of antibodies against gp120 of HIV at 1:40 and 1:80 dilutions using ELISA. Negative control constisted of nonspecific antibodies and positive control consisted of a known source of antibodies against gp120, both of which were supplied along with the kit for the ELISA assay.

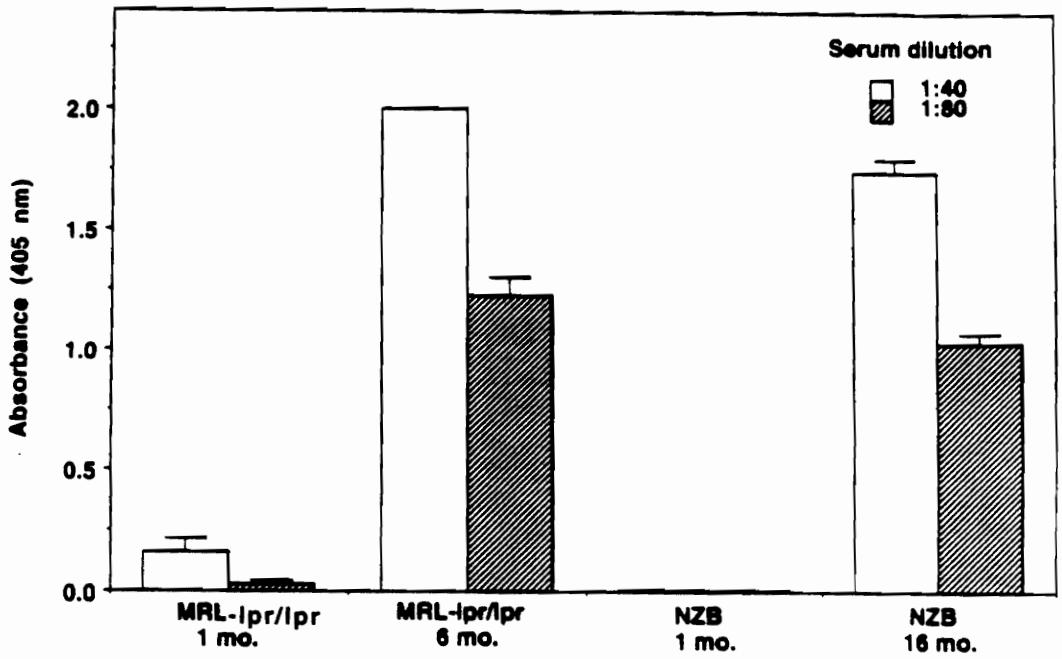

Fig 6.2 **Induction of antibodies against gp120 of HIV following the onset of autoimmunity.** Serum samples were screened for anti-gp120 activity from MRL-*lpr/lpr* and NZB mice before (1 month of age) and after (6 and 16 months, respectively) the onset of autoimmunity.

Alloimmunizations involving differences in class II MHC, trigger anti-gp120 antibodies:

To investigate further whether the antibodies against gp120 represent anti-class II MHC antibodies, we screened several reference allo-antisera obtained from NIH, generated by immunization of mice with allogeneic cells differing only at the class II MHC. All such sera tested, such as, those bearing anti-I^k, anti-I^b or anti-I^d antibodies, exhibited anti-gp120 activity, using ELISA assay (Fig. 6.3). These data suggested that the anti-gp120 activity seen following alloimmunization may represent the antibodies directed against class II MHC molecules.

Effect of immunizations with autoreactive T cell clone on the induction of antibodies against gp120:

Previously it has been reported that MRL-*lpr/lpr* mice but not MRL-+/+ mice exhibit strong autoreactive T cells prior to the appearance of autoimmune disease (14). Thus it was possible that the antibodies to gp120 could indirectly result from an antibody response directed against the autoreactive T cell receptor. To this end, we immunized MRL-+/+ mice with 4 weekly injections of autoreactive T cells isolated from MRL-*lpr/lpr* mice. The data demonstrated in Fig. 6.4 suggested that unimmunized MRL-+/+ mice or mice receiving two weekly immunizations with autoreactive T cells, failed to exhibit antibody response to gp120. However, upon 4 immunizations, there was a strong anti-gp120 antibody response in these immunized mice. Together, these studies demonstrated that repeated autosensitization using the autoreactive T cells may also trigger antibodies which can bind to gp120 of HIV.

