TUMOR CELL-IMMUNE CELL INTERACTIONS: A LETHAL TWO WAY STREET

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(Abstract)

We investigated the role of Fas ligand in the development of anti-tumor immunity. The LSA tumor specific cytotoxic T lymphocyte (CTL) clone, PE-9, expressed both Fas and Fas ligand. This CTL clone upregulated Fas and Fas ligand expression upon activation through the T-cell receptor and induced apoptosis in Fas^+, LSA tumor cells using the FasL-based pathway. However, LSA and EL-4 tumor cells constitutively expressed Fas ligand and killed Fas^+ PE-9 CTLs and Fas^+, but not Fas-negative (Fas^-) activated T cells and thymocytes. These data suggested that T cells and cancer cells can kill each other and that cancer cells may use Fas ligand to evade the action of the immune T cells.

In addition to the expression of membrane-bound form, $FasL^+$ LSA and EL-4 tumor cells produced a soluble form of Fas ligand when they grew in vivo and in vitro. Serum from EL-4 or LSA-bearing wild type mice contained significant levels of Fas ligand. The soluble FasL induced apoptosis in liver and thymus of C57BL/6 wild type (Fas^+) mice, but not C57BL/6 *lpr/lpr* (Fas^-) mice. The detection of apoptosis in the liver of C57BL/6 *gld/gld* (FasL-defective) mice suggested that the source of Fas ligand found in the sera of EL-4 or LSA-bearing mice was from the tumor cells rather than the host cells.

CTL or NK cells used FasL-based apoptosis to kill the target cells when activated. To this end, we tested whether constitutive expression of Fas on tumor cells generate enhanced anti-tumor immunity. IL-2 or poly-I-C induced/ activated NK/LAK cells displayed higher cytotoxicity against L1210 Fas^+, but not L1210 Fas^- tumor cells.

Furthermore, growth of L1210 Fas^+, but not Fas^- tumor, in vivo, generated Fas-specific cytotoxic T lymphocytes. Therefore, mice bearing L1210 Fas^+ tumor cells survived for a longer time than mice bearing L1210 Fas^- tumor cells.

To determine the role of the Fas, FasL, and perforin in the initiation of tumor, C57BL/6 +/+ ($FasL^+$, Fas^+), C57BL/6 *lpr/lpr* (Fas^-), C57BL/6 *gld/gld* ($FasL^-$), and perforin knock-out (PKO) ($FasL^+$, Fas^+, but perforin-deficient) mice were injected with methylcholanthrane (MCA). Tumor development in lpr or gld mice was faster and uncontrollable, compared to C57BL/6 (wild-type) and PKO mice. However, wild-type and PKO mice showed delayed tumor appearence and were able to suppress tumor growth. In addition to the deficiency of Fas or FasL, high levels of TGF-β and IL-10 expression detected in *lpr* and *gld* mice were also responsible for the early tumor development.

Together these data suggested that interactions between Fas and Fas ligand, expressed on immune cells and tumor cells, play an important role in the generation of anti-tumor immunity. Tumor cells use FasL to evade the action of the immune system, and upregulation of FasL makes T cells more cytolytic. Tumor growth may depend on the number of cancer cells vs. the number of cancer specific T cells.

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List of Abbreviations

Fas	Fas receptor
FasL	Fas Ligand
PKO	Perforin knock-out
CTL	Cytotoxic T lymphocyte
APC	Antigen presenting cells
bp	Base pair
BCNU	1,3-Bis(2-chloroethyl)-1-nitrosourea
CD	Cluster of differentiation
Con A	Concanavalin A
Can A	Concanamycin A
FITC	Fluorescein isothiocyanate
IL-10	Interleukin-10
TGF-β	Transforming Growth Factor Beta
MCA	20-Methylcholanthrene
LAK	Lymphokine activated cells
NK	Natural killer cells
mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
PCR	Polymerase chain reaction
RT-PCR	Reverse trancribed PCR
TCR	T cell receptor
rIL-2	Recombinant interleukin-2
TUNEL	Terminal transferase mediated dUTP nick end labeling
sFasL	Soluble form of Fas ligand
mFasL	Membrane bound Fas ligand
ELISA	Enzyme linked immunoabsorbant serological assay
H&E	Hemotoxylin and eosin
gld	Generalized lymphoproliferative diseases (Fas ligand deficient mice)

lpr	Lymphoproliferative diseases (Fas deficent mice)
DN	Double negative

Chapter 1: Introduction and Specific Aims

Introduction:

Immunology is the science of study of the immune system. The main role of the immune system is to protect the host from infections and the development of cancer. The structures of the immune system and mechanisms of function have lately been discovered, but the practice of immunology started in Turkey and China 2000 years ago by transferring pustule from smallpox infected individuals to healthy people. The mechanisms by which smallpox pustules provided protection was not understood until Edward Jenner and later on Louis Pasteur used aged, thus attenuated bacteria to protect individuals from subsequent development of infectious diseases (Austyn and Wood, 1994; Kuby, 1994).

The immune system consists of organs, cells, and molecules. Immunity is divided into two groups: innate and acquired. Innate immunity includes the anatomical and physiological barriers, and phagocytic cells, including macrophages, monocytes, neutrophils, basophils, and eosinophils. Even though phagocytic cells do not have memory, they become more active after subsequent exposure to the same pathogen. In contrast to the innate immunity, acquired immunity has specificity, diversity, memory, and self/non-self recognition. The immune system is so specific that it is capable of distinguishing a single mutation, or minor differences between two molecules. Because of this specificity, the immune system can also generate enormous diversity to respond to any molecule found in nature or artificially synthesized. Cells involved in the specific immune system are educated in such a way that they can distinguish self molecules from non-self or foreign molecules. Therefore, immune responses are only generated against non-self molecules, and after the secondary exposure to the same molecules, a much stronger reaction is produced in a short time (Kuby, 1994; Abbas et al., 1994; Austyn Wood, 1994).

B and T lymphocytes are the predominant cells in the acquired immune system and originate from stem cells found in the bone morrow. B cells stay in bone morrow until they become mature. When mature B cells leave to the bone morrow, they express a unique antigen specific receptor, a membrane bound antibody. These receptors include IgD and IgM molecules. B cell receptors only recognize soluble antigens preserved in their native form. Therefore, degraded proteins are not recognized by B cells in conventional ways. However, the production of antibodies against double stranded DNA indicated that these cells can respond to many molecules, including lipid and carbohydrates. Following exposure to antigens, B cells undergo immunoglobulin class-switching and differentiate into memory B and plasma cells. Plasma cells actively secrete one of five classes of antibodies. Plasma cells in their short life span produce a large number of antibodies which have a single antigenic specificity. IgG is the most common antibody found in the serum. IgA is involved in mucosal immunity, and therefore when IgAs are passing through the epithelial cells, they are further modified to become a dimer. Thus, secreted IgA molecules exist as dimers. IgE binds to the Fc receptor on mast cells and is associated with allergies.

In contrast to the B cells, some stem cells migrate to the thymus in response to the chemokines secreted by stromal cells which are located in the thymus. As they interact with cells and molecules in thymus, T cells differentiate by expressing unique receptors, T-cell receptor (TCR), and CD3, a co-receptor. The TCR is heterodimer, consisting of either $\alpha\beta$ or $\gamma\delta$ polypeptide chains. The majority of T cells express $\alpha\beta$-TCR and only 3-5 % of T cells carry the $\gamma\delta$ TCR. Following the expression of TCR and CD3 receptor, the T cells differentiate into two groups. Those T cells which express CD8 molecules are known as cytotoxic T lymphocytes (CTLs), and those T cells that express CD4 molecules are designated as T helper cells. During the subsequent maturation stages in thymus, most self antigens are processed and presented in association of Major Histocompatability Complex (MHC) to the T cells. T cells which can not recognize MHC and self molecules or respond strongly to the self molecules, are eliminated

through apoptosis. Mature self-restricted $CD4^+$ or $CD8^+$ T cells are released into the circulation.

The sources of the antigen determine their processing and presention pathways. Proteins expressed in the nucleus, or viral antigens, are the endogenous antigens. These molecules are processed by antigen presenting cells (APC) and presented by MHC class I molecules. Such complexes are recognized by $CD8^+$ T cells. Other kinds of antigens enter the cells from outside. Such antigens are known as exogenous antigens. The exogenous antigens are presented in association with MHC class II molecules and activate the $CD4^+$ T helper cells.

The expression and distribution of Class I and Class II MHC encoded molecules are different. Class I MHC molecules are expressed on nucleated cells so that $CD8^+$ T cells can perform immunosurveillance. However, class II MHC molecules are more restricted to the professional antigen presenting cells (APC) including, dendritic cells, macrophages, monocytes, and B cells.

In response to an antigen, both CD4 and CD8 T cells are activated, but outcomes of the activation are different. CD4 T cells start secreting a variety of cytokines that stimulate cells involved in innate and acquired immunity, whereas activated CD8 T cells instead upregulate the expression of cytolytic genes used for killing infected cells or cancer cells.

Immune responses generated by B cells or antibodies is known as the humoral immune response, whereas cell-mediated immunity results from activation of T cells. To generate efficient immunity to infections or cancer, the cells and molecules involved in innate, humoral, and cell-mediated immunity have to closely work.

Development of Cancer:

In higher eukaryotes, the growth and division of somatic cells, in response to growth regulatory molecules, are tightly regulated in such a way that steady state levels of any given cell stays constant. Communication of a cell with its vicinity and whole body is provided by signalling through receptor-ligand interactions. When normal cells respond to inappropriate growth molecules or generate inappropriate response to normal growth molecules (Israel, 1995), they undergo abnormal development, turning into cancer cells. How many factors or years are necessary for the normal cells to transform into tumor cells? Obviously, dysregulation of one or two molecules may not be sufficient to drive normal cells into cancerous state and several years may have to pass from beginning to clinical level of cancer. Malignant cells usually develop from undifferentiated normal cells which are programmed to be differentiated into mature cells.

Development of cancer consists of 3 distinct stages: initiation, promotion, and progression (Stuart, 1989). During the initiation stages, normal cells become unresponsive to growth inhibitory molecules due to mutations of the cell cycle regulatory proteins or some other unknown factors. Small molecules found in environmental pollutants may interfere with endocrine pathways and act as growth regulatory hormones or block the growth inhibitory factors. p53 and retinoblastoma proteins (RB) are two well known tumor suppressor molecules which regulate cell proliferation (Levine, 1993). The expression and accumulation of the p53 protein is increased in response to cellular stress (Wiman, 1997), such as exposure to UV or other DNA damaging reagents. In this case, the p53 protein stops the cell cycle at G1 phase until damage on DNA is repaired, otherwise cells undergo programmed cell death. Attenuation of p53 protein function in cancer cells leads to accumulation of mutated cells, some of which lose the control of cell proliferation that regulates normal cells with consequent generation of cancer. Therefore, p53 mutations are the most common genetic alteration in cancers of humans (Levine, 1994). In addition to mutation, some viral proteins and E1A protein of adenovirus directly bind and inactivate the wild type p53 protein. Therefore, virus mediated transformation of lymphocytes is very common *in vivo* and *in vitro*. Defective forms of the p53 gene can also be inherited (Levine, 1994). We now know that a large percentage of breast carcinomas, osteosarcomas, chronic myelogenous leukemia, lung, skin, and

colon cancers are associated with mutation in the p53 gene (Greenblatt et al., 1994; Levine, 1993).

RB, another tumor suppressor molecule, controls progression of the cell cycle at the end of G1 and lets cells enter S phase to finish cycle (Weinberg, 1995). RB controls the check-point at G1 phase by activating or inactivating cell cycling proteins (Bartek et al., 1997). Mutations inherited or induced later in life, or inactivation by viral-gene product(s) deregulate RB functions (Kiyono et al., 1998). This leads to loss of this check point of the cell cycle and accelerates cell proliferation. A defective form of the RB gene was detected in osteosarcomas, retinoblastomas, bladder carcinomas, leukaemia, lung, prostate, and breast cancer (Levin, 1993). Cells that start displaying abnormal proliferation, are recognized and killed by cytotoxic T lymphocytes (CTLs). CTLs induce lysis through cell to cell contact. Such interactions require membrane bound cytolytic molecules or Fas ligand. During the initial stages of tumor growth, Fas expression is reduced or parts of this receptor are mutated. Therefore, the immunesurveillance capability of CTLs is impaired and a single cancerous cell is clonally expanded. However, it is believed that mutations or inactivation of one or two of these molecules may not still be enough to induce cancer in human.

There are some other important molecules, known as oncogenes that play a role in the initiation and promotion of cancer. Products of oncogenes include growth regulatory molecules, growth receptors or ligands. They can be an endogenous gene of the host or a gene inserted by virus into the host genome. Fms, erb-A, erb-B and neu are some growth factor receptors, sis and cytokines are growth factors, and bcl-2 and myc are anti-apoptotic genes. Overexpression of myc, erb-A, erb-B were found to be associated with progression of neuroblastomas, ganglioneuroblastoma, and sarcomas (Mares et al., 1998). The integration of Hepatitis B virus erb-A- and erb-B- (Zhang et al., 1992) like DNA was suggested to be involved in liver oncogenesis. Constitutive expression of IL-2, a T cell growth cytokine, IL-2 receptor, IL-4 and IL-4 receptor by tumor cells enhanced abnormal proliferation and transformation of normal T cells, and addition of blocking antibodies to these molecules inhibited the proliferation and tumor progression

(Nagarkatti et al., 1994). Furthermore, high levels of expression of IL-8 and IL-10 were detected in adult T cell leukaemia (Mori, 1996 and 1995). Some other immunosuppressive cytokines, such as transforming growth factors and IL-10, are secreted by tumor cells and are involded in malignancy of cancer. Some cytolytic molecules promote malignancy of cancer when they are expressed by cancer cells. For example, we have lately demonstrated that expression of Fas ligand, a cytolytic molecule, by tumor cells results in the killing of tumor specific T cells, thereby enhancing tumor growth.

The bcl-2 family of genes were first cloned from a B cell lymphoma and recently were found to be overexpressed by multiple myelomas (Egle et al., 1997) and malignant glioma cells (Weller et al., 1995). Bcl-2 is an anti-apoptotic molecule. It is located in the membrane of the mitochondria and blocks the signal for apoptosis at a part of down stream of the caspases. It had been shown to protect tumor cells from apoptosis mediated by cytotoxic immune cells (Egle et al., 1997), chemotherapeutic drugs and therapeutic irradiation (Weller et al., 1995).

In summary, cancer cells usually arise from undifferentiated cells resulting in alteration of two or more cell cycle regulatory or apoptosis regulatory molecules in a duration of the time. Also, growth factors and/or cytokines stimulate tumor development in an autocrine fashion if their receptors are expressed on cancer cells.

Generation of Immune Response to Cancer:

As a result of phenotypic and genotypic alterations, tumor cells express molecules different from self molecules. These are known as tumor specific antigens. Tumor specific antigens are unique to the tumor cells and do not occur on other cells in the body. Tumor-associated antigens are not unique to the tumor cells, they are also expressed by the normal cells under certain conditions, or these are produced by high levels by the tumor cells, but not by normal cells. Tumor-specific and tumor-associated antigens can generate strong immune responses. In animal models and human patients, tumor-specific

T cells can be isolated thereby suggesting that cell mediated and humoral immune response against some tumor exist in vivo. Because of the immune responses generated against tumor-specific antigens, many potential cancerous cells are possibly deleted before they reach to malignant stages . However, some of them can still escape from immune destruction and clonally expand.

The immune response elicited against cancer results from collaboration between a wide variety of cells including, T, B, NK (Natural Killer) cells, macrophages, dendritic cells, basophils, and neutrophils.

According to the conventional belief, T cells perform immunosurvillance and delete any cancerous or virus infected cells. Despite the lack of T cells, nude mice spontaneously develop cancer with a frequency similar to at seen in wild-type mice (Parker and Klubes 1985), thereby suggestion that this hypothesis may not be valid for all tumors. However, generation of effective cytotoxic T lymphocytes against some tumors, particularly progressive and metastatic tumors, may be crucial for anti-tumor immunity. To produce a CTL response, first of all tumor specific antigen(s) and class I MHC molecules have to be expressed on tumor cells because $CD8^+$ T cells recognize antigens in association with class I MHC molecules. Class I MHC molecules are so important for T cell activation that even transfecting of tumor cells with class I gene induced strong CTL response, leading to killing of these tumor cells. The tumor cells used in this experiments did not expressed class I MHC molecules (Elliott et al., 1988). Virally induced tumors express viral associated tumor antigens that can evoke strong T cell response. However, these cells do not express MHC antigens, or viral proteins interrupt the association of MHC complex (Ellem et al., 1998). A strong immune response is generated against virus induced tumor cells. These responses may still not be to eliminate cancer cells. The final outcome may depend on the nature of the immune response and the ability of tumors to develop resistanc. Spontaneously developed tumors may not exhibit any tumor specific antigens, and thus they are invisible to immune system, but express high level of MHC antigens. The expression of tumor specific antigens and MHC molecules vary on carcinogen induced cancers. These data suggest that the expression of

tumor antigen and MHC Class I molecules on the cancer cells vary. Those cells that express immunogenic antigens in association with MHC class I are more likely to be killed before entering into the malignant stages and those cells that do not have one or both of them may progress towards clonal expansion.