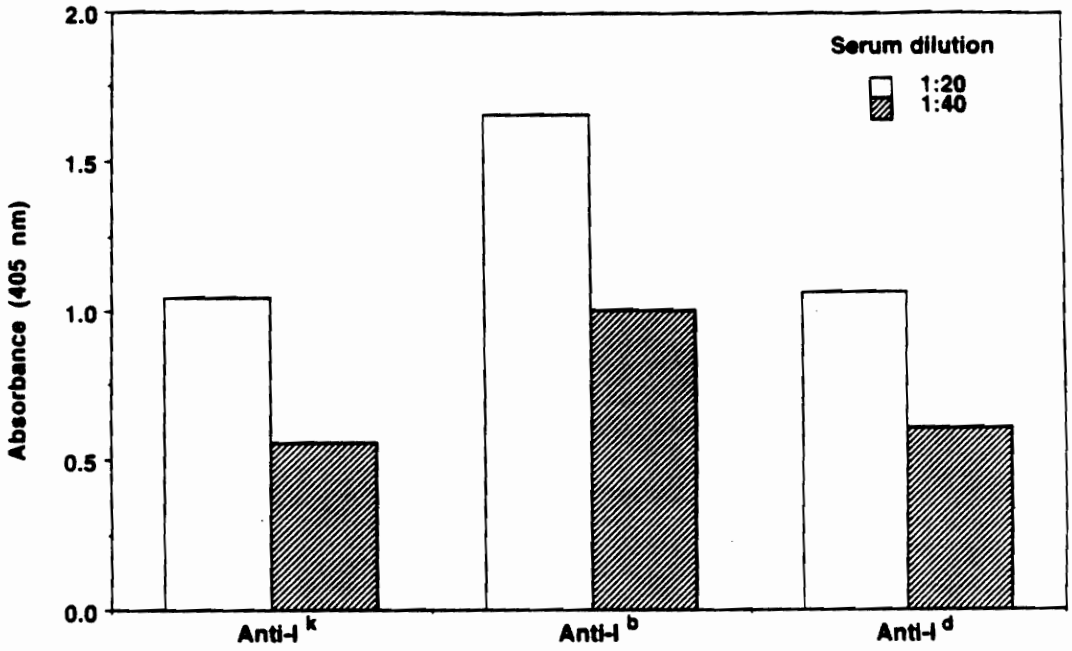

Fig 6.3 **Demonstration of antibodies against gp120 in alloimmune sera.** Serum samples obtained from alloimmunizations containing antibodies against I^k, I^b and I^d were screened for antibodies against gp120 as described in Fig. 6.1.