Co-stimulatory molecules, B7-1 and B7-2, play a role for T cell polarization and generation of primary immune response against tumor cells. If antigens in association with MHC is presented in the absence of costimulatory molecules, T cells become anergic or deleted. In addition to the lack of MHC, cancer cells do not carry B7 molecules. Transfection of melanoma or EL-4 T cell lymphoma with B7-1 generated not only strong a CTL response to the transfectants, but also conferred resistance to their parental, non-B7-1 transfectant tumor cell lines (Townsend et al., 1994). These studies suggested that the reconstitution of B-7 in immunogenic tumor cells was able to stimulate T cells and these results are important for designing therapeutic protocols to treat the cancer patients.

In addition to the downregulation of MHC and co-stimulatory molecules, tumor cells secrete immunosuppressive molecules. For example, melanomas secrete many cytokines including IL-10 and TGF-β which cease the CTL activity. Suppression of the immune system by TGF-β facilitates tumor cell expansion.

NK cells do not express either immunoglobulin or TCR receptor and thus mediate killing independent of MHC molecules. They use receptors such as Fc, CD16, and CD69 to contact the target cells. NK susceptibility increased in low MHC expression. Yac-1, a tumor cell, which do not express MHC class I molecules and thus is efficiently killed by NK cells. Transfection of Yac-1 cells with MHC class I molecules totally abrogated the NK-mediated killing (Carlow et al., 1989). Therefore, NK cells play an important role in killing MHC^- tumor cells.

Tumor antigens sometimes leak out from the cytoplasm and activate B cells to produce antibodies. The antibodies bind to tumor and trigger complement mediated lysis

ocells or help NK cells to bind tumor cells through Fc receptor. However, the role of the antibodies for tumor development and regression are controversial. In some cases they promote, and other cases, they regress tumor growth.

Cell death, immune system and cancer:

Cell death is a natural and necessary process observed in all living organisms and plays an important role in development, defense, aging, and homeostasis (Vaux and Strasser 1996). Cell death in bacteria occurs as suicide when they are infected by phages so that phages are not spread to the other bacteria. For the same reason, virus infected T cells also kill themselves, but the mechanisms used for suicide in bacteria and in T or vertebrate cells are totally different (Shub, 1994).

Cell death in vertebrates is developmentally regulated. For example, during the development of the nervous system in fetus as many as 85 % of the developing neuron, 95% of T cells, in thymus (Horvitz et al., 1994), and 80% of B cells in bone morrow, die even without leaving the primary site. The webbed structure of duck foot is due to the lack of cell death (Gilbert, 1988). Mammary development seen in female is not common in male because testosterone secretion in male ceased the mammary development by upregulating apoptosis at this site (Gilbert, 1988). Also, steady state levels of cells is so tightly regulated that the number of the cells stays constant. For example, every day at a given time a person carries $50x10^9$ neutrophils in the circulation and at the end of the average one day life span, they are eliminated by apoptosis (Cohen et al., 1992). During the infection, an individual may need large numbers of immune cells. Following the clearing of the infection, those activated cells are killed, otherwise they may pose a danger to self tissues.

There are two kinds of cell death seen in mammals: Necrosis and Apoptosis. Necrosis is initiated by overwhelming cellular injury resulting in swelling, bursting of cells, and finally releasing of cytolytic granules. These cytotoxic molecules cause inflammation,

redness and pain (Steller, 1995; Kuby, 1995). In contrast, apoptosis is induced by gene products and characterized by relatively intact membrane, cell shrinkage, chromosomal condensation, cleavage of DNA into oligonuclesomal fragments, and packaging of the cytoplasmic contents into small granules. After undergoing apoptosis, cells are immediately phagocytosed by macrophages and thus, intracellular molecules are not released, and no inflammation occurs.

Apoptosis or programmed cell dead is programmed in each individual cell and encoded by genes or gene families of the host, including hormones, receptors, ligands, and cytolytic molecules. The apoptosis program can be triggered by many intrinsic or extrinsic signals, such as starvation, infection by virus, radiation, aging, hormones, drugs, stress, alcohol etc., leading to activation of internal death signals.

The nematode *Caenorhabditis elegans* is an excellent model system to understand developmentally regulated cell death and the role of mutation in abnormal cell death (Horvitz et al., 1994) because *C. elegans* has 1090 somatic cells, 131 of which die at a certain stage of development (Ellis et al., 1991) and has similar genes and molecules that control programmed cell death as seen in humans (Horvitz et al., 1994) and mouse. There are 11 genes involved in cell death, 3 of them, ced-3, ced-4, and ced-9, (ced, **C**. **E**legans **d**eath gene) are used for killing and 7 of them (ced-1, ced-2, ced-5, ced-6, ced-7, and ced-8) control for phagocytosis and one gene, nuc-1, functions in the degradation of engulfed cells (Horvitz et al., 1994). All ced gene products are premade and stored inside of the cell as an inactive form. Ced-3 and ced-4 are required to induce apoptosis. Ced-9 promotes survival and can inactivate ced-3 by binding ced-4 (Steller, 1995). Mutation in ced-3 and ced-4 totally eliminates apoptosis in 131 somatic cells (Horvitz et al., 1994; Steller, 1995).

Ced-3 and ced-9 genes are functionally related to mammalian ICE-1, (Interleukin-1β-converting enzyme) or caspases (Thornberry and Lazenbik, 1998) and bcl-2, respectively (Adams and Cory, 1998). Overexpression of ICE or caspases caused excessive death. Bcl-2 promotes cell survival by inhibiting the activation of caspases (Adams and Cory,

1998). Caspases cleave the proteins that prolong survival of the cells and activate nucleases that degrade the host DNA (Thornberry and Lazebnik,1998).

Apoptotic genes even in *C. elegans* reveal some information about how their mammalian counterparts work, particularly the extracellular signalling to activate caspases. In mammals, this signal is initiated by receptor-ligand interaction or by cross-linking of receptor or directly releasing cytolytic molecules onto the membranes of target cells. These include Fas receptor, Fas ligand, TNF receptor and its ligand, Apo3L and receptor, Apo2L and DR4 or DR5 receptor (Ashkenazi and Dixit, 1998). The number of existing receptors increases as new genes are discovered. UV light can aggregate Fas receptor and activate caspases independent on Fas ligand binding. Despite many different mechanisms described, T cells in immune system primarly use two major mechanisms to kill cancer and virally infected cells. These include perforin- and Fas ligand-mediated.

TNF (Tumor Necrosis Factor) is secreted by activated macrophages, cytotoxic T cells, and Th1 cells (Kuby, 1995). It induces apoptosis by binding TNFR1 (Tumor necrosis Factor Receptor), but TNF-related factors are slow-acting, inducing apoptosis in sensitive cells only after 24 hour or more exposures, when compared to CTLs that induce DNA fragmentation in minutes (Cohen et al., 1992). Inflammation observed in the necrosis is also a sign of TNF-mediated apoptosis.

Many life threatening diseases today are associated with either inhibited or excessive apoptosis. Cancer, including breast, prostate and ovarian cancer, follicular lymphoma, hormone-driven tumor, autoimmune diseases, and viral infection are characterised by accumulation of cells resulting from either increased proliferation or failure of cells to undergo apoptosis in response to appropriate stimuli (Thomson, 1995; Hsu et al., 1994). Diseases resulting from increased apoptosis include AIDS, neuron damage after stroke and other ischemic injury, Alzheimer's disease, Parkinson's disease, cerebellar degeneration, toxin induced liver disease and alcohol mediated liver destruction (Thomson, 1995; Barinaga, 1998).

The survival of normal cells depend on the survival signal received from other cells and environment, whereas tumor cells overcome this obstruction so that they can grow without dependence on each other or at distinct sites where primary tumor cells arise and ignore the survival and environmental signals. The secretion of growth factor and expression of their receptors by tumor cells are possibly the main reason for cells to grow independently. However, fast growth is not the only problem to neoplastic transformation since immature cells are programmed to proliferate and differentiate to become mature cells, otherwise they undergo programmed cell death. Almost all tumors arise from undifferentiated cells. There are positive correlations between tumor growth and inhibition or decrease in apoptosis. Apoptotic and growth regulatory genes are directly involved in the pathogenesis of cancer. Tumor cells become resistant to cell death by not expressing apoptotic molecules or producing large amount of anti-apoptotic molecules.

Bcl-2 located in the membrane of mitochondria, endoplasmic reticulum and nucleus blocks the apoptosis by controlling Ca^{+2} influx into the cells and organelles. In tumor cell lines, overexpression of bcl-2 provides resistance to apoptosis induced by lymphocytes, chemotherepeutic agents, and radiation. Bcl-2 transgeneic mice were found to have 30% more B cells in circulation and co-operation of this molecule with other oncogenes increases the susceptibility of mice to naturally occurring tumors.

Tumor suppressor genes arrest the cell cycles at G1 phase if there are some mutations or damages in DNA. When damage has occurred beyond repair, cells commit suicide; otherwise these cells most likely transform into cancerous stages. The p53 protein is not functional in most human tumor cells and mutation in the RB gene is associated with development of osteosarcomas, retinoblastamas, carcinoid tumor, and small lung cancer (Sherr, 1996). There is positive correlation among the upregulation of apoptotic molecules and tumor suppressor genes in response to somr drugs. Addition of bleomycin to hepatoma cells, p53 +/+HepG2 but not p53-/-Hep3B cells , upregulated Fas expression on membrane of the cells and increased susceptibility to Fas mediated apoptosis (Muller

et al., 1997). These data clearly showed that positive correlation exists among apoptotic molecules, tumor suppressor genes, and apoptosis.

Inadequacy of Fas-Fas ligand interactions, mutations at the cytoplasmic parts of Fas, and deficiency of caspase activation can all contribute to resistance to apoptosis.

Programmed cell death is also important for establishing and maintaining a healthy immune system. Premature T cells migrate to the thymus and begin to rearrange T-cell receptor (TCR) receptor genes. $CD4^+CD8^+$ T cells that carry a productive TCR gene are further selected. Those T cells that have high affinity to self MHC and self antigens associated with MHC are eliminated by negative selection. Mature single positive T cells ($CD4^+$ or $CD8^+$) are released into the circulation whereT cells are further selected in the periphery based on the recognition of the self antigens. During the immune response, the number of CD4 and CD8 T lymphocytes increase so that they can attack the foreign invader efficiently. Some of the activated T cells become memory cells and rest of them are removed from the immune system, otherwise, they keep secreting cytokines that could be toxic to the host. Those T cells that do not have a productive TCR or fail to recognize self MHC, or carry TCR receptors that show high affinity to self antigens in association with MHC molecules are removed by apoptosis.

B cells are also selected under the same pressure and those B cells that fail one of these steps are removed by programmed cell death. The consequence of failing to undergo apoptosis in one of these steps is the development of the autoimmune diseases. Rheumatoid arthritis, systemic lupus erythematosus, multiply sclerosis, autoimmune diabetes, and other autoimmune diseases, are associated with inhibition of apoptosis (Kuby, 1995; Thompson, 1995). *Lpr* and *gld* mice are good example to demonstrate the failing of apoptosis and the development of autoimmune diseases. The defect in Fas or Fas ligand in *lpr* and gld mice (Nagata, 1995; Fukanaha, 1993), respectively, results in accumulation of double negative T cells in secondary lymphoid organs and in developing a disease similar to human lupus. The detection of higher levels of circulating auto-reactive antibodies in *lpr* and *gld* mice indicates the existence of a large number of

autoreactive B cells. The impairing of Fas-mediated apoptosis by deletion in the cytoplasmic domain of Fas (Rieux-Laucat et al., 1995) or by soluble Fas (Cheng et al., 1994) was associated with human lymphoproliferative syndrome and systemic lupus erythematosus. These data suggest that apoptosis, particularly Fas-mediated apoptosis, is important for development and maintaining of immune system homeostasis. The inhibition or downregulation of cell death leads to deleterious autoimmune diseases and cancer.

To prevent the propagation of virus, infected cells commit suicide by apoptosis or are killed by cytotoxic T cells following the presentation of viral proteins on host MHC class I molecules. Viruses depend on host cells to produce their viral proteins, and therefore they develop a mechanism to interfere with apoptotic pathway in infected cells. The viral gene products E1B or BHRF1 from adenovirus and Epstein-Barr virus, respectively were shown to block apoptosis using similar mechanisms as used by bcl-2 (Thompson, 1994). The CrmA, a protease inhibitor from cowpox virus, disrupts apoptosis by inhibiting ICE, interleukine-1β-converting enzymes or cyteine proteases (Thompson, 1994). Some other viral products from Ebstein-Bar virus or Chronic Sindbis virus promote the survival of cells by upregulating bcl-2 expression (Thompson, 1994). These data demonstrated that inhibition of apoptosis in infected cells causes the establishment of viral colonies or latencies in the host. In contrast to other viruses, HIV infects and causes depletion of the CD4+ T cells by inducing apoptosis.

It is believed that several neurological diseases including Alzheimer's diseases, Parkinson's diseases, spinal mascular atrophy, and cerebellar degeneration are caused by apoptosis of neurons (Thompson, 1994). Furthermore, the morphologic characteristics of dying cells after stroke were found to be similar to apoptosis observed in normal cells (Marcia, 1998). These data suggest that excessive cell death can be reduced with caspase inhibitors following the stroke.

Programmed cell death can be divided into two phases: transferring extracullular signals into the cells and activation of caspases in mammals or ced genes in *C. elegans*.

Activation of caspases is sufficient for a cell to commit suicide. However, for delivering the cell mediated apoptosis, which is important for the immunotherapy of cancer, cell to cell contact provided by receptors and ligands, is necessary. Cytotoxic T cells use two major pathway to kill targets cells: Perforin and Fas ligand.

Perforin Mediated Cell Death:

Human and mouse perforin protein consists of 555 amino acid residues with a molecular weight ~ 66-70kDa, but the mature perforin protein loses 20 amino acids after cleavage of a signal sequence (Lowin et al., 1995; Liu et al., 1995). Eighty-five percent of amino acid residues of mouse and human perforin protein are identical. The amino acid sequence and mode of action of perforin are also similar to complement components, C6-C9, (Lowin et al., 1995).

Perforin is synthized in the endoplasmic reticulum and post-translational modification is done in the Golgi apparatus. Mature proteins are packaged into lysosomal-like cytoplasmic granules (Peters et al., 1991) which stay in the cytoplasm until released. These data suggest that perforin is a pre-formed molecule and during the initiation of cell death, no new gene expression is required.

When killer cells contact target cells, monomeric perforin molecules are released which directly bind to the membrane of the target cells. Perforin opens a hole on the membrane and to be more effective, monomers aggregate to ensure that the holes are not repaired. Perforin by itself does not induce DNA fragmentation, but other molecules including Granenzyme B, a serine esterase, enter into the cells through the hole created by perforin and cause protein and nucleic acid degradation (Liu et al., 1995). Using of calciun chealator, such as EGTA , during the cytotoxic assay totally abolishes perforin-mediated killing suggested that the binding of perforin to the target cells membrane may depend on the presence of the extracullular Ca^{+2}.

A transcriptional regulatory mechanism controls perforin expression to ensure that it is restricted to immune cells (Lui et al., 1995; Lowin et al., 1995). However, the detection of perforin and granzyme B by human kerotinocytes indicated that its expression is broader than that previously thought (Berthou et al., 1997). Perforin expression is detectable in T lymphocytes in circulation, spleen, lymph node, and peritoneal exudase. In addition perforin is also expressed by NK cells, cells in uterus, lung, and salivary gland of the pregnant mice (Lowin et al., 1995). Virus specific $CD4^+$ T cell clones and Th2 type of helper cells were also found to carry perforin molecules (Yasukawa et al., 1993; Lancki et al., 1991).

CTLs recognize viral or tumor antigens associated with the MHC class I molecules and kill the virally infected or tumor cells. The induction of cell death by purified perforin molecules indicated that perforin is a potent and effective cytolytic toxin. Therefore perforin-mediated lysis is an important arm of CTLs to induce lysis on target cells, particularly one that has no Fas molecule on the membrane.

Natural Killer Cells (NK) are important lymphocytes that provide immunosurveilance against cancer because most tumor cells downregulate MHC class I expression or tumor specific antigen is not presented on MHC. NK cells are effective against such targets because they recognize targets independent of MHC molecules. Also, LAK cells (Lymphocyte Activated Killer Cells) recognize antigens in the same fashion as NK cells and both of them constitutively express perforin.

Perforin knock-out (PKO) mice have been generated by three different groups and these mice exhibit normal phenotype. The CTLs and NK cytotoxicity to different target cells is slightly reduced. (Kagi et al., 1994; Lowin et al., 1994; Walsh et al., 1994). However, when target cells were activated to express Fas antigen, $CD8^+$ T cells from PKO mice induced significant lysis (Walsh et al., 1994). In agreement with these data, we also demonstrated that activated NK cells from PKO mice killed the Fas^+ targets (Bradly et al., 1998), thereby suggesting that CTL and NK cells express both FasL and perforin simultaneously and based on the targets, they use one or both of them.

Fas and Fas Ligand:

The induction of cytotoxicity by two monoclonal antibodies on certain cells led to discovery of Fas receptor (Yonehara et. al., 1989). Mouse Fas antigen consists of 306 amino acids with molecular weight 35 kDa and is located on chromosome 19 (Watanabe-Fukunaga et al., 1992). The size of human Fas antigen is 319 amino acids with 36 kDa molecular weight which is slightly larger than that of the murine counterpart (Itoh et al., 1991). The membrane bound Fas consists of three parts: transmembrane, extracullular , and cytoplasmic. The size of these are 17, 141, and 145 amino acids in mouse and 17, 157, and 145 amino acids in human, respectively (Itoh et al., 1991; Watanabe-Fukunaga et al.,1992). The location of the carboxyl end of the protein in the cytoplasm and cysteine rich residues in the extracellular parts indicate that Fas belongs to the type I receptor family. This family includes Tumor Necrosis Factor receptor (TNFR), CD40, OX40 and Nerve Growth Factor receptor (NGFR) (Nagata, 1997). In addition to the cysteine rich extracellular part, Fas and TNFR-I carry the similar death domain in the cytoplasmic part. Despite the similar molecular structure, mouse and human Fas antigen display 65.7% and 49.3% homology at the nucleotide and protein levels, respectively (Watanabe-Fukunaga et al., 1992).

. The orientation of Fas ligand on the membrane is different from Fas. The C-terminus of Fas ligand is located on the outside of the cells. Therefore, it belongs to the Type II TNF ligand family (Nagata, 1997). This family includes TNF, lymphotoxin, CD40 ligand and Fas ligand. A mature human and mouse Fas ligand consists of 3 parts: cytoplasmic, transmembrane and extracellular. Mouse, human, and rat Fas ligand have more homology when compared to the Fas from the same species. For example mouse and human Fas ligand contain 271 and 281 amino acids with 76.9 % homology and more interestingly, 91.4% of amino acids in mouse and rat Fas ligand are identical (Nagata, 1994; Takahashi et al., 1994). Both human and mouse FasL gene is located on chromosome 1. In contrast to the Fas, the cytoplasmic part of FasL does not have any

significance for binding and inducing lysis of target cells, based on studies performed thus far. Therefore, of 281, 179 amino acids make up the extracellular part of Fas ligand (Nagata, 1994). The last 80 amino acids of the C-terminus of Fas ligand play an important role in folding and binding to the Fas (Schneider et al., 1997).

Binding FasL to Fas causes apoptosis in target cells and this is a major cytolytic mechanism utilized during the development and maintenance of the immune system. The expression of Fas and Fas ligand are varied in the body. Fas expression, when compared to the Fas ligand expression, is less restricted. Similarly, Fas expression in humans is strictly controlled in contrast to the Fas expression in mice.

Those tissues, such as liver, thymus, and uterus, which have high cell turnover rate, express Fas antigen at very high levels. In contrast, cells in the testis, brain and muscle which do not divide or are not replenished, do not express any detectable levels of Fas. Other organs, such as spleen, lymph nodes and pancreas carry moderate levels of Fas receptor. It is also reported that skin or the keratinocyte, particularly during disease progression, carries significant amounts of Fas antigen (Viard et al., 1998; Aragane et al., 1998).

The tolerance to self antigens is induced during the maturation of T and B cells. Therefore, in the thymus, T cells from double negative ($CD4^-$ -$CD8^{-)}$, double positive ($CD4^+$ $CD8^+$) to single positive ($CD4^+$ or $CD8^+$) stages, express very high levels of Fas. During the process of negative selection, those T cells which display high affinity to self antigen in association with self MHC, are deleted through the Fas-mediated apoptosis (Noble et al., 1998). Similarly, B cells also express Fas receptor during the maturation process in the bone morrow and autoreactive B cells or those B cells which have unproductive immunoglobulin gene rearrangements, are removed through Fas-mediated apoptosis. Some self antigens expressed in rare tissues are not presented in bone morrow and thymus. The self antigen reactive mature T and B lymphocytes in the periphery express excessive Fas and are killed by FasL-mediated lysis.

Activated $CD4^+$ and $CD8^+$ T cells express both Fas and Fas ligand. The purpose of activation of $CD4^+$T cells is to produce cytokines and of $CD8^+$ T cells is to become cytotoxic T lymphocytes. Activated $CD4^+$ T cells are killed by $CD8^+$ T cells, however at this stage CTL is resistant to Fas-mediated apoptosis (Noble, 1998). Furthermore, bystander naïve $CD8^+$ T cells that are activated through the TCR-ligand interaction are also sensitive for Fas mediated apoptosis (Nagata, 1997; Hornung et al., 1997). The CTL response is often suppressed by an autocrine Fas-mediated apoptosis as seen in Jurkat cells (Dhein et al., 1995) or tumor specific Fas^+ CTL (Zeytun et al, 1997). These data suggest that Fas is expressed at varying levels at different stages of the activation of T cells. This facilitates the deletion of non-specifically activated T cells as well as antigen-specific activated T cells, so that tissue destruction is prevented.

Mast cells constitutively express Fas antigen and their number is strictly controlled by Fas-mediated mechanisms (Hartmann et al., 1997). Mature neutrophils have the shortest life span and die by apoptosis. The expression of both Fas and Fas ligand simultaneously by the neutrophils indicates that Fas-Fas ligand mediated apoptosis via autocrine fashion keeps the neutrophil population at the steady-state levels (Liles and Klebanoff, 1995). Abnormal Fas regulation in some patients results in accumulation of the double-negative T cells and development of hypereosinophilia (Simon et al., 1996), thereby suggesting that Fas plays an important role the regulating the number of leukocytes, including polymorphonuclear cells in the circulation.

The expression levels of Fas and sensitivity to Fas-mediated apoptosis vary among tumor cells. The sensitivity of cancer cells to the physiological effects of Fas-Fas ligand signaling contribute to the efficacy of the natural immune surveillance against the malignant cells. Fas is expressed on both hematologic and non-hematologic tumor cells, including B- and T cell leukemias and lymphomas, colon carcinoma, breast cancer, melanoma, prostate cancer and pancreatic adenocarcinoma (Yonehara et al., 1989; Owen-Schaub et al., 1994). However, they are resistant to Fas-mediated apoptosis. Detection of a point mutation in the cytoplasmic part of the Fas of the myeloma cells (Landowski et al., 1997) contributed to the pathogenesis of the disease. These data implied that detection

of the Fas on the membrane of the tumor cells may not necessarily indicate the sensitivity to cell to FasL-mediated apoptosis. In vitro, constitutive expressing of Fas in L1210 and LSA tumor cells, makes them more sensitive to CTL and NK mediated lysis. In vivo, injection of IL-2 increased the survival rate of L1210 Fas^+ tumor-bearing mice (Bradley et al., 1998). Treatment of the myeloma cells with INF-α (Egle et al., 1996) or hepatacellular carcinoma cells with INF-γ (Yano et al., 1996) upregulated Fas expression on cancer cells and then they became sensitive to Fas-mediated apoptosis. UV light induced Fas-mediated apoptosis is independent of Fas ligand (Aragane et al., 1998). The regression of the tumor from patients treated with chemotherapeutic drugs or radiation results from increased expression of Fas and induction of apoptosis. Treatment of tumor cells with cytokines such as interferons, increases Fas expression and make tumor cells more sensitive to CTL or NK cells mediated killing. Similarly, administration of IL-2 into cancer patients upregulates Fas ligand expression on T cells, thereby facilitating the killing of tumor cells. More importantly, anti-cancer drugs which specifically elevate Fas-induced apoptosis, would be an effective way to treat the cancer patients. These data indicate that tumor cells are sensitive to Fas mediated apoptosis if they carry functional Fas receptor.

Fas ligand was initially thought to be expressed by activated $CD8^+$ T cells. However, $CD4^+$ T lymphocytes, natural killer cells (Arase et al., 1995), dendritic cells (Suss snd Shortman, 1996), phagocytic macrophages and human monocytes (Kiener et al., 1997) also express cytoplasmic, membrane bound, or soluble forms of Fas ligand. A subclass of Dendritic cells killed the $CD4^+$ T cells by membrane bound FasL-mediated apoptosis. Both macrophages and monocytes express high levels of cytoplasmic Fas ligand and kill bystander leukocytes by release of these molecules. In thymocytes, only stromal and antigen presenting cells express membrane Fas ligand, but not T cells (French et al., 1997). Cytolytic $CD4^+$ T cells do not express perforin, and Fas ligand is the only pathway that they can use to kill the target cells. More interesting data came with an observation that FasL expression is not restricted to lymphoid cells, and that non-lymphoid organs known as immune privilege sites, such as eye (Griffith et al., 1995), testis (Bellgrau et al., 1995), uterus and placenta (Hunt et al., 1997) also carry Fas ligand

to protect themselves from activated leukocytes. Suprisingly, many tumor cells also express Fas ligand as the means of defending against infiltrating leukocytes. LSA and EL-4 T-cell lymphomas constitutively expressed Fas ligand and killed tumor specific cytotoxic T lymphocytes (Zeytun et al., 1997). Similarly melanoma (Hahne et al., 1996), colon carcinoma (O'Connel et al., 1996), human oesophageal carcinoma (Bennett et al., 1998), and human colonic adenocarcinomas (Shiraki et al., 1997) induced apoptosis in Fas expressing cells and tissues. The FasL expression by tumor cells can also have other consequences that are beneficial to the host. FasL transfected tumor cell lines were rejected as a result of neutrophil infiltration (Seino et al., 1997). The injection of adenoviral vector encoded Fas ligand into the locally grown colon carcinoma eliminated the tumor masses. The investigators concluded that Fas L causes inflammation and neutrophils infiltration at the tumor sites (Arai et al., 1997). Many of these tumors that we studied constitutively express Fas ligand and a low level of Fas. In contrast, the expression of Fas on T cells is induced after activation with mitogens or cytokines. Therefore, the final outcome of battle between tumor cells and T cells in vivo depends on the microenvironment of the tumor and competence of the host immune system.

FasL expression by CTL is always detectable by RT-PCR, but it is dramatically upregulated after stimulation with PMA and Ca-ionophore. Hepatitis or HIV infection also increases FasL production. Soluble TNF (Tumor Necrosis Factor) downregulates FasL, and thus facilitates the infiltration of T cells and destruction of these immune privilege sites.

The marked interest in Fas ligand in transplantation arises from the fact that organs expressing Fas ligand kill the infiltrating T lymphocytes which are the main mediator of destruction of foreign tissues. Attack of T cells on pancreatic islet cells causes diabetes mellitus. Transplantation of H-2^k allogeneic islets injected with syngeneic myoblast transfected with FasL into H-2^b mice was not rejected for more that 80 days while the control cells were rejected in ~10 days (Lau et al., 1996). Similarly, injection of a plasmid containing FasL into inflamed thyroid inhibited infiltration of T lymphocytes (Batteux et al., 1999), through destruction of thyroid is mediated by autocrine rather that

infiltrating lymphocytes (Stassi et al., 1999). In contrast to these studies, transplantation of a heart constitutively expressing Fas ligand under α-myosin promoter is rapidly rejected due to increased neutrophil infiltration observed in transplanted FasL-expressing transgeneic heart (Takeuchi et al., 1999).

The functions of the endogenously expressed Fas ligand could be different from FasL that are transfected and constitutively expressed. The natural expression of FasL by the tumor cells may not cause inflammation and neutrophils infiltration. Such FasL may promote tumor growth by killing the immune cells. However, FasL-transfected tumor cells overexpress these molecules which may lead to neutrophil infiltration and cause inflammation.

Both Fas and Fas ligand are expressed on the membrane and are involved in signalling pathways. The proliferation of $FasL^-$ CTL in response to alloantigen was lower than that of $FasL^+$ CTL. Therefore, it was suggested that reverse FasL signalling is required for the maximum activation of T cells. In agreement with these data, we were not able to establish tumor specific CTL clones from Fas- or FasL-deficient mice. These data suggest that Fas ligand is necessary for the activation of T cells as well as apoptosis.

Tumor necrosis factor gene family is expressed as membrane bound and soluble form. The soluble form is very active and responsible for tissue destruction during the infection. Therefore, both Fas and Fas ligand which belong to the TNF family are generated in membrane bound and soluble forms. The alternative RNA splicing or loosening of the transmembrane parts result in the creation of the soluble Fas molecules. Increased levels of soluble Fas molecules in systemic lupus erythematosus patients and the development of autoimmune-like diseases in mice injected with the soluble Fas molecules suggested that this form of Fas interferes with the functions of its membrane bound form (Cheng et al., 1994). Similarly, the expression of soluble form of Fas was associated with malignant disease pathogenesis (Owen-Schaub et al., 1995). These data indicated that the secreted form of Fas blocks apoptosis by binding to the Fas ligand.

The cleavage of membrane bound Fas ligand by metalloproteinase generates a soluble form of it. The cleavage site of the enzyme is between Ser-126 and Leu-127, located in exon two of the extracullular part of Fas ligand (Schneider et al., 1998). If FasL is carried on the membrane, it is effective during cell to cell contact. However, if it is secreted, it enters into the circulation and binds and destroys the Fas expressing tissues such as liver, lung, and heart. The increase in the amount of FasL is often associated with diseases. Fas ligand was detected in serum of patients with large granular lymphatic (LGL) leukemia, natural killer cells lymphomas (Tanaka et al.,1996), nasal lymphomas (Sato et al., 1996), hemophagocytic syndrome and daimon-blackfan anemia (Hasegawa et al., 1998), and rheumatoid arthritis. After head injury, the cerebrospinal fluid was also found to contain detectable concentration of Fas ligand. FasL expressing tumor cells, including EL-4 and LSA, also secrete FasL which induced apoptosis in liver, thymus, and lungs in vivo (Zeytun et al., 1999). Furthermore, there was a positive correlation between the amount of FasL found in serum and invasive capability of the nasal lymphoma (Sato et al., 1996). Interestingly, the concentration of Fas ligand in the serum dramatically decreased during the recovery period from the cancer. The cytolytic capability of sFasL was reduced compared to the membrane bound form (Scheider et al., 1998), but it is not clear whether the high levels of sFasL is as functional as membrane bound form. However, the existence of other molecules which facilitate the crosslinkage of sFasL to the Fas receptor have not been investigated. The purposes of the shedding Fas ligand by T cells and $FasL^+$ tumor cells may be different. Activated T cells upregulate FasL expression and carrying Fas ligand for a long time on the membrane may cause non-specific destruction of self tissues. Tumor cells release large amount of Fas ligand in circulation and destroy the Fas and Fas ligand expressing tissues and cells so that they can easily metastasize.

Mutation in the expression of Fas or its ligand results in lymphoproliferative and autoimmune disorders such as those seen in mice expressing homozygous *lpr* and *gld* mutation (Watanabe-Fukunaga et al., 1992; Takashi et al., 1994). The *lpr* and *gld* mice exhibit large numbers of unique T lymphocytes which are $\alpha\beta$-TCR^+, CD4-, CD8- designated as double- negative T cells (DN). These mice developed hypergamma-

globulinemia and lymph-node enlargement as well as autoantibody production and die early from autoimmune disease, which is similar to the human lupus. Such data suggest that Fas and Fas ligand interactions are important for normal functioning of the immune system and therefore may play a significant role in the induction of peripheral tolerance.

CTLs have been shown to a play major role in the regulation of tumor growth. However the exact role played by Fas and Fas ligand in the anti-tumor immunity is not clear. Studies in our lab and elsewhere have suggested that Fas and Fas ligand are important immunomodulatory molecules. Because these molecules are necessary for preventing autoimmune disease in mice, activation of T cells, and apoptosis, further investigation on the role played by these molecules in the tumor cell-host cell interactions may shed new light in developing effective treatment against cancer.

Specific Aims:

Fas and Fas ligand are the immunomodulatory molecules. Defective Fas-FasLigand signaling results in the development of autoimmune diseases and cancer. Fas and FasL have been shown to be expressed both by the tumor cells and by the immune cell of the host. Thus, in the current study we hypothesized that Fas-FasL interactions play an important role in the generation of an effective immunesurveillance against cancer. The following specific aims were investigated in the current study:

Aim: 1- LSA tumor cell specific $CD8^+$ T cells were developed from wild-type and perforin knock-out mice to investigate whether tumor specific $CD8^+$ T cells express and use FasL and/or perforin to mediate lysis. Also, the expression of Fas and Fas ligand by LSA and EL-4 tumor cells and their role in immune evasion will be studied.

Aim:2- To determine the role of the Fas in the induction of anti-tumor immunity in vivo. The transfected tumor cells with sense or antisense Fas or Fas ligand are injected into syngenic mice in the presence or absence of T cell stimulatory molecules and the growth characteristics of tumor was studied.

Aim: 3- To investigate whether $FasL^+$ tumor cells secrete FasL, and the biological significance of this form in tumor bearing host.

Aim: 4- To study the presence and role of the alternate forms of FasL expressed by T cells.

Aim: 5- To test the sensitivity of the Fas, Fas ligand, and perforin-deficient mice in the induction of tumor by methyl cholanthrene (MC). The secretion of immunosuppressive molecules by cancer cells was also addressed.

Chapter 2: Fas-Fas ligand-based interactions between tumor cells and tumor specific T lymphocytes

Abstract:

In the current study we investigated the repercussions of the interaction between tumor cells (LSA) and the tumor-specific CTL (PE-9), when both expressed Fas and Fas ligand (FasL). The CTL clone, PE-9, expressed high levels of Fas and FasL upon activation through the TCR. Furthermore, the activated PE-9 cells used both perforin and FasL-based pathways to kill the Fas^+ LSA tumor cells. Interestingly, the LSA tumor cells also constitutively expressed FasL but not perforin, and killed Fas^+ PE-9 CTL as well as Fas^+ but not Fas^- activated T cells and thymocytes, as detected using the JAM test. The PE-9 CTL, cultured for 24 hours in the presence of cell-lysates of FasL-bearing LSA but not FasL-deficient P815, exhibited significant apoptosis as detected using TUNEL method. Moreover, another $FasL^+$ T cell lymphoma line, EL-4, induced apoptosis in Fas^+ but not Fas^- T cells in a similar fashion. The current study demonstrates for the first time that not only can the tumor-specific CTL mediate Fas-based killing of the tumor cells, but also the $FasL^+$ tumor cells can kill the Fas^+ tumor-specific CTL. Thus, the survival of the tumor or the host may depend on which cell can accomplish this task more efficiently. The current study also suggests that FasL-based killing of CTL by specific tumor cells may constitute a major limiting factor in successful immunotherapy.