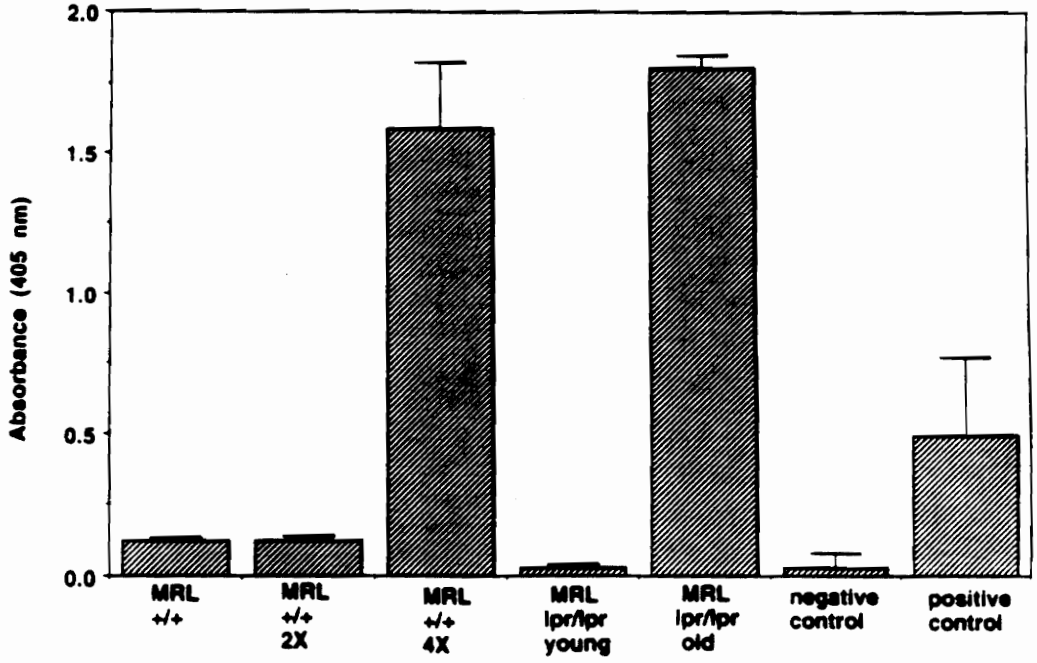

Fig 6.4 **Immunizations with autoreactive T cells triggers anti-gp120 activity.** Autoreactive T cell clone (Auto K1.4) was injected weekly into MRL-+/+ mice. Mice were sacrificed after 2 or 4 immunizations (2X and 4X) and serum samples were screened for anti-gp120 activity. The control +/+ mice (first column) were injected with PBS. Sera from young and old MRL-*lpr/lpr* mice were screened as controls along with the negative and positive controls supplied with the ELISA kit. All serum samples were tested at 1:80 final dilution.

Discussion

The data presented in these studies demonstrate that autosensitization and allosensitization directed against class II MHC molecules can lead to the induction of antibody response against gp120 of HIV. These studies corroborate the hypothesis proposed by Hoffman and colleagues (Hoffman and Kion, 1991; Kion and Hoffman, 1991) that autoimmune and alloimmune mice may produce antibodies against the MHC-image (MI), which may resemble antibodies to gp120 of HIV. Furthermore, our studies demonstrate that the antibodies to gp120 are detected in autoimmune susceptible mice only after the onset of autoimmune disease and similar antibodies can also be demonstrated in the sera of mice which have been immunized against class II MHC differences as well as in mice which have been immunized with syngeneic autoreactive T cells which recognize the self-class II MHC molecules.

We wish to propose three hypotheses to explain the production of anti-gp120 antibodies in autoimmune susceptible mice following the onset of autoimmune disease. Firstly, the autoimmune mice may produce anti-self class II MHC antibodies and because gp120 resembles MHC-image (Hoffman and Kion, 1991;Andrieu *et al.*,1986;Ziegler and Stites, 1986), the anti-self MHC antibodies may be able to bind to gp120. Secondly, the autoimmune mice have large proportions of autoreactive T cells (Cohen *et al.*, 1986). These are $CD4^+$ T helper cells recognizing self Ia molecules (Weksler *et al.*, 1981). In MRL-*lpr/lpr* mice heightened autoreactive T cell responses can be demonstrated at the beginning of

autoimmune disease (Cohen *et al.*, 1986). We have previously shown that such autoreactive T cells can induce the proliferation of both syngeneic T cells as well as B cells (Nagarkatti *et al.*, 1985a; Nagarkatti *et al.*, 1985b; Nagarkatti *et al.*, 1989; Udhayakumar *et al.*, 1988). Thus it is possible that against the increased production of autoreactive T cells during autoimmune disease, the autoimmune mice may produce antibodies directed against the idiotypic determinants of the autoreactive T cell receptors. Such antibodies would have Class II MHC-image and therefore resemble gp120 and in turn, can trigger anti-MHC-image antibodies or antibodies capable of binding to gp120. The third possibility involves T cell response directed against the autoreactive T cells. We and others have shown that autoreactive T cells can induce the proliferation of naive $CD4^+$ T cells (Udhayakumar *et al.*, 1988; Suzuki *et al.*, 1986). Such anti-autoreactive T cells could represent those that are responding to the idiotypic determinants on autoreactive T cells based on the observation that the proliferative response of $CD4^+$ T cells was independent of Ia^+ APC and was not blocked by monoclonal antibodies against class I or class II MHC molecules (Nagarkatti et al, 1985a). Thus, inasmuch as, the autoreactive T cells have receptors against self-Ia molecules, the anti-autoreactive T cells may express receptors bearing class II MHC-image. The antibodies against the anti-autoreactive T cells should resemble anti-MHC-image and therefore should bind to gp120. It should be noted that although CD4 which has complementarity to class II is not conserved between mice and humans with respecto to its ability to bind gp120 of HIV, the image of MHC might be conserved across species. This may explain why anti-MHC-image antibodies react with gp120 (Kion and Hoffman, 1991).