Introduction:

Recent studies have demonstrated that CTL can kill target cells using two distinct lytic pathways, first, the degranulation pathway which uses perforin possibly in combination

with granzymes and secondly, the Fas-based pathway in which the interaction between Fas ligand (FasL) expressed on the CTL and Fas on target cells, triggers apoptosis and target cell death (Kagi et al., 1996). It is believed that cross-linking of Fas by an appropriate ligand is sufficient to trigger cell death (Itoh et al., 1991; Suda et al., 1993). This raises an interesting question of whether tumor targets that express FasL, can kill a Fas^+ tumor-specific CTL following interaction between these two cells.

FasL is known to be expressed in both membrane-bound or soluble form. Recently, FasL was shown to be expressed by melanomas, hepatocellular carcinomas and colon cancer and furthermore, such cells were found to induce apoptosis in Fas-sensitive but not Fas-resistant lymphoma cells (O'Connell et al., 1996; Hahne et al., 1996; Strand et al., 1996). These data suggested that FasL expression by tumor cells may contribute towards creating immune privileged site and immunosuppression. Despite such recent reports, whether FasL-bearing tumor cells can directly kill tumor-specific CTL has not been previously tested. CTL are relatively more resistant than tumor targets, in their susceptibility to cell-mediated cytotoxicity (Kranz and Eisen, 1987). Also, not all Fas^+ target cells are susceptible to FasL-based killing (Strand et al., 1996). Thus it is necessary to directly test whether FasL-bearing tumor cells can kill tumor-specific CTL.

To this end, we used a T cell lymphoma line, LSA and the tumor-specific CTL clone, PE-9, previously characterized in our laboratory (Hamman-McKibben et al., 1995; Seth et al., 1991). The current study demonstrates for the first time that tumor cells that express FasL can kill the specific effector CTL and other activated T cells that express Fas. Such a mechanism may help the tumor cells evade the specific immune surveillance and induce immunosuppression in the host.

Materials and Methods:

Mice:

Adult female C57BL/6 (+/+) and C57BL/6 *lpr/lpr* mice were purchased from Jackson Laboratories (West Grove, PA) and maintained in our animal facility (Hammond et al., 1993).

Tumor Cell lines:

LSA, is a radiation leukemia virus-induced T cell lymphoma and EL-4 is a chemically induced T cell lymphoma, both syngeneic to C57BL/6 strain. L1210 Fas^+ and L1210 Fas^- cell lines represent murine lymphoma lines derived from DBA/2 strain, transfected with sense and antisense Fas cDNA (Rouvier et al., 1993). AutoD1.4T is a T cell line that underwent spontaneous transformation *in vitro* (Nagarkatti et al., 1994). P815 is a mastocytoma syngeneic to DBA/2 mouse. All tumor cell lines were grown in syngeneic mice or maintained in culture with RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 2 mM glutamine, 50 μM 2-mercaptoethanol, 10 mM Hepes, 1 mM glutamine, 40 μg/ml gentamicin sulfate, and 10% fetal bovine serum (Atlanta Biologicals Norcross, GA).

CTL line:

PE-9, is a $CD8^+$ αβ TCR^+ CTL clone isolated from C57BL/6 mice rejecting the LSA tumor (Hamman-McKibben et al., 1995; Seth et al., 1991). PE-9 CTL mediates antigen-specific killing of LSA tumor cells but not other syngeneic or allogeneic targets (Hamman-McKibben et al., 1995; Seth et al., 1991). The PE-9 cells were maintained in culture by supplementing the medium with 50 U/ml rIL-2 and occasional stimulation with irradiated (2500 rads) or mitomycin-C treated LSA.

Activation of PE-9 T cells through the TCR:

Prior to studying the expression of Fas, FasL or the cytotoxicity, the PE-9 cells were cultured with irradiated LSA tumor cells for 48 hours and the viable cells were purified by centrifugation on ficoll-hypaque (Seth et al., 1991). In addition, in some experiments the PE-9 cells were stimulated with immobilized anti-CD3 or anti-αβ TCR mAbs for 48 hours (Matzinger, 1991).

Antibodies:

Monoclonal anti-Fas (Jo2) and anti-mouse FasL Abs were obtained from Pharmingen (San Diego,CA). FITC-labeled $F(ab')_2$ fragments of anti-hamster IgG and anti-mouse IgG were purchased from Jackson Immunoresearch Lab, Baltimore, MD. The hybridomas, 145.2C11 (anti-CD3 mAb, hamster IgG) and H57-597 (anti-αβTCR, hamster IgG) were grown in our laboratory and purified as described elsewhere (Hammond et al., 1993).

Detection of Fas, Fas ligand, and perforin expression using reverse transcriptase(RT)-PCR:

RT-PCR was carried out to detect membrane-bound Fas, FasL and perforin gene expression in a similar way as previously described (Hammond et al., 1993) . The primers used were as follows: Fas sense primer, 5'-GCACAGAAGGGAAGGAGTAC-3'; Fas antisense primer, 5'GTCTTCAGCAATTC-TCGG GA-3' (amplified fragment, 455 bp); FasL sense primer 5'-GAGAAGGAAA-CCCTTTCCTG-3'; FasL anti-sense primer, 5'- ATATTCCTGGTGCCCATGAT-3' (amplified fragment, 940 bp); perforin sense primer, 5'-GGTCAGAATGCAAGCAGA-AGCACAA-3'; perforin anti-sense primer, 5'-TTGGAGGTGAGGTGGAACTGAAGTT-3' (amplified fragment 499 bp); mouse β–actin sense primer, 5'-ATCCTGACCCTGAACTACCCCATT-3'; β-actin anti-sense primer, 5'-GCACTGTAGTTTCTCTTCGACACGA-3' (amplified fragment, 464 bp). PCR amplification products were visualized in 1.5% agarose gels after staining with ethidium bromide.

Flow cytometric analysis of Fas and FasL:

The activated PE-9 cells, thymocytes or various tumor cells were incubated with antibodies against Fas or FasL, washed, followed by incubation with FITC-conjugated secondary antibody. The cells were analyzed for fluorescence using Coulter Epics V flow cytometer (Seth et al., 1991).

Specific killing using ^{51}Cr-release assay:

^{51}Cr-release assay was performed to study the killing of tumor cells by CTL as described elsewhere. Briefly, tumor targets or LPS blasts (Seth, 1991) were labeled with 100 μCi of ^{51}Cr, by incubating at 37 ^{o}C for 60 min. Various ratios of effector:target cells in triplicate were mixed in 96-well round-bottom plates (Costar, Cambridge, MA) and incubated at 37 ^{o}C for 4 hr. Spontaneous release was measured by incubating ^{51}Cr-labeled cells alone, and total release was determined by incubating target cells with 0.1% sodium dodecyl sulfate. After 4 hr incubation, the supernatants were harvested and radioactivity was measured using a γ-counter (TmAnalytical, Elk Groove Village, IL). The cytotoxicity was calculated as follows: % specific killing = E - S / T - S X 100, where E = experimental release, S = spontaneous release, and T = total release.

Use of concanamycin A to inhibit perforin-based cytotoxicity:

In some assays, concanamycin A was used to study the relative role played by perforin and FasL expressed by T cells, in the cytotoxicity of various targets as described by Kataoka et al. (1996) . The cytotoxicity was performed using ^{51} Cr release assay as described above, except that the effector cells were preincubated with concanamycin A (ICN Pharmaceuticals Inc, Costa Mesa, CA) at a concentration of 100nM, for 2 hours in a final volume of 100 μl in microtiter plates, followed by addition of labeled target cells.

DNA fragmentation and specific killing using JAM test:

JAM test was used to study the ability of tumor cells to kill T cells. DNA fragmentation was assessed by labeling the target T cells with ^{3}H-thymidine as described

by Matzinger (1991) and modified in our laboratory (Kamath et al., 1997). Briefly, target cells consisting of PE-9 cells, con A-activated spleen cells (Hammond-McKibben et al.,1995) or thymocytes, were labeled with 5 μCi/ml ^{3}H-thymidine (ICN, Irvine, CA) by incubating in a humidified atmosphere with 5% CO_2 at 37 ^{o}C for 6 hours. The excess ^{3}H-thymidine was washed off and 5x10^{3} target cells were mixed with varying numbers of effector tumor cells in 96-well plates (Costar, Cambridge, MA). The plates were incubated at 37 ^{o}C for 2.5 hours and the cells were harvested onto glass fiber filters using a semi-automatic cell harvester. Filters were dried and added to liquid scintillation fluid and radioactivity was counted in a β–counter (Tm analytical, Elk Grove Village, IL). The mean percentage of DNA fragmentation was calculated from triplicate culture using the following formula:
% DNA fragmentation = (S-E) / S X 100, where S = retained DNA in the absence of killers (spontaneous) and E = experimentally retained DNA in the presence of killers.

Detection of apoptosis using TUNEL method:

Apoptosis in the CTL clone, PE-9 was detected using terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP, referred to as TdT-mediated nick end labeling or TUNEL technique (Kakkanaiah et al., 1991) which labeling of DNA strand breaks using(Boehringer Mannheim, Indianapolis, IN). The PE-9 cells (1 x10^{6}) were cultured in tissue culture plates with cell lysates of (1x10^{6}) LSA, EL-4 or P815 cells in 2ml medium. Twentyfour hours later, the PE-9 cells were washed twice with medium containing phosphate buffered saline (PBS) and fixed with 4% p-formaldehyde for 30 minutes at room temperature (Kakkanaiah et al., 1991). The cells were next washed with PBS, permeabilized on ice for 2 minutes and incubated with FITC-dUTP for an hour in the incubator. Fluorescence of the cells was measured by flow cytometry as described (Hammond-McKibben et al.,1995). The analysis was performed by a Coulter Epics V flow cytometer. Five thousand cells were analyzed per sample.

Results:

Expression of Fas and FasL by PE-9 CTL and LSA tumor cells:

PE-9 is a CTL clone isolated from C57BL/6 mice rejecting syngeneic LSA tumor. The PE-9 cells are $CD8^+$, $\alpha\beta TCR^+$ and mediate tumor-specific MHC-restricted lysis of LSA (Seth, 1991). To further investigate whether Fas-FasL interactions play a role in the cytotoxicity mediated by PE-9 cells against LSA, we first analyzed this cell line for the expression of Fas and FasL mRNA. The PCR data shown in Fig. 2.1 suggested that PE-9 cells stimulated through the TCR expressed FasL gene (Fig. 2.1A) and perforin (Fig. 2.1B). Interestingly, when tumor cell lines were screened for the expression of these molecules, only LSA and EL-4 but not AutoD1.4T constitutively expressed FasL (Fig. 2.1C). Also, AutoD1.4T cells but not LSA or EL-4, were found to constitutively express the perforin gene (Fig. 2.1C). The TCR-activated PE-9 cells and the LSA tumor cells were also found to express Fas (Fig. 2.1D).

The surface expression of Fas and FasL on PE-9 and LSA cells was further corroborated using flow cytometry. PE-9 cells maintained in culture with irradiated LSA and IL-2 were found to express significant levels of Fas (Fig. 2.2A). The wild-type LSA tumor cells expressed significant but lower levels of Fas (Fig. 2.2B). However, by cloning we were able to isolate a stable cell line that exhibited higher levels of Fas, and was designated, LSA (Fas^+) cell line (Fig. 2.2C).

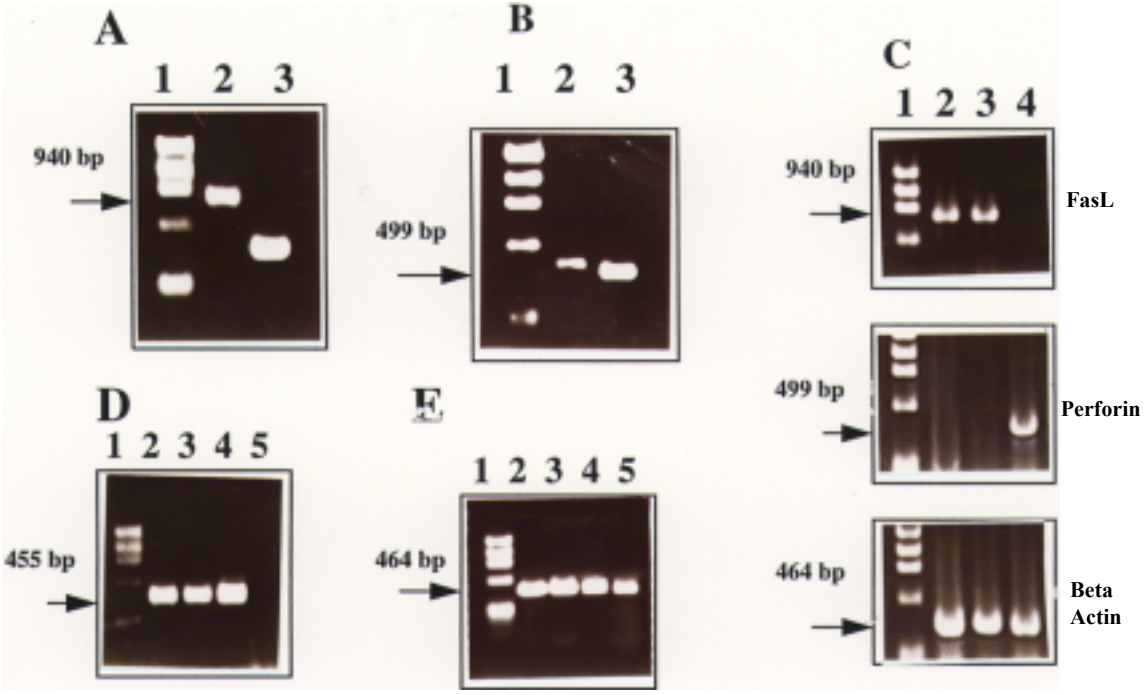


Figure 2.1: Detection of Fas, FasL and perforin mRNA using RT-PCR. PE-9 cells were stimulated with immobilized anti-CD3 mAbs prior to PCR analysis. (A) represents PE-9 cells analyzed for FasL expression with Lane 1 being a molecular marker; lane 2, FasL and lane 3, β-actin as a control. (B) represents PE-9 cells analyzed for perforin expression in which lane 1 represents molecular marker; lane 2, perforin and lane 3, β-actin. (C) depicts FasL and perforin expression by tumor cell lines, in which lane 1 is a molecular marker; lane 2 is LSA tumor; lane 3, EL-4; and lane 4 is Auto D1.4 tumor. (D) depicts expression of Fas in which lane 1 is a molecular marker; lane 2, PE-9 cells; lane 3, LSA; lane 4, C57BL/6 thymocytes and lane 5 is L1210 Fas^- cell line. (E) represents β-actin controls for Fig. 1D with lanes identical to those depicted in D.

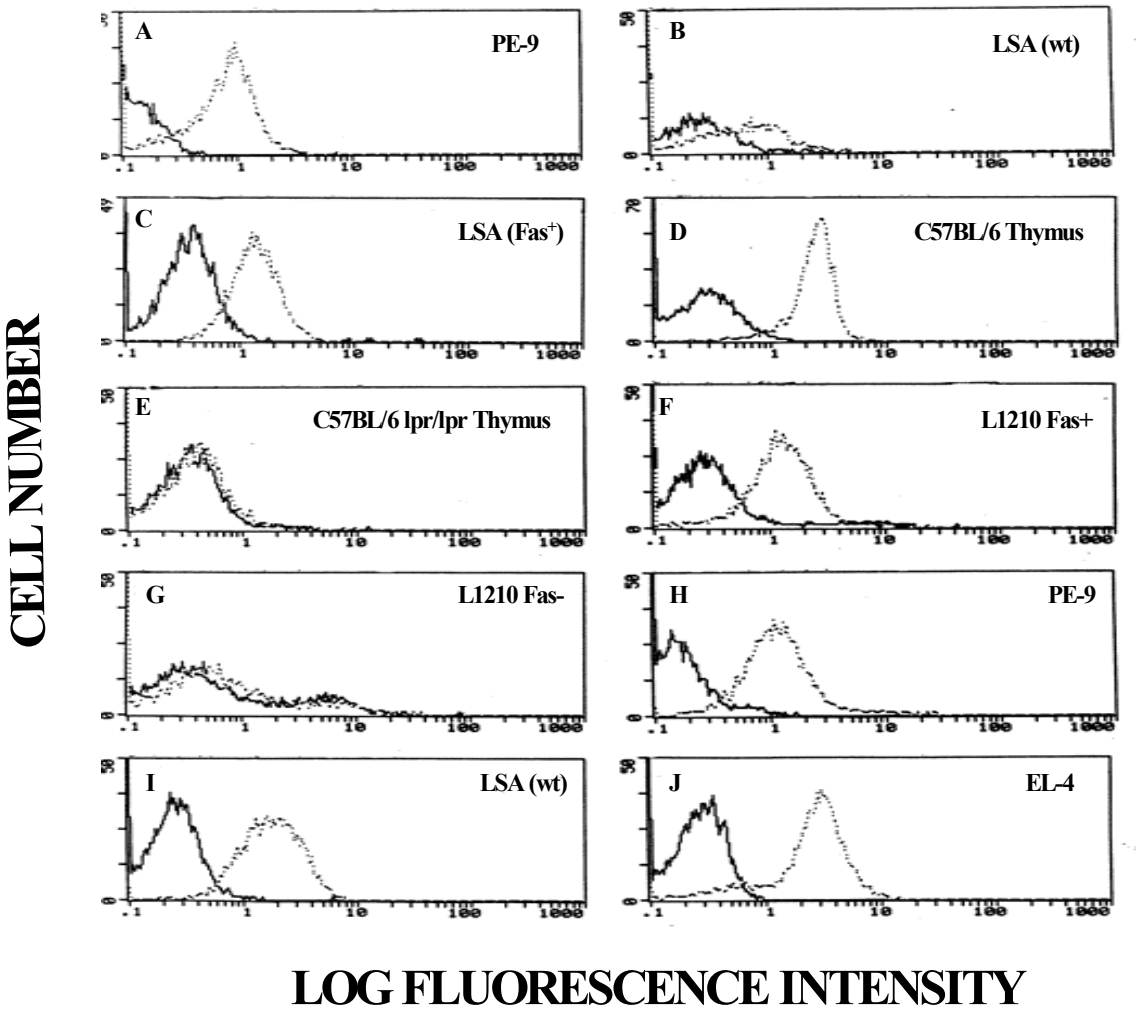


Figure 2.2: Detection of Fas and FasL using flow cytometry. Various cells were stained for expression of Fas or FasL as described in methods. Histograms A through G, represent Fas expression and H through J represent, FasL expression. Bold histograms represent cells incubated with FITC-conjugated secondary Abs only and broken histgrams depict cells stained with anti- Fas or anti-FasL Abs + FITC-conjugated secondary Abs.

The Fas expression by this and the PE-9 cell line, was comparable to that exhibited by thymocytes from C57BL/6 (+/+) mice (Fig. 2.2D) and L1210 Fas^+ cell line (Fig. 2F). Also, as expected, thymocytes from C57BL/6 (lpr/lpr) mice and the L1210 Fas^- cell line failed to exhibit significant levels of Fas (Fig. 2.2E and 2.2G).

When FasL expression was studied flow cytometrically, PE-9 cells demonstrated low levels of expression (data not shown), however, upon activation either using LSA or mAbs against the TCR, they expressed higher levels of FasL (Fig. 2.2H) consistent with recent studies which demonstrated that FasL is upregulated following activation through the TCR (Hammond et al., 1993) In addition, LSA and EL-4 tumor cells were found to express high levels of FasL constitutively (Fig. 2.2I and J). Also, Auto D1.4 T and P815 tumor cells failed to exhibit FasL when analyzed flow cytometrically (data not shown).

PE-9 CTL can mediate FasL-based cytotoxicity:

We next addressed whether PE-9 cells use FasL-dependent cytotoxicity to kill the specific LSA targets. The data shown in Fig 2.3 demonstrated that activated PE-9 CTL mediated increased cytotoxicity of mutant LSA Fas^+ cells that expressed higher levels of Fas when compared to wild-type LSA cells (Fig. 2.3A,B). Furthermore, addition of mAbs against Fas (Jo2) caused significant inhibition in the cytotoxicity of LSA tumor cells (data not shown). When LPS-activated B cell blasts from C57BL/6+/+ and C57BL/6lpr/lpr mice were used as targets, in a redirected cytotoxicity assay, in the presence of mAbs against the αβTCR or anti-CD3 mAbs (Seth et al., 1991), Fas^+ targets were found to be more susceptible to lysis when compared to Fas^- targets. (Fig.2.3 C,D). Furthermore, identical results were obtained when Fas^+ L1210 targets were compared to Fas^- L1210 targets (Fig. 2.3E,F). These data together demonstrated that the PE-9 cells when activated were using FasL-dependent pathway to kill the Fas^+ target cells.

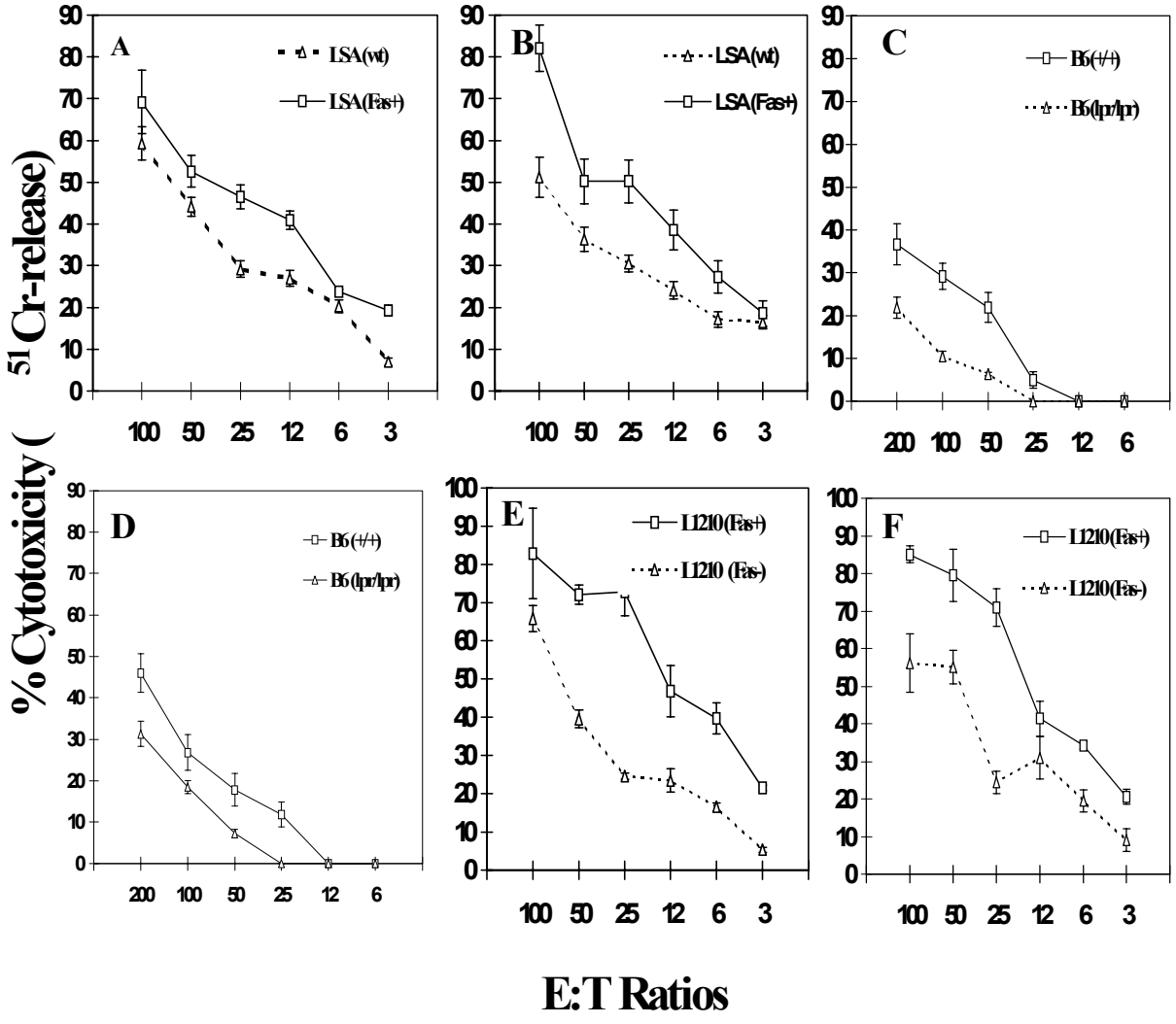


Figure 2.3: Cytotoxicity mediated by PE-9 cells against LSA and other targets: PE-9 cells were tested for their lytic activity against various ^{51}Cr labeled targets. PE-9 CTL were activated with mAbs against the TCR either prior to the cytotoxicity assay or the mAbs against the TCR were added while performing the cytotoxicity assays to facilitate activation of T cells as well as redirected lysis. The mAbs used were against αβTCR in panels A, C and E and against CD3 in panels B, D and F. In A and B, LSA wild type (wt) and a clone expressing higher levels of Fas (LSA Fas$^+$) were used as targets. In C and D, LPS blasts from the spleens of +/+ and lpr/lpr mice were used. In panels E and F, L1210 Fas$^+$ and Fas$^-$ tranfectants were used as targets.

However, the fact that Fas^- targets were also killed by PE-9 cells, suggested that perforin also played a significant role. To further address the relative contributions of perforin and FasL, the ability of PE-9 cells to kill various target cells was studied in the presence of concanamycin A, which has been shown to inhibit perforin-based but not Fas-based cytotoxicity (Kataoka et al., 1996). The cytotoxicity was studied using various targets as described in Fig 2.3. The data shown in Fig 4 indicated that concanamycin A markedly inhibited the cytotoxicity mediated by PE-9 cells against most Fas^- targets, whereas, significant cytotoxicity was still demonstrable against Fas^+ targets. It should be noted that the inhibitory effect of concanamycin A against Fas^- targets was more pronounced at lower E:T ratios consistent with an earlier study (Kataoka et al., 1996). Also, the differences in the susceptibility of Fas^+ and Fas^- targets to PE-9 cell-mediated killing was more striking in the presence of concanamycin A (Fig 2.3 versus Fig 2.4) which together suggested that PE-9 cells were using both Fas-based and perforin-based lytic pathways. It should be noted that several recent studies have demonstrated that in an acute short term cytotoxicity assay, the lysis of target cells can be attributed completely to perforin and FasL (Glass et al., 1996), thereby ruling out the possible involvement of other factors.

LSA tumor cells can kill tumor-specific CTL in a FasL-dependent manner:

Inasmuch as, the LSA tumor cells also expressed FasL, we next investigated whether these tumor cells could kill the tumor specific Fas^+ PE-9 CTL as well as other Fas^+ T cells. To study this cytotoxicity, we used JAM test for several reasons: First, unlike tumor cells, T cells and thymocytes do not label well with ^{51}Cr. Secondly, JAM test is more sensitive and detects DNA fragmentation which occurs before membrane damage (Duke et al., 1983). Moreover, when tumor cells and PE-9 cells were mixed, one would

expect not only the tumor cells to kill the PE-9 cells but also the vice versa. Thus, a sensitive assay in which DNA fragmentation can be detected within 2 hours clearly provided an advantage.

The data depicted in Fig 2.5A demonstrated that LSA and EL-4 tumor cells mediated efficient killing of tumor-specific CTL, PE-9. Furthermore, LSA and EL-4 tumor cells induced DNA fragmentation in +/+ but not in *lpr/lpr* thymocytes (Fig. 2.5B) and Con-A blasts (Fig 2.5C). It should be noted that the LSA and EL-4 cells failed to express perforin (Fig. 2.1) and thereby ruling out the possible involvement of perforin. Also, AutoD1.4T tumor cell line which expressed perforin but not FasL failed to kill any of the T cells screened (data not shown).

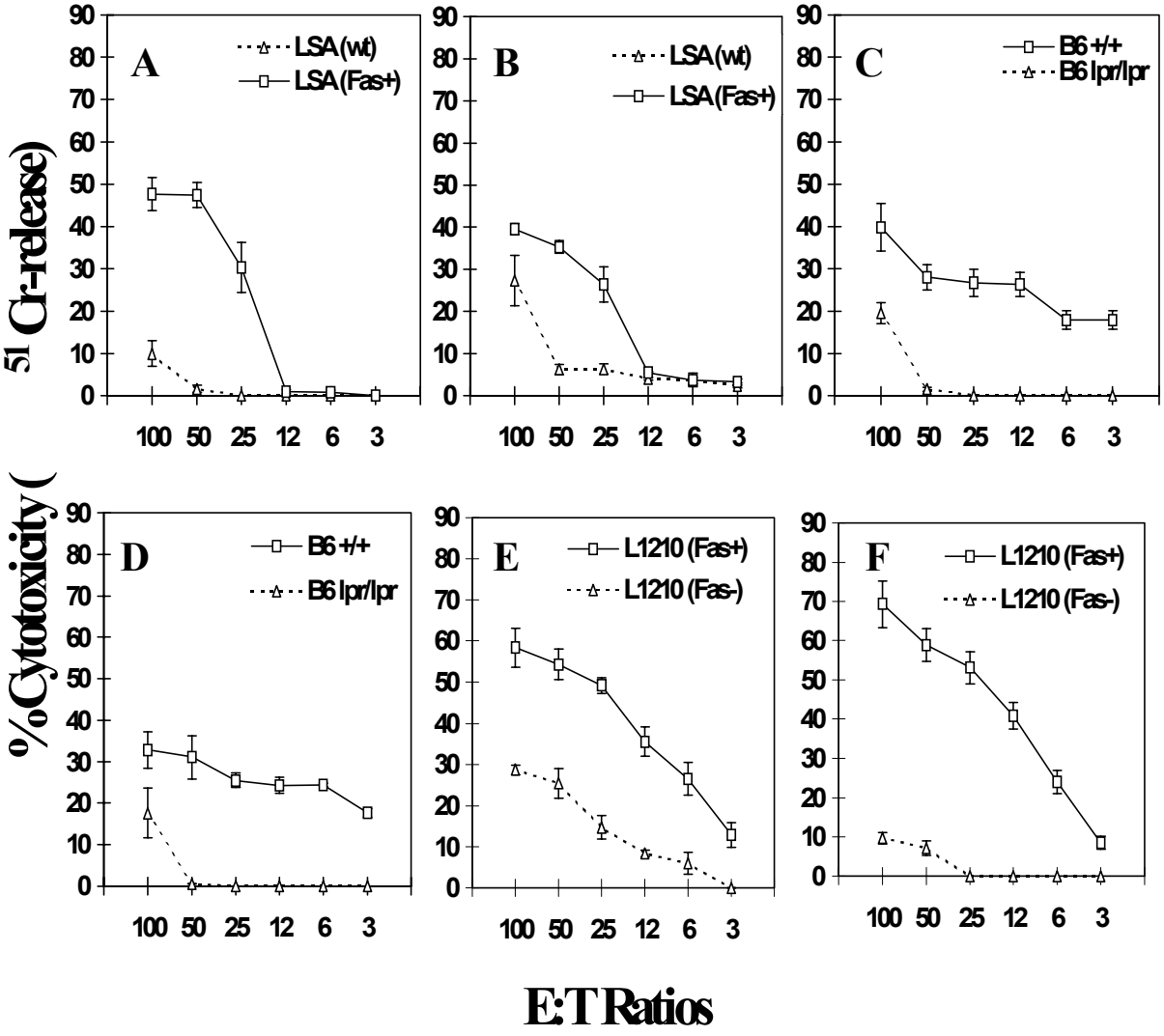


Figure 2.4: Effect of concanamycin A on cytotoxicity mediated by PE-9 cells against Fas^+ and Fas^- targets. PE-9 cells were tested for their lytic activity against various ^{51}Cr labeled targets, exactly as described in Fig 3, except for the addition of concanamycin A, as described in methods. The cytotoxicity assay depicted in panels A-F was carried out in a similar fashion, as described for their respective panels in Fig 2.3

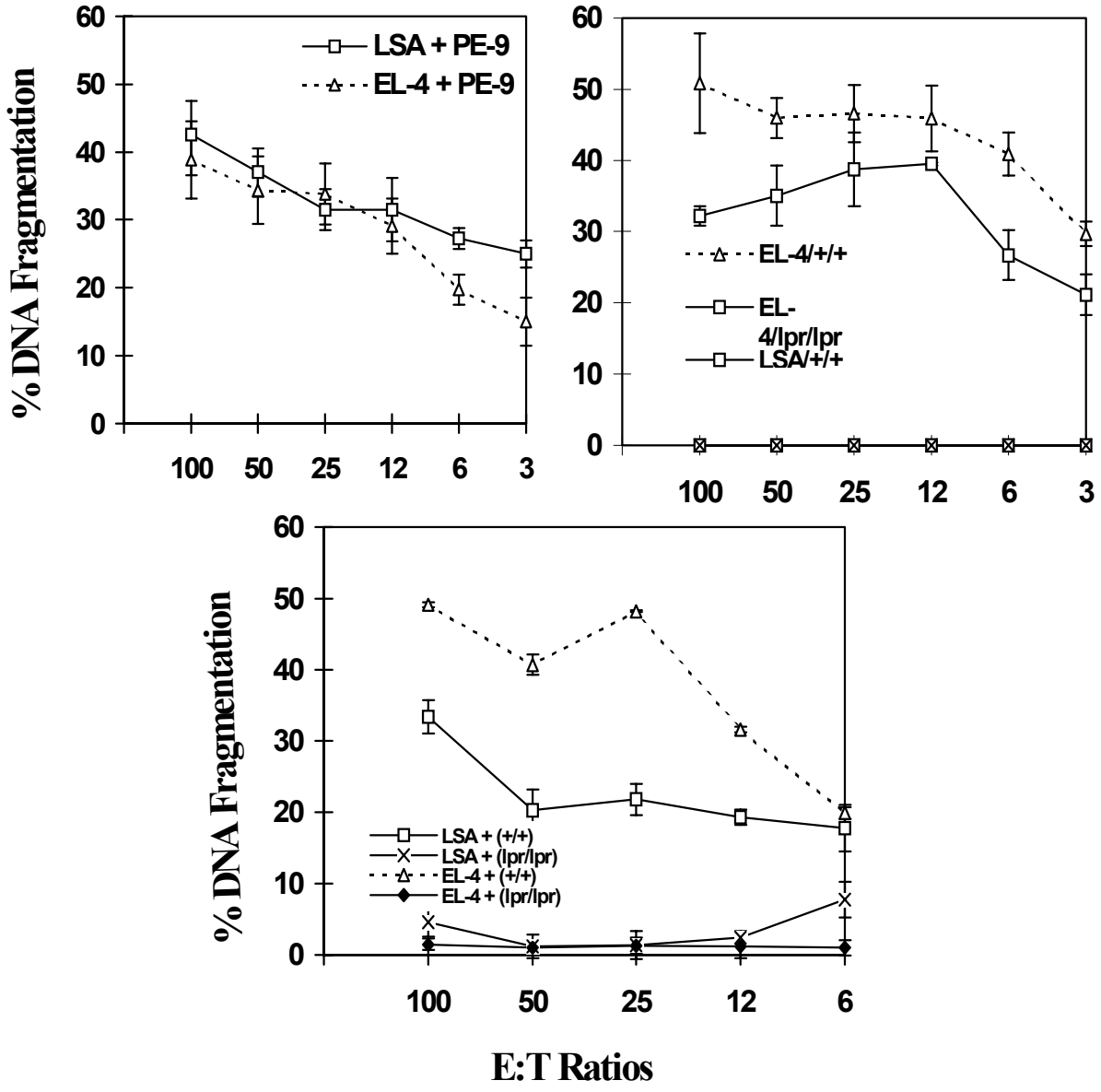


Figure 2.5: LSA and EL-4 tumor cells can mediate DNA fragmentation in Fas^{+} PE-9 and other T cells. PE-9 T cells (A), thymocytes (B) or con A activated T cell blasts (C) were labeled with ^{3}H-thymidine and used as targets to study the ability of LSA and EL-4 tumor cells to mediate cell death.