In addition to autoimmunity, allosensitization has also been shown to trigger anti-gp120 antibodies. The exact mechanism by which such immunizations lead to anti-gp120 antibody production is not clear. There are two possible ways which could account for anti-gp120 response. First, there may be an antibody response (MHC-image response) against the idiotypic determinants expressed on alloreactive T cells. These alloreactive T cells may be of donor or host origin. Such an MHC-image response can in turn trigger anti-MHC-image antibodies capable of reacting against gp120. Secondly, the host may directly mount an antibody response against the donor class II MHC, which may mimic anti-MHC-image and therefore recognize gp120 of HIV.

In summary, the current study demonstrates that auto- or allo-sensitizations may lead to a series of network interactions between T and B cells involving class II MHC molecules and their interactive ligands, leading to the induction of antibodies against gp120 of HIV. Alternatively, exposure to gp120 of HIV could lead to production of autoantibodies bearing the class II MHC-image or anti-class II MHC-image. Such antibodies, by virtue of being able to bind to Ia+ APC or T helper cell idiotypes may cause a depletion or inhibition in the interaction thereby resulting in immune dysfunction.

Chapter 7: Conclusions

Mice homozygous for *lpr* or *gld* mutations develop severe lymphadenopathy and autoimmune disease resulting from the accumulation of unique T cells which do not express the adhesion molecules CD4 and CD8. Such T cells have been designated double negative (DN) T cells. The nature, origin, and functions of these T cells is not known. Based on the fact that the DN T cells do not produce IL-2 and fail to proliferate when stimulated through the T cell receptor (TCR), it has been suggested that these DN T cells are anergic and do not exhibit any functions *in vivo*. It has also been hypothesized that the *lpr* and *gld* mutations which cause a defect in the expression of Fas and Fas-ligand, respectively, lead to defective apoptosis of self-reactive T cells and the accumulation of DN T cells may in these mice may result from failure of these cells to undergo apoptosis.

The T lymphocytes are classified functionally as T helper cells which provide help to the B lymphocytes and macrophages, or the cytotoxic T lymphocytes which participate in killing of virally infected target cells and cancer cells. In the current study, we investigated the functions of these unique DN T cells found in *lpr* and *gld* mice. Our data demonstrated for the first time that DN T cells from *lpr* mice exhibit spontaneous cytotoxic activity against certain tumor targets as well as participate

in redirected lysis of a variety of tumor targets when stimulated not only through the CD3/αßTCR complex, but also when activated through certain adhesion molecules, such as, CD44 and gp90^{MEL-14}. Unlike normal T cells, the *lpr* DN T cells constitutively transcribed a variety of cytokine genes, such as, perforin, TNF-α, and IFN-γ. The fact that these DN T cells could mediate cytotoxicity when activated through adhesion molecules, such as, CD44 and gp90^{MEL-14} suggested that the *lpr* DN T cells may also be able to closely interact with endothelial cells which express ligands for CD44 and gp90^{MEL-14} and that such an interaction may play a significant role in the induction of vasculitis that is seen in these autoimmune susceptible mice.

It was interesting to note that unlike *lpr* DN T cells, similar cells from mice bearing a homozygous *gld* mutation failed to exhibit cytotoxic activity despite the fact that the *gld* DN T cells expressed similar levels of adhesion molecules as well as spontaneously transcribed perforin, TNF-α, and IFN-γ genes. Further research into the mechanism, demonstrated that the reason why the *gld* DN T cells were not capable of mediating cytotoxicity was due to the fact that these cells did not express a functional Fas-ligand. Furthermore, *lpr* DN T cells could not mediate cytotoxicity of Fas$^-$ target cells. Together these data demonstrated that the cytotoxicity mediated by the *lpr* and *gld* DN T cells was totally dependent on the interaction between the Fas-ligand, expressed by the effector cells and Fas, expressed on the target cells. This mechanism was different from the cytotoxicity exhibited by alloreactive CTL, which were found to be Fas-independent. These data together suggested that the DN T cells may represent unique T cells which are different from the CD8$^+$ αßTCR$^+$ cytotoxic T lymphocytes.