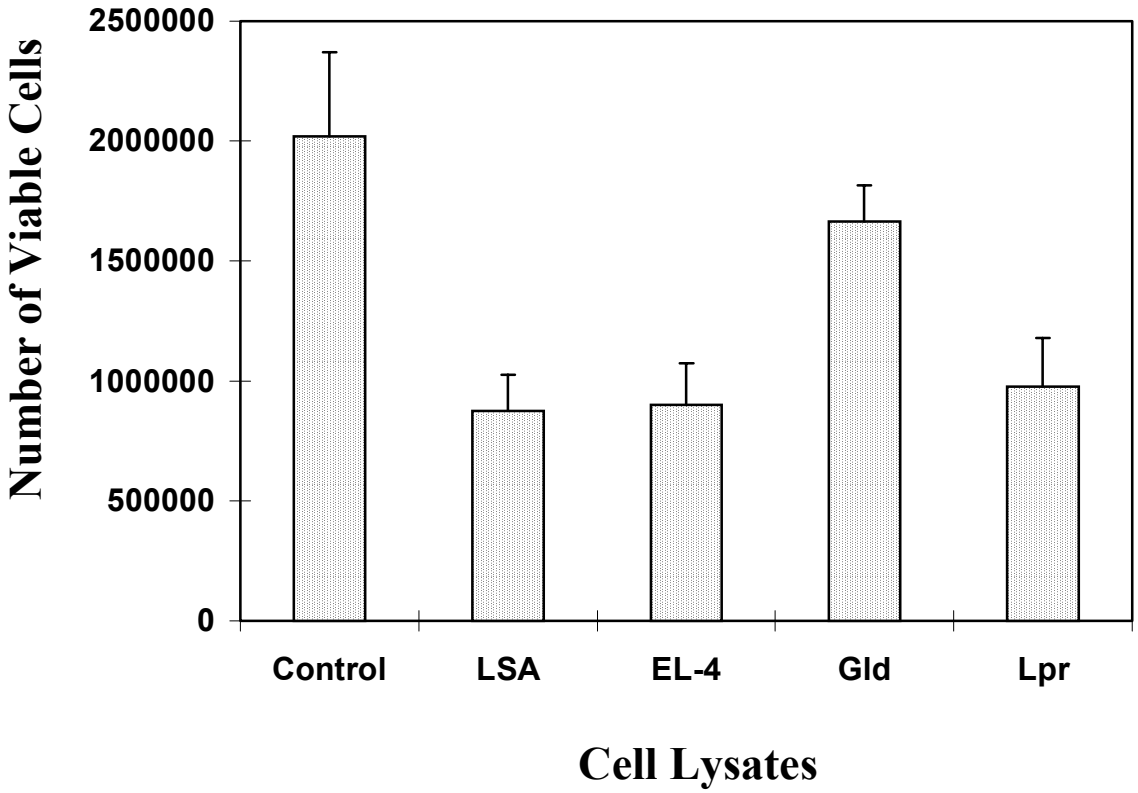


Figure 2.6: PE-9 CTL cultured in the presence of lysates of LSA or EL-4 tumor cells exhibit decreased cell viability. Fas^{+} PE-9 cells were cultured *in vitro* for 24 hours in the presence of medium alone (control), or lysates of LSA, EL-4 or lymph node cells from gld or lpr mice. The cell viability was measured by trypan blue dye exclusion. The vertical bars represent mean viable cell counts in triplicate cultures ± S.D.

Cell lysates of FasL-bearing tumors can induce apoptosis in PE-9 CTL:

Fas^+ PE-9 cells were cultured *in vitro* in the presence of cell lysates from $FasL^+$ LSA or EL-4 for 24 hours, and the cell viability was measured using trypan blue dye exclusion assay. The data shown in Fig. 2.6 suggested that within 24 hours, there was ~50% decrease in number of viable cells when compared to the medium controls. In these studies we used lysates of lymph nodes from 4 month old gld/gld or lpr/lpr mice which have been previously shown to express massive amounts of FasL (Watanabe et al., 1995) However, the FasL from gld/gld mice is functionally defective (Watanabe et al., 1995). As shown in Fig. 2.6, lysates from lpr but not gld cells could induce significant decrease in the viability of PE-9 cells.

To further corroborate that FasL-bearing LSA and EL-4 cells induce apoptosis in PE-9 CTL, the cells cultured in a similar fashion as described above were stained with TdT and FITC-dUTP. The data shown in Fig. 2.7 indicated that PE-9 cells cultured with lysates of LSA or EL-4 induced marked apoptosis (Fig. 2.7C and 2.7D), whereas, similar concentrations of lysates from FasL-deficient P815 tumor cells failed to induce significant apoptosis (Fig. 2.7B). In these experiments thymocytes subjected to radiation (2000rads) and cultured for 24 hours *in vitro* served as a positive control (Fig. 2.7A).

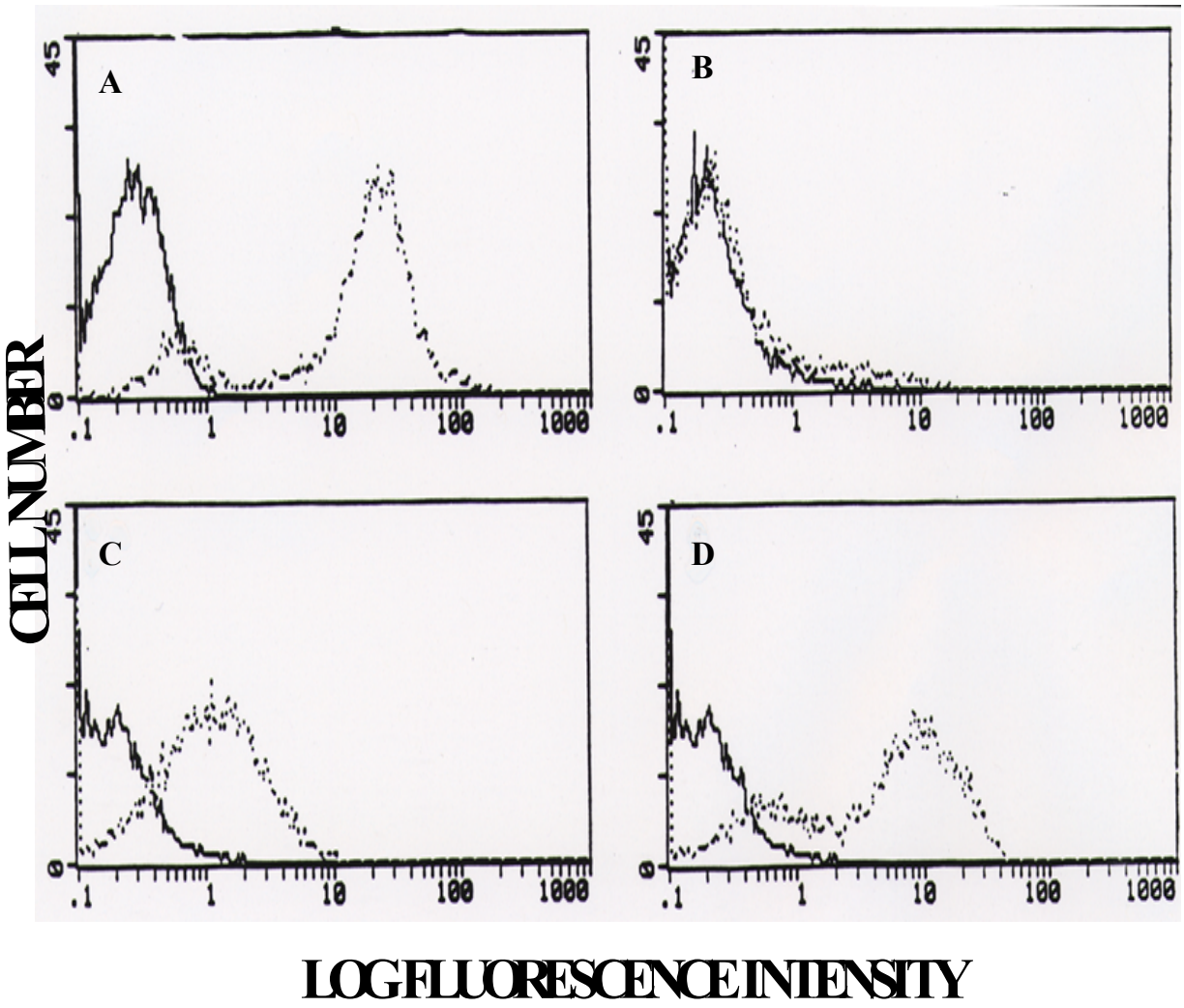


Figure 2.7: PE-9 CTL cultured with lysates of LSA or EL-4 cells exhibit apoptosis. The PE-9 cells were cultured in the presence of cell lysates as described in Fig. 2.5. The PE-9 cells were stained with TdT + FITC-dUTP and analyzed using a flow cytometer. (A) represents irradiated thymocytes as a positive control for apoptosis. PE-9 cells cultured with lysates from FasL-deficient P815 (B), or $FasL^{+}$ LSA (C) or EL-4 (D) have been depicted. The bold histograms represent negative controls consisting of cells cultured in the presence of medium and broken histograms show the cells irradiated (A) or cultured with cell lysates (B, C and D).

Discussion:

In the current study we demonstrated using a tumor cell line and a tumor-specific CTL clone that when both the effector and the target cells express Fas and FasL, they can induce apoptosis in each other. The ability of $FasL^+$ tumor cells to kill tumor-specific CTL is particularly interesting and suggests that such tumor cells may evade the action of the CTL by their ability to induce apoptosis in CTL. Such a mechanism may explain why immunotherapy with tumor-specific CTL clones may fail to eradicate the tumor. This is consistent with our earlier observation that PE-9 cells can afford protection only in 50% of the LSA tumor-bearing mice and that IL-2 administration does not further improve the efficacy (Hammond-McKibben et al., 1995).

FasL was originally thought to be expressed only on CTL and NK cells. However, recent studies demonstrated that FasL may be expressed by immunologically privileged tissues such as stroma cells of the eye and Sertoli cells of the testis (Bellgrau et al., 1995; Lau et al., 1996; Griffith et al., 1995). Furthermore, a variety of cancers such as large granular lymphocytic leukemias, colon cancer, hepatocellular carcinomas and melanomas were found to express FasL (Nagata, 1996). Such FasL-bearing tumor cells have been shown to kill other tumor target cells bearing Fas (O'Connell et al., 1996; Hahne et al., 1996; Strand et al., 1996), thereby suggesting that FasL is functional in cancer cells and that such a mechanism can explain how some tumor cells can evade immune attack by CTL and NK cells. The data from the present study provides direct evidence to this effect.

In the current study, we noted that not only LSA but also a nonspecific tumor such EL-4 could kill PE-9 cells consistent with the observation that ligation of Fas is enough

to trigger apoptosis (Itoh et al., 1991; Suda et al., 1993). The ability of EL-4 to kill PE-9 cells or those of LSA and EL-4 cells to kill a variety of non-specific Fas^+ targets, can be explained by the fact that the effector and target cells may be brought in contact with each other during the *in vitro* cytolytic assay thereby facilitating Fas-FasL interactions. Alternatively, the tumor cells produced soluble FasL that triggered cell death. However, it is important to note that the killing of PE-9 cells by LSA, can occur naturally, following antigen-specific interaction between PE-9 and LSA cells.

Several studies have shown that engagement of Fas by its ligand is sufficient to trigger cell death (Itoh et al., 1991; Suda et al., 1993). If this is true, culture of a cell line that expresses both Fas and FasL should trigger cell death. This may indeed explain why *in vitro* cultured LSA tumor cells rapidly die after attaining higher cell densities. On the other hand, Fas-FasL interactions may not always be sufficient to induce cell death (Strand et al., 1996; Glass et al., 1996). For example, in an earlier study, $FasL^+$ EL-4 cells failed to kill Fas^+ target cells (Glass et al., 1996). The reason for the discrepancy of this finding with the current study is not clear. The authors speculated that the EL-4 cell line used in their study may have expressed a mutant or nonfunctional form of FasL, or alternatively may lack an accessory molecule critical for the induction of lysis. Other molecules by which tumor cells escape from self-inflicted injury may include acquisition of an intracellular mechanism that renders the cells resistant to Fas-induced apoptosis (Nagata, 1996).

Some types of tumor have also been shown to produce soluble FasL. A clinical case of a nasal lymphoma was accompanied by high serum levels of soluble FasL (Sato et al., 1996). These levels correlated with marked liver damage and pancytopenia. Such data suggested that FasL expression by tumor cells may also lead to systemic toxicity and multiorgan failure often seen in cancer patients.

In summary, the current study demonstrates that when tumor cells and the antigen-specific CTL express both Fas and FasL, there can be a bidirectional interaction and killing in both cell types. The outcome may depend on the stage of activation of cells and degree of expression of signaling molecules and cytolytic factors. On the other hand, this may constitute an important mechanism by which tumor cells may evade the destroying forces of the host's immune system and may explain why LSA-tumor bearing mice fail to exhibit tumor-specific CTL activity and do so only following chemotherapy which eliminates majority of the tumor cells (Nagarkatti and Kaplan, 1985; Nagarkatti et al., 1989). Expression of FasL by tumor cells may constitute a major obstacle for successful immunotherapy using tumor-specific CTL

Chapter 3: The role of Fas in the rejection of tumor cells: The in vivo response generated by Cytotoxic T lymphocytes (CTLs) and Natural Killer (NK) cells

Abstract:

In the current study, we investigated whether the naive, poly I:C or IL-2-induced NK/LAK cells use perforin and/or Fas ligand (FasL) to mediated cytotoxicity. We correlated these findings with the ability of mice to reject syngeneic Fas^+ and Fas^- tumor cells either spontaneously or following IL-2 treatment. The spontaneous NK cell-mediated cytotoxicity was primarily perforin-based, whereas, the poly I:C and IL-2 induced NK/LAK activity was both FasL- and perforin-dependent. L1210 Fas^+ tumor targets were more sensitive than L1210 Fas^- targets, to poly I:C and IL-2-induced cytotoxicity in wild-type, *gld/gld*, and perforin knockout (KO) mice. When L1210 Fas^+ and Fas^- tumor cells were injected subcutaneously (s.c.) or intraperitoneally (i.p.) into syngeneic mice, Fas^- tumor cells caused mortality earlier than Fas^+ tumor cells. Also, ~20% of the mice injected s.c. with L1210 Fas^+ tumor cells, survived the challenge(>60 days), while all mice injected similarly with L1210 Fas^- tumor cells, died. When immunotherapy using IL-2 (10,000 U, three times/day for a week, followed by once/day for an additional week) was attempted in mice injected subcutaneously with tumor cells, IL-2 treatment was very effective against mice bearing L1210 Fas^+ (40% survival) but not L1210 Fas^- (0% survival) tumor. These data correlated with the finding that the LAK cells from IL-2 injected mice caused increased cytotoxicity against L1210 Fas^+ when compared to L1210 Fas^- targets. Also, L1210 Fas^+ tumor-bearing mice exhibited

increased tumor-specific CTL activity when compared to those bearing L1210 Fas^- tumor cells. Together our studies demonstrate for the first time that expression of Fas on tumor targets makes them more immunogenic as well as susceptible to CTL and IL-2 induced LAK activity. The Fas^+ tumor cells are also more responsive to immunotherapy with IL-2.