The fact that *lpr* DN T cells could mediate lysis when activated through

homing receptors, such as, CD44 and gp90^{MEL-14} suggested that the DN T cells may be able to mediate cytotoxicity of endothelial cells because these cells express the ligands for CD44 and gp90^{MEL-14}. Experiments directed to test this hypothesis demonstrated that *lpr* DN T cells were indeed able to mediate spontaneous cytotoxicity of syngeneic endothelial cells but not fibroblast cells. These data therefore suggested that the *lpr* DN T cells may contribute towards the induction of vasculitis by closely interacting with the endothelial cells and by virtue of being able to spontaneously produce a variety of cytokines, such as, perforins, TNF-α, and IFN-γ. Such an interaction might trigger an inflammatory type of response which in turn could give rise to vasculitis seen in these autoimmune susceptible mice.

It should be noted that the *lpr* and *gld* DN T cells may not be able to mediate cytotoxicity *in vivo* in the natural host because of lack of Fas or Fas-ligand due to *lpr* and *gld* mutations, respectively. However, these findings are highly significant because they indicate that the DN T cells may not be able to mediate cytotoxicity of autoreactive B cells and this may lead to continuous production of autoreactive B lymphocytes capable of producing autoantibodies thereby giving rise to the autoimmune disease.

To further elucidate the mechanism of interaction between the T lymphocytes and the endothelial cells, we undertook systematic analysis of the mechanism by which a tumor specific CTL clone, designated PE-9, which expressed high levels of CD44 and gp90^{MEL-14} mediated the cytotoxicity of endothelial cells. During these studies, we made several important observations which would influence the ability of the CTL clone PE-9 to mediate successful immunotherapy against the syngeneic LSA tumor. Importantly, we observed that the cytotoxic T

cell clone exhibited a cycling pattern of lytic activity with peak cytolytic activity at 48 hours after subculture and virtually no cytolytic activity at 96 hours after subculture. The success of immunotherapy against the tumor also depended on the cycling pattern of lytic activity exhibited by the CTL clone. The activated CTL clone was able to mediate cytotoxicity against syngeneic endothelial cells and this cytolytic activity was found to be TCR-independent, MHC-unrestricted and was mediated through adhesion molecules such as CD44 and its interaction with the well characterized hyaluronate ligand. The cytotoxic T cell clone was not only able to mediate lysis of endothelial cells *in vitro*, but also when adoptively transferred into irradiated mice and administered with IL-2, was found to trigger vascular leak syndrome (VLS) resulting from significant damage to endothelial cells. These data therefore suggested that IL-2 may upregulate CD44 expression on the CTL clones and such cytotoxic T cells could then cause significant damage to the endothelial cells leading to the induction of VLS.

The above studies are clinically significant because similar observations have been made in patients who were treated with T lymphocytes or LAK cells along with high doses of IL-2. Such treatments have been shown to be highly toxic resulting in VLS and pulmonary edema, as well as respiratory distress. This has therefore limited the use of immunotherapy to treat cancer successfully. Our data suggested that the alternate pathways of CTL activation may be responsible for the induction of VLS. Therefore, it should be possible to block such toxicity using mAbs against adhesion molecules, such as, CD44 and $gp90^{MEL-14}$.

Normally it is believed that cytotoxic T cells mediate lysis of specific target cells when activated through the TCR. This specificity enables the cytotoxic T cells

to carefully recognize virally infected target cells or transformed cells and destroy them, sparing those cells that are normal. If this is true, what is the significance of our study demonstrating that cytotoxic T cells can be activated independent of the TCR through certain adhesion molecules, such as, CD44? It is possible that the alternate pathway of CTL activation may enable the destruction of virally infected or cancer cells which may downregulate the expression of MHC molecules. Thus, while the TCR-dependent cytotoxicity may be able to destroy the virally infected or cancer cells which express normal levels of MHC, the alternate pathway of CTL activation may help to destroy those mutants from virally infected or transformed cells which down-regulate MHC expression, thereby affording significant protection to the host. However, such a mechanism should be very carefully regulated and there could be a number of factors which may regulate such alternate pathways of CTL activation. These include, up or downregulation of CD44 or its ligand, presence of soluble CD44 or hyaluronate in the serum, etc. Furthermore, any dysregulation in the alternate pathway of CTL activation may cause nonspecific lysis of endothelial cells or any other cell which expresses the ligand for the adhesion molecules which participate in the alternate pathway of CTL activation. Such nonspecific cytotoxicity can also cause significant damage resulting in the induction of the vascular disease seen in *lpr* and *gld* mice.

In the current study we also observed that anti-gp120 antibodies were found in the sera of several strains of autoimmune and alloimmune mice, even though these mice had never been exposed to HIV. The production of anti-gp120 antibodies in autoimmune mice correlated with the onset of autoimmune disease. Furthermore, injection of normal mice with a syngeneic autoreactive $CD4^+$ T_H cell

clone triggered anti-gp120 antibody production. Thus, anti-gp120 antibodies in autoimmune mice may be the result of an antibody response to the idiotype on autoreactive T cell clones which would have MHC-image (MI) and therefore resemble gp120. MI antibodies could in turn elicit an antibody response producing anti-MI which would bind to gp120.

We also demonstrated that alloimmune sera resulting from differences in the I region of MHC, contained anti-gp120 antibodies. Pervious studies for Hoffman and colleagues demonstrated that alloimmune sera contained both anti-foreign and anti-anti-self MHC antibodies. Thus, anti-gp120 antibodies could result from two different mechanisms in alloimmune mice. Firstly, anti-gp120 could result from an antibody response to idiotypic determinants on alloreactive T cells (MI) of donor or host origin. Secondly, the host may mount a response to the portion of MHC class II which cross-reacts with gp120 and thereby mimic anti-MI.

The studies presented here, provide further evidence that HIV may not be the sole cause of Acquired Immune Deficiency Syndrome (AIDS). HIV determinants may mimic MHC II and thereby perturb the normal network of class II MHC molecules and their interactive ligands. Thus HIV may trigger an autoimmune disease in which the immune system attacks itself and commits suicide, thereby indirectly causing AIDS.

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List of Abbreviations

lpr	lymphoproliferative
gld	generalized lymphoproliferation
DN	double-negative
DP	double-positive
TCR	T cell receptor
T_H	T helper
CTL	cytotoxic T lymphocyte
LGL	large granular lymphocyte
NK	natural killer
LAK	lymphokine activated killer
TAL	tumor associated lymphocyte
TIL	tumor infiltrating lymphocyte
PEC	peritoneal exudate cells
FcR	Fc Receptor
TNF	tumor necrosis factor
IFN	interferon
MHC	major histocompatibility complex
SLE	systemic lupus erythematosus
HIV	Human Immunodeficiency Virus

AIDS	Acquired Immune Deficiency Syndrome
CsA	cyclosporin A
IL	interleukin
PMA	phorbol myristate acetate
HEV	high endothelial venule
ELISA	enzyme linked immunosorbant assay
MI	major histocompatibility complex image
PCR	polymerase chain reaction
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
LPS	lipopolysaccharide
HA	hyaluronate
MST	mean survival time
S.E.M.	standard error of the mean

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Research

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Grants/Scholarships/Awards

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Virginia Tech Biology Department matched funds $500

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Professional Experience

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Student Contractor, Chemical Research Development and Engineering Center, Aberdeen Proving Ground, Maryland. Semester Breaks and summers May 1988 to August 1989. (Gas Chromatography, help with soil leaching and tree toxicology studies)

Presentations at Meetings

1) Hammond-McKibben, Denise M., and Prakash S. Nagarkatti. Role of Cytotoxic T cells in the Induction of Vascular Leak Syndrome (VLS) during cancer immunotherapy. Paper presented at Virginia Academy of Science 72nd Annual Meeting, James Madison University, Harrisonburg, VA, May, 1994.

2) Hammond-McKibben, Denise M., and Prakash S. Nagarkatti. Mechanism of Vascular Leak Syndrome (VLS) induced during cancer immunotherapy. Paper presented at The Fourteenth Annual American Cancer Society, Virginia Division Symposium on Basic and Clinical Cancer Research, Virginia Tech, Blacksburg, VA, March, 1994.