Introduction:

NK cells comprise an important component of the immune system involved mainly in surveillance against cancer and viral infections (Trincheri, 1989). NK cells exhibit spontaneous cytolytic activity against certain tumor cells and virally infected target cells in an MHC-unrestricted manner. In addition, NK cells can be activated by binding of Abs through CD16 receptor to mediate lysis of target cells called Ab-dependent cell-mediated cytotoxicity. The NK cells can also be activated by IL-2 and IFN-γ inducers such as polyinosonic-polycytdylic acid (poly I:C), to mediate increased lysis of NK-sensitive targets as well as kill a broader panel of target cells, including NK-resistant cells (Trinchieri, 1989; Oehler and Herberman, 1978). Such IL-2 induced cytolytic property exhibited by NK and T cells has been designated , lymphokine-activated killer (LAK) cell activity (Grimm et al., 1983).

Recent studies have demonstrated that cytotoxic T lymphocytes (CTLs) and NK cells can kill target cells using two distinct lytic pathways. First, the degranulation pathway which uses perforin possibly in combination with granzymes (Podack et al., 1991; Smyth and Trapani, 1995) and secondly, the Fas-based pathway in which the interaction

between Fas-Ligand (FasL) expressed on cytolytic lymphocytes and Fas on target cells, triggers apoptosis and target cell death (Kagi et al., 1996; Kojima et al., 1994). Moreover, all cytotoxic activity measured in a 4 hour assay can be attributed to perforin and FasL (Glass et al., 1996; Lee et al., 1984).

Although NK cells have been known to mediate increased lysis of target cells including those that are NK-resistant when activated with IL-2 or IFN-γ inducers *in vivo,* it is not clear whether such lytic activity results from upregulation of perforin and/or FasL. Inasmuch as, IL-2 is used to activate LAK cells in the immunotherapy of certain types of cancer (Mule et al., 1984), it is important to investigate whether such treatment augments both perforin and FasL-based pathways and therefore whether it is effective against both Fas^+ and Fas^- tumors.

Although the role played by FasL in the cytotoxicity mediated by CTL and NK cells is well established, the outcome of expression of Fas on the tumor cells and its ability to trigger the anti-tumor immunity in the host is not clear. In the current study, we used perforin-deficient and FasL-defective mice to address the role of perforin and FasL in LAK cell-mediated cytotoxicity as well as used Fas^+ and Fas^- L1210 tumor cells to delineate the role played by Fas in tumor-growth and induction of anti-tumor immunity. The data demonstrated that IL-2 and Poly I:C, triggered the induction of both FasL- and perforin-based cytolytic activity. Also, the survival rate in mice injected with Fas^+ L1210 tumor cells was better than that seen in mice receiving Fas^- L1210 cells, due to the fact that the Fas^+ tumor cells induced stronger NK/LAK and tumor-specific CTL activity, when compared to Fas^- tumor cells.

Materials and Methods:

Mice:

C57BL/6 +/+ (+/+) and DBA/2 mice were purchased from Charles River (Boston, MA). C57BL/6 *gld/gld* (*gld*) mice were purchased from Jackson Labs (Bar Harbor, ME) and perforin KO mice were generously provided by Dr. W.R. Clark (University of California). The perforin KO and *gld* mice were of C57BL/6 origin. The *gld* and perforin KO mice were bred in our facilities (Nagarkatti et al., 1988). All mice used in the current study were female and were 3-4 weeks old.

Cell Lines:

The tumor cell lines used were as follows: YAC-1, a Moloney virus induced lymphoma sensitive to NK cells; P815, a mastocytoma resistant to NK cells; L1210 (Fas^+ and Fas^-), an NK resistant DBA/2 derived mouse lymphoma transfected with sense and antisense Fas cDNA (Hammond et al., 1993). All cell lines were maintained in tissue culture medium, RPMI-1640 (GIBCO , Grand Island, NY) supplemented by 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2mM glutamine, 50 μM 2-mercaptoethanol, 10mM HEPES, 1mM glutamine and 40 μg/ml gentamycin, as previously described (Nagarkatti et al., 1989).

In vitro growth characteristics of L1210 Fas^+ and L1210 Fas^- tumor cells:

L1210 Fas^+ and L1210 Fas^- tumor cells were cultured in tissue culture flasks at a concetration $2x10^5$ cells/ml. At 12, 24, 36, 48, 60, and 72 hours following *in vitro* culture, the viable cell count was carried out using trypan blue dye exclusion.

Flow cytometric analysis of Fas:

The tumor cell lines were screened for the expression of Fas by incubating the cells with normal mouse serum initially to block the Fc receptors. Next, the cells were washed and incubated with anti-Fas mAbs (Jo2, Pharmingen, San Diego, CA) in cold for 30 min. The cells were washed and stained with FITC-conjugated anti-hamster IgG [$F(ab')_2$]. The negative controls were stained with normal isotype matched antibody and the FITC-conjugated secondary antibody. The cells were analyzed using a flow cytometer (Epics V, Model 752; Coulter Corp, Miami, FL).

LAK/NK Cells:

NK cells were purified as previously described (Nagarkatti and Kaplan, 1985). Briefly, single cell suspensions of the spleen were prepared in RPMI-1640 medium supplemented with 5% fetal calf serum (Zeytun et al., 1997) using a homogenizer (Stomacher, Tekmar Co., Cincinnati, OH). Plastic adherence for one hour at 37 ^{o} C, was used to deplete macrophages. The cells were then passed over nylon wool columns and the nonadherent cells used as a source of spontaneous NK activity. To study the

inducible NK activity, the nonadherent cells were cultured for approximately 48 hours with 1000 U/ml of IL-2 (kindly provided by Hoffman LaRoche, Nutley, NJ). In some experiments, NK activity was induced by intraperitoneal injection of poly I:C (Sigma Chemical Company, St. Louis, MO), at 15μg/kg suspended in PBS (Oehler et al., 1978). In other experiments, NK cells were activated *in vivo* by i. p. injection of 10,000U of IL-2 three times a day for 5 days.

Isolation of Tumor-specific CTL:

DBA/2 mice were injected with 1 x 10^6 L1210 Fas^+ or L1210 Fas^- live tumor cells in 0.2ml PBS subcutaneously. On day 5, these mice were injected with 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), an anti-cancer drug, at a concentration of 20 mg/kg body weight as described, so as to enhance the tumor-specific immune response (Lee et sl., 1996; Yagita et al., 1995). Five days later, the mice were sacrificed, T cells were purified and $5x10^6$ cells were cultured in 24 well tissue culture plates with irradiated(2000 R) L1210 Fas^+ or L1210 Fas^- tumor cells ($5x10^3$)in 2 ml medium. After 5 days, the cells were harvested and viable cells were purified on Ficoll-Hypaque density gradient centrifugation. The cells were tested for cytotoxicity against ^{51}Cr-labeled L1210 Fas^+ or L1210 Fas^- tumor cells. The specificity of the CTL was confirmed by testing the cytotoxicity against YAC-1 and P815 targets.

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

The cytotoxicity mediated by NK cells was studied using ^{51}Cr-release assay (Nagarkatti et al., 1989). Target cells (YAC-1, P815, L1210 Fas^+, and L1210 Fas^-) were

labeled with 100 μCi ^{51}Cr in the form of $Na_2{}^{51}CrO_4$, incubated at 37° C for one hour, washed three times, then seeded in 96-well plates (Costar, Cambridge, MA) at 5 x 10^3 cells/well along with varying numbers of effector cells. The plates were incubated at 37 °C for 4 hours. Following the incubation, the plates were harvested with the Titertech collecting system (Skatron Inc., Sterling, VA). The amount of ^{51}Cr released by the target cells was measured using a γ-counter (TmAnalytic, Elk Grove Village, IL). Percent cytotoxicity was calculated as: (experimental release - control release)/(total release - control release) x 100. In these experiments, the control release was measured in the presence of target cells alone, which was usually less than 15%. Total release was measured by incubating target cells in the presence of 0.1% sodium dodecyl sulfate.

In blocking studies, the assays were performed in the presence of 2ug/ml of anti-Fas mAbs (Jo2, Pharmingen, San Diego, CA) or 100 nmol/L of concanamycin A (ICN Pharmaceuticals Inc., Costa Mesa, CA) (Oshimi et al., 1996). In these experiments, the control release was measured both in the presence or absence of the inhibitors to ensure that the Abs or reagents alone did not alter control release.

In vivo growth characteristic of L1210 tumor cells and immunotherapy:

Groups of five DBA/2 mice were injected subcutaneously (s.c.) or intraperitoneally (i.p.) with either 1 x 10^6 L1210 Fas^+ or L1210 Fas^- tumor cells suspended in 0.2 ml PBS. These mice were injected i.p. with 10,000 U IL-2 (kindly provided by Hoffman-LaRoche) suspended in 0.1 ml PBS, 3 times/day for 7 days, followed by once a day, for an additional 7 days. The control mice received PBS in a similar fashion. The mice were observed for tumor growth and survival, for 60 days, after which the experiment

was terminated. These experiments were repeated with consistent results. The mean survival time (MST) was calculated for each group and compared statistically using t-test. The MST for mice which were alive on day 60, was considered to be 60.

Results:

It should be noted that part of data presented was generated by Mike Bradley who coautored this work published in blood. These include data shown in Figure 3.1, A and 5. These figures have been included to maintain the continuity of the results presented.

Spontaneous, poly I:C- activated and IL-2- activated NK/LAK activity in wild-type, perforin deficient and FasL-defective mice:

To study the role of perforin and FasL in NK/LAK cell activity, we used wild type, perforin-deficient or FasL-defective (*gld/gld*) mice. Also, to investigate the effect of activation, the spontaneous NK cell activity was compared to that seen following *in vitro* culture of cells with IL-2 or following *in vivo* administration of poly I:C. maximum cytolytic activity against NK sensitive YAC-1 targets in all three groups of mice, followed by poly I:C-activated and spontaneous lytic activity. Furthermore, FasL-defective mice showed a similar degree of spontaneous lysis when compared to the wild-type mice. However, perforin-deficient mice exhibited virtually no spontaneous cytolytic activity.

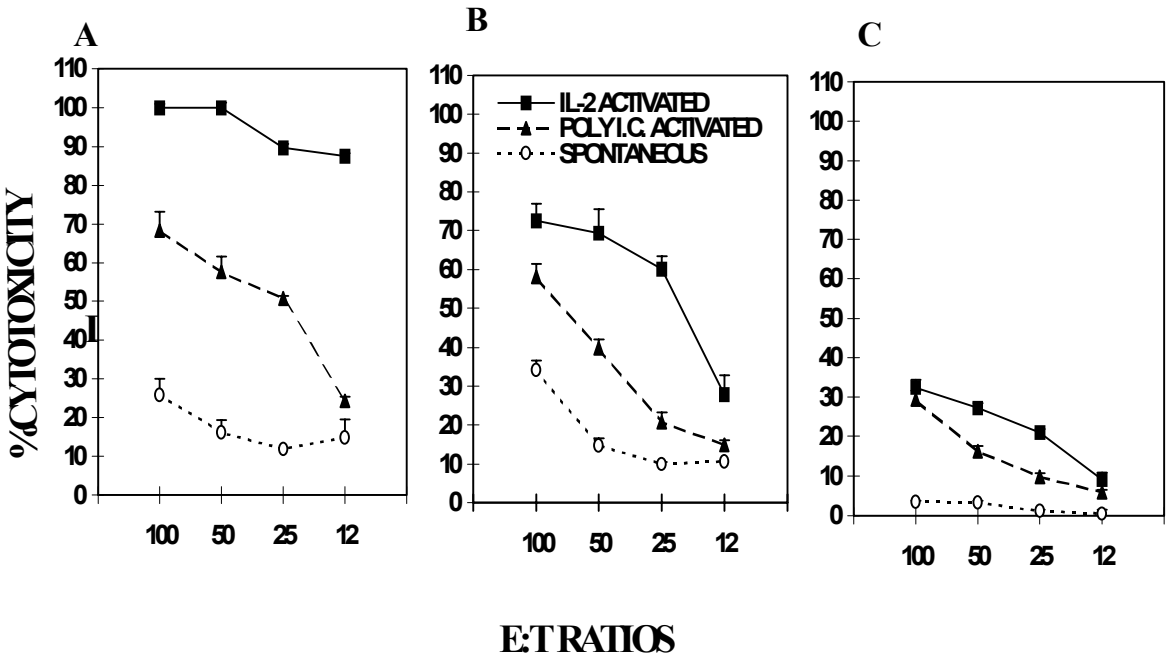


Figure 3.1: Comparison of spontaneous, poly I:C, and IL-2 induced NK/LAK cell activity in C57BL/6 wild-type, *gld/gld*, and perforin KO mice. NK/LAK cells from wild-type (A), *gld/gld* (B), and perforin KO (C) mice were tested for cytotoxicity against NK-sensitive YAC-1 tumor targets. Same symbols were used in panel A, B, and C to depict different types of cytotoxicity. The cytotoxicity was studied using ^{51}Cr-release assay and the mean % cytotoxicity ± S.E.M of triplicate culture were plotted.

The data shown in Fig. 3.1(A,B,C) demonstrated that IL-2 activated NK cells showed. It should be noted that YAC-1 tumor cells were Fas^+ (Fig. 3.2). These data demonstrated that the spontaneous NK activity was primarily perforin-based. The fact that *gld/gld* mice had similar levels of spontaneous NK activity as the wild-type mice, suggested that FasL was not critical during spontaneous lysis.

When poly I:C and *in vitro* IL-2 activated NK/LAK cell activity were compared in the 3 groups of mice, it was noted that the wild-type mice exhibited the highest level of cytotoxicity followed by the *gld/gld* mice and perforin-deficient mice. The poly I:C - induced NK activity was comparable between wild-type and *gld/gld* mice, whereas, it was markedly reduced in perforin-deficient mice. These data suggested that in poly I:C-induced NK activity , perforin played a more important role than FasL. In contrast, the IL-2 induced NK activity was based both on FasL and perforin, inasmuch as, both perforin-deficient and *gld/gld* mice exhibited significant cytotoxicity and the wild-type mice showed the highest level of cytotoxicity. However, even in IL-2 induced NK activity, perforin played a more important role because perforin-KO mice had much lower level of cytotoxicity than *gld/gld* mice.

Further studies were conducted to investigate the role of perforin and FasL in cytotoxicity against NK-resistant P815 tumor cells as targets, which were also found to be Fas^+ (Fig. 3.2). The results shown in Fig. 3.3 suggested that P815 tumor targets were resistant to spontaneous and poly I:C-induced cytotoxicity. However, *in vitro* IL-2 activated LAK cells were able to kill P815 cells in all 3 groups of mice tested.

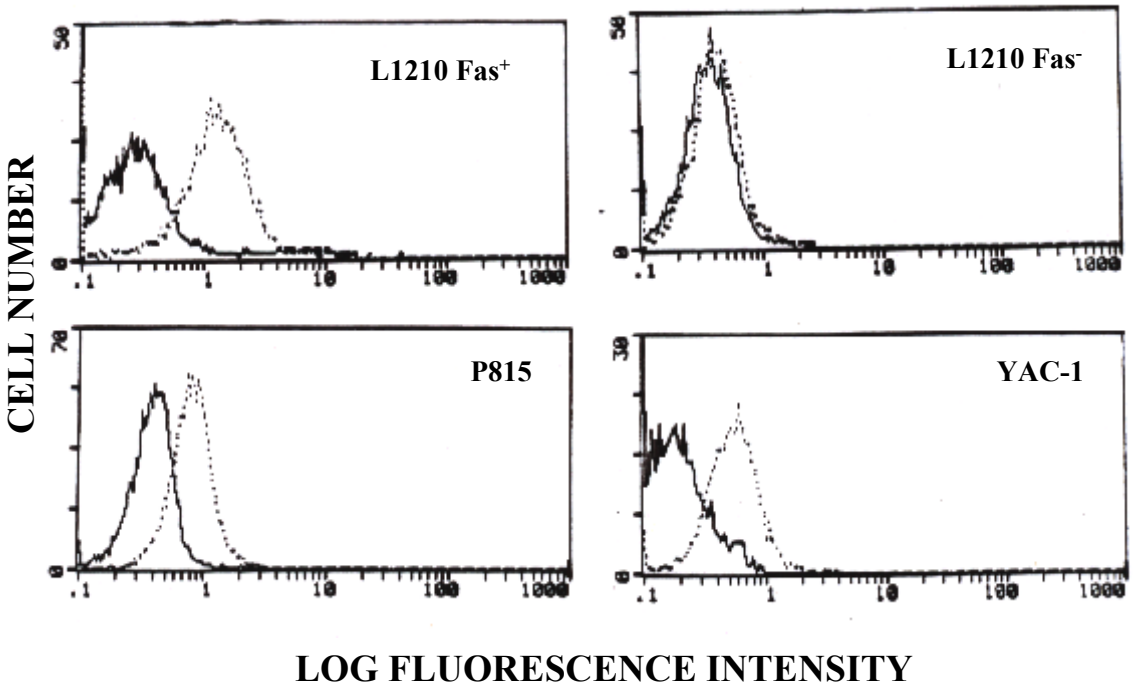


Figure 3.2: Flow cytometric analysis of tumor targets for the expression of Fas. Tumor cells lines were stained with isotype control (bold histograms) or anti-Fas mAbs (broken histograms), followed by FITC-conjugated secondary antibodies and analyzed flow cytometrically.