3) Hammond, Denise, Prakash S. Nagarkatti, and Mitzi Nagarkatti. Role of Adhesion Molecules Expressed By Tumor-Infiltrating Lymphocytes In Successful Immunotherapy of Cancer. Poster presented at The New York Academy of Sciences conference on Current Trends in Cancer Therapy, Washington, D.C, January, 1993.

4) Hammond, Denise, Prakash S. Nagarkatti. Regulation of Successful

Immunotherapy of Cancer. Paper presented at the American Cancer Society Regional Meeting on Cancer Research, Richmond, Virginia, April, 1993.

5) Hammond, Denise, Prakash S Nagarkatti, and Mitzi Nagarkatti. Role of Adhesion Molecules Expressed by Tumor-Infiltrating Lymphocytes in Successful Immunotherapy of Cancer. Poster presented at the Graduate Student Assembly's Graduate Student Research Symposium, March 30, 1993, Virginia Tech.

6) Hammond, Denise, Prakash Nagarkatti, and Mitzi Nagarkatti. Characterization of Abnormal T cells from the Autoimmune mouse strain MRL-lpr/lpr as Cytotoxic T Lymphocytes. Paper presented at the Virginia Academy of Sciences Annual Meeting, May 20, 1993, Norfolk, Virginia.

7) Hammond, D., P.S. Nagarkatti. Role of adhesion molecules expressed by tumor-infiltrating lymphocytes induced following chemotherapy in successful immunotherapy of cancer. Paper presented at the 18th International Congress of Chemotherapy, Stockholm, Sweden, June 27, 1993.

8) Nagarkatti, Prakash S., Denise Hammond-McKibben. Problems associated with immunorehabilitation or therapy with IL-2: Mechanism of IL-2 mediated toxicity. Paper presented at International Congress of Immunorehabilitation, July, 1994, Sochi, Russia.

9) Hammond-McKibben, Denise, Prakash S. Nagarkatti, and Mitzi Nagarkatti. Lysis of endothelial cells by abnormal, double-negative T cells found in autoimmune MRL-lpr/lpr mice. Poster presented at Experimental Biology 95 (FASEB meeting), Atlanta Georgia, April, 1995.

10) Kao, Henry, Denise Hammond-McKibben, Prakash S. Nagarkatti, and Mitzi Nagarkatti. Factors regulating successful immumotherapy using a tumor-specific cytotoxic T cell clone. Paper presented at Experimental Biology 95 (FASEB meeting), April, 1995.

11) Nagarkatti, Prakash S., Denise Hammond-McKibben, and Mitzi Nagarkatti. Interaction between fas and its ligand is essential for double-negative T cell-mediated cytotoxicity. Poster presented at Experimental Biology 95 (FASEB meeting), April, 1995.

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1) Hammond-McKibben, Denise M., Aruna Seth, Prakash S. Nagarkatti, and Mitzi

Nagarkatti. Characterization of factors regulating successful immunotherapy using a tumor-specific cytotoxic T lymphocyte clone: Role of Interleukin-2, cycling pattern of lytic activity and adhesion molecules. *Int. J. Cancer*, 1994 60:828-836.

2) Hammond, Denise M., Prakash S. Nagarkatti, Lisa Gote, Aruna Seth, Mona Hassuneh, and Mitzi Nagarkatti. Double-negative T cells from MRL-*lpr/lpr* mice mediate cytolytic activity when triggered through adhesion molecules and constitutively express perforin gene. *J. Exp. Med.* 178:2225-30; 1993.

3) Nagarkatti, P.S., D. Hammond, A. Seth, and M. Nagarkatti. Role of adhesion molecules in successful immunotherapy of cancer. *Recent Adv. Chemotherapy*, 999-1001, 1993.

Papers Submitted

1) Hammond-McKibben, D., M. Nagarkatti, P. Nagarkatti. Double-negative T cells from mice homozygous for *lpr* but not *gld* mutation exhibit cytotoxic activity: Role of Fas and Fas-ligand interactions. Submitted March, 1995.

2) Hammond-McKibben, D., M. Nagarkatti, P. Nagarkatti. Demonstration of antibodies against gp120 of Human Immunodeficiency Virus (HIV) following auto-sensitization in mice. Submitted March, 1995.