Moreover, in such IL-2 induced cytolytic activity, both perforin and FasL played a significant role, inasmuch as, both perforin-deficient (Figure 3.3 C) and FasL-defective (Figure 3.3 B) mice exhibited cytolytic activity against P815. However, perforin-KO mice expressed lower levels of cytotoxicity than the *gld/gld* mice, thereby suggesting that P815 cells were more sensitive to perforin than FasL.

To further address the role of FasL and perforin in the killing of P815 tumor targets by IL-2 activated LAK cells, the cytotoxicity was measured in the presence of concanamycin A which in known to inhibit perforin-based but not FasL-based cytotoxicity (Oshimi et al., 1996; Arase et al., 1995) and anti-Fas mAbs (Jo2) known to inhibit FasL-based cytotoxicity. The data shown in Fig 3.3 (D, E, F) indicated that anti-Fas antibody caused significant inhibition in the lysis of P815 targets by LAK cells from wild-type and perforin-deficient mice but failed to inhibit the lysis mediated by LAK cells from *gld/gld* mice. Moreover, concanamycin A inhibited the LAK activity from wild-type and *gld/gld* mice but not from perforin-deficient mice. Together, these data corroborated the observation that the lysis of P815 target cells by LAK cells was both perforin and FasL based, although perforin played a more important role than FasL.

To ensure that the IL-2 induced cytolytic activity was primarily dependent on LAK cells (T cells + NK cells) but not macrophages, the cells were analyzed for CD3, NK1.1 and Mac-3 in all 3 strains of mice. The data shown in Table 3.1 indicated that wild-type, *gld/gld* and perforin-deficient mice had similar level of T cells and NK cells before and after IL-2 activation. Also, such cultures were devoid of macrophages.

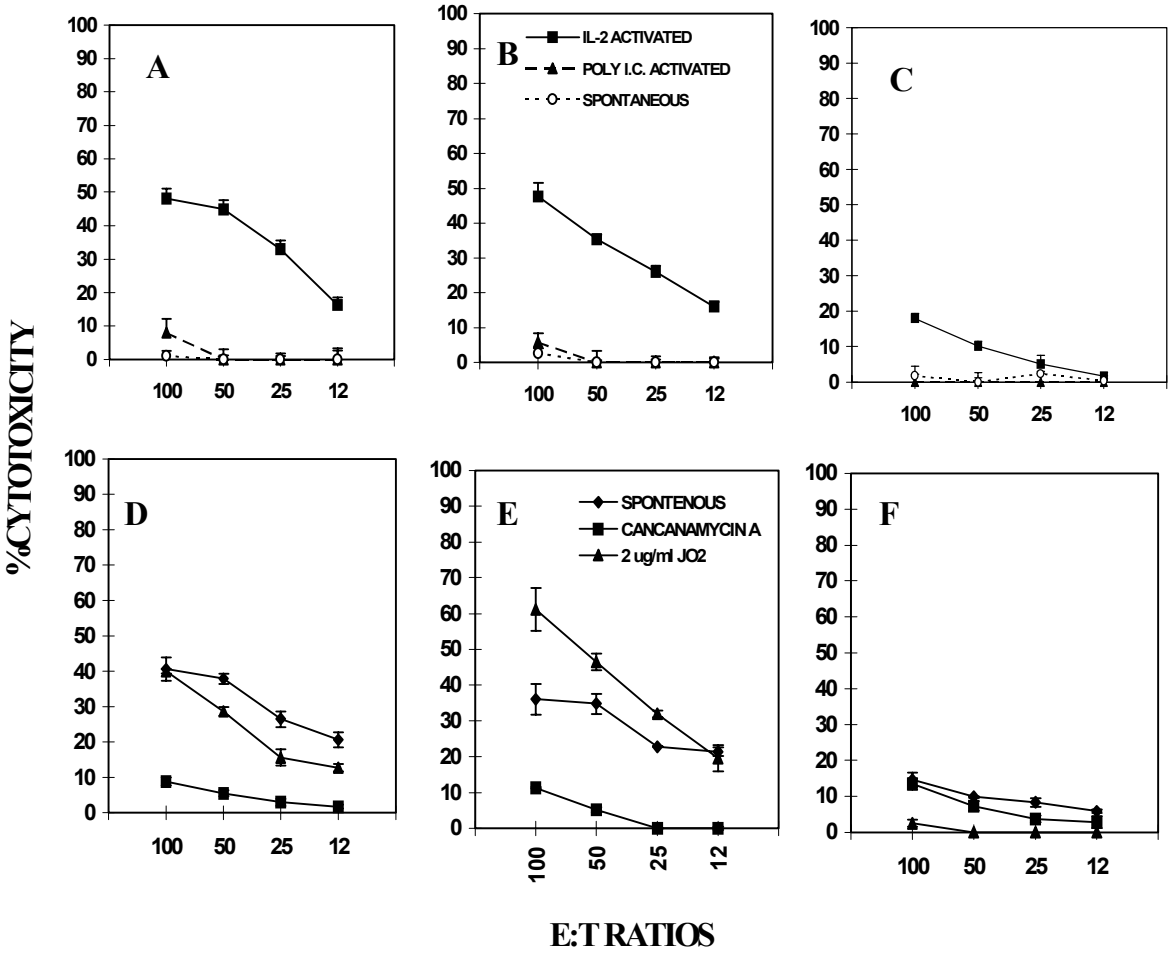


Figure 3.3: Spontaneous and induced NK/LAK activity against P815 tumor targets. NK cells from wild-type (A, D), *gld/gld* (B, E), and perforin KO (C, F) mice were tested for cytotoxicity against NK-resistant P815 tumor targets as described in Figure 1. In panels D, E and F in vitro IL-2 activated LAK cells were used as effectors and cytotoxicity was performed in the presense of mediun, concanamycin A (100 nmol/L) or anti-Fas mAbs (2ug/ml). The symbols used in panel B are common to panel A and C. Also, symbols shown in panel E are similar to those used in panel D and F.

Table 3.1Expression of NK1.1, MAC-3 and CD3 in the Spleen cells after IL-2 stimulation[a]

	Medium			IL-2		
Strain	**CD3**	**NK1.1**	**MAC-3**	**CD3**	**NK1.1**	**MAC-3**
Wild-type	76.85±2.85	14.75±2.25	0.2±0	79.35±6.35	11.75±3.25	1.5±1.2
PD	83.7±10.8	9.05±0.15	0.9±0.5	85.15±5.05	6.1±2.1	0.65±0.35
GLD	83.15±8.05	13.9±4.3	0.75±0.35	85.85±2.35	15.2±1.1	0.35±0.25

[a] Splenic nylon wool purified T cells from wild-type, perforin-deficient (PD) or *gld* mice were cultured with medium or IL-2 *in vitro* and stained for NK1.1, MAC-3 and CD3 markers and analyzed flow cytometrically. The data are depicted as mean percent positive cells±S.E.M. Data is representative of three experiments. There was statistically no significant difference between various groups.

The data shown in Table 3.1 also indicated that perforin-deficient and *gld/gld* mice had similar proportion of T and NK cells when compared to the wild-type mice. These data are consistent with recent studies from our lab in which it was noted that IL-2 treatment in vivo caused identical phenotypic changes in lymphocytes from wild-type, *gld/gld*, and perforin-KO mice.

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

To exclude the possible variations seen in target cell-susceptibility and to address the role of Fas, we used L1210 tumor cells which had been transfected with Fas-sense (Fas^+) or antisense (Fas^-) (Rouvier et al., 1993). The expression of Fas on these cell lines was confirmed by flow cytometric analysis (Fig 3.2). As shown in Fig 3.4 (A,B,C), Fas^- L1210 tumor targets were resistant to spontaneous and poly I:C activated killing by NK-cells in all 3 groups of mice, similar to the NK-resistant P815 cells (Fig 3.2). However, following IL-2 activation, LAK cells from wild type (Figure 3.4 A) and *gld/gld* (Figure 3.4 B) mice mediated significant lysis whereas, similar cells from perforin-knockout mice (Figure 3.4 C) failed to exhibit lytic activity. In fact, LAK cells from *gld/gld* mice mediated increased cytotoxicity when compared to the wild type mice, the reason for which was not clear. they were resistant to spontaneous killing by the NK cells similar to Fas^- L1210 targets in all 3 groups of mice. Interestingly, when NK cells activated with poly I:C were tested, the Fas^+ targets became susceptible to cytotoxicity.

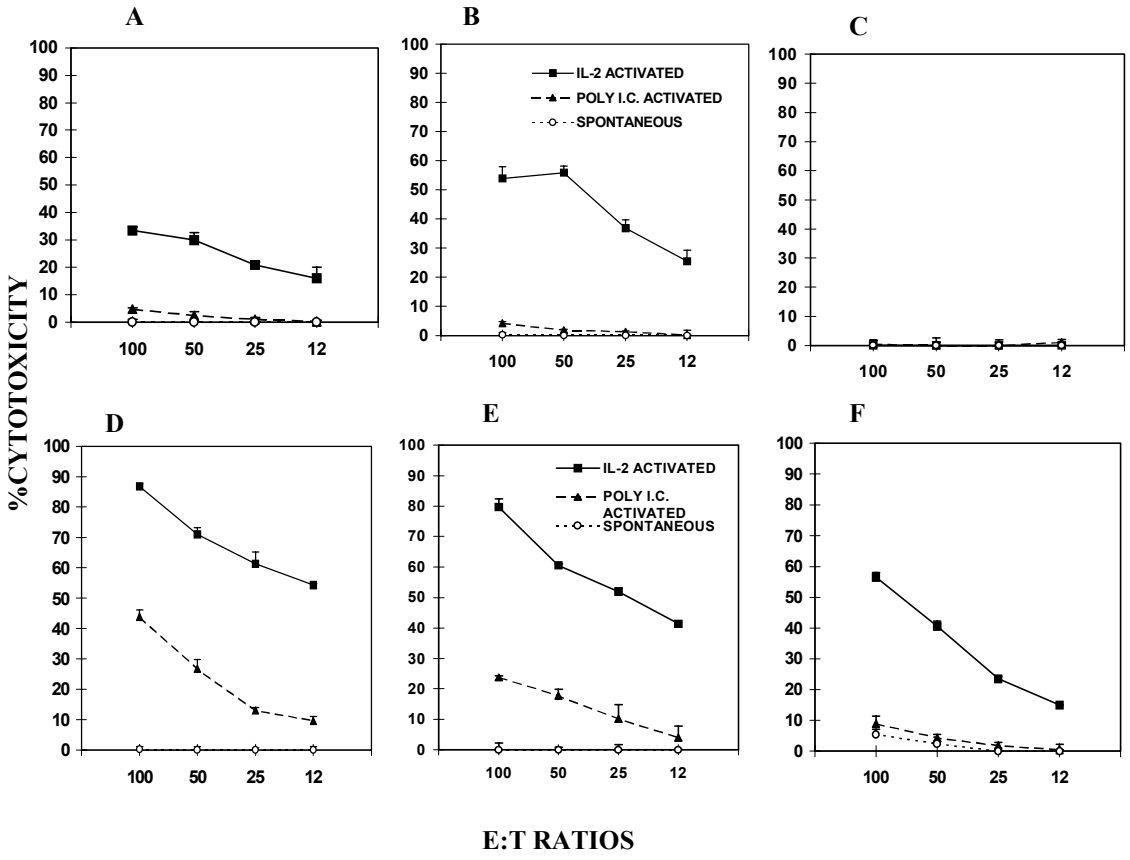


Figure 3.4: NK/LAK cell-mediated cytotoxicity against Fas^+ and Fas^- tumor targets. NK/LAK cells from wild-type (A, D), *gld/gld* (B and E), and perforin-KO (C and F) mice, were tested for cytotoxicity against L1210 Fas^- (A, B, C) or L1210 Fas^+ (D, E, F) tumor targets. The cytotoxicity was performed as described in Figure 3.1.

When L1210 Fas^+ targets were similarly tested (Fig 3.4, lower panel), it was noted that. Such poly I:C-induced cytotoxicity was both FasL- and perforin-based because both *gld/gld* (Figure 4 E) and perforin-knockout (Figure 3.4 F) mice exhibited significant cytotoxicity. Such cytotoxicity was, however, less than that seen in wild type mice (Figure 3.4 D). Similar results were also seen using LAK cells activated with IL-*2 in vitro* (Fig 3.4 D,E,F). Overall, when L1210 Fas^+ and Fas^- targets were compared (Fig 3.4 lower versus upper panel), Fas^+ targets were found to be more susceptible to lysis following poly I:C or IL-2 activation of NK/LAK cells, thereby suggesting that activation of NK/LAK cells triggers not only the perforin-based but also the FasL-based pathway.

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

To further corroborate *in vivo* that IL-2 activation upregulates both perforin and FasL-based cytolytic activity, wild-type, *gld/gld*, and perforin KO mice were administered with IL-2 for 5 days and the spleen cells were tested for lytic activity against Fas^- and Fas^+ L1210 tumor cells. The data shown in Fig. 3.5 demonstrated that Fas^+ targets were markedly more sensitive to cytotoxicity by *in vivo* IL-2 activated LAK cells from wild-type mice when compared to the Fas^- L1210 tumor cells (Figure 3.5 A). Also, the wild-type mice (Figure 3.5 A) exhibited highest level of cytotoxicity when compared to *gld/gld* (Figure 3.5 B) or perforin-deficient (Figure 3.5 C) mice, which demonstrated moderate degree of lysis.

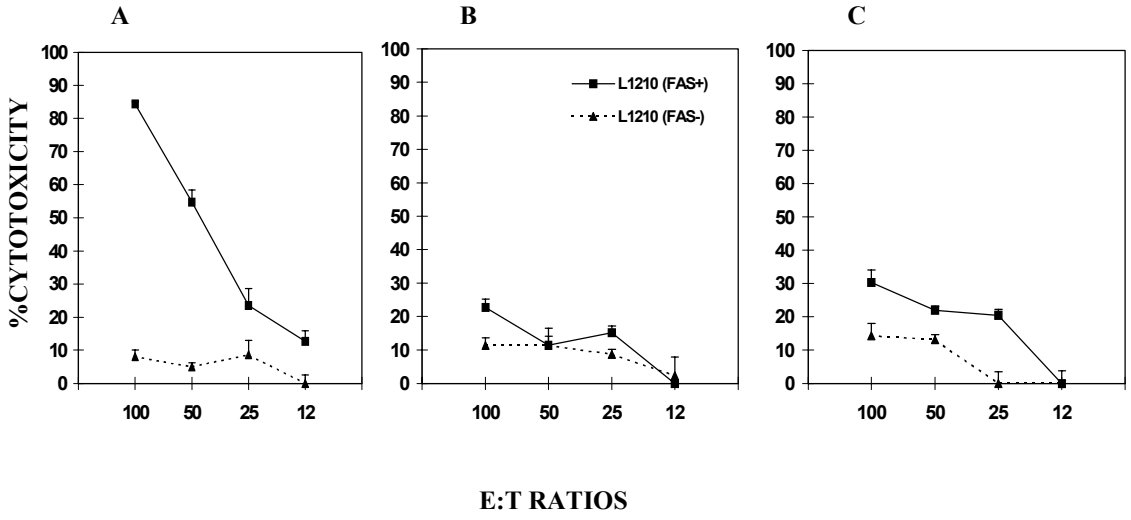


Figure 3.5: Cytotoxicity mediated by LAK cells activated *in vivo* with IL-2. Wild-type (A), *gld/gld* (B) or perforin-KO (C) mice were injected with 10,000 units of IL-2 twice a day for 4 days and the LAK cells were tested for cytotoxicity against L1210 Fas^- and L1210 Fas^+ tumor targets. Cytotoxicity was studied as described in Figure 3.1.

These data indicated that *in vivo* IL-2 administration triggered both perforin and FasL-based cytotoxicity and that such cytotoxicity was more effective against Fas^+ tumor targets.

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

Inasmuch as, the expression of Fas caused increased susceptibility of tumor cells to lysis by activated LAK cells, we next addressed whether Fas^+ and Fas^- L1210 tumor cells would exhibit differential ability to induce tumors in the syngeneic host and whether IL-2 immunotherapy would have varying effects on the growth of these tumor cell lines *in vivo*. To this effect, 1 x 10^6 Fas^+ or Fas^- L1210 tumor cells were injected s.c. and were administered either with PBS as a control, or IL-2 (10,000 U three times a day for one week, followed by once a day for an additional week). The survival of the mice was monitored for ~60 days. As shown in Figure 3.6, all L1210 Fas^- tumor cell + PBS injected mice, died with a MST=18.2 ± 6.0 days. In contrast, IL-2-treated L1210 Fas^--bearing mice survived for longer periods (MST=27.4± 4.6 days). This increase in MST resulting from IL-2 treatment was significant ($p<0.05$) when compared to the MST in PBS-treated Fas^--tumor bearing mice. Interestingly, mice bearing Fas^+ L1210-induced tumor, survived for longer periods (MST=37.9± 6.1 days), with a significant proportion (~20%) surviving for more than 60 days at which time period, the experiment was terminated. Thus, the MST in mice bearing Fas^+ L1210 tumor was greater than those bearing Fas^- tumor ($p<0.05$). Moreover, IL-2 treatment of Fas^+ tumor-bearing mice led to increased survival rate (40%) with a MST of 43.8± 15.5.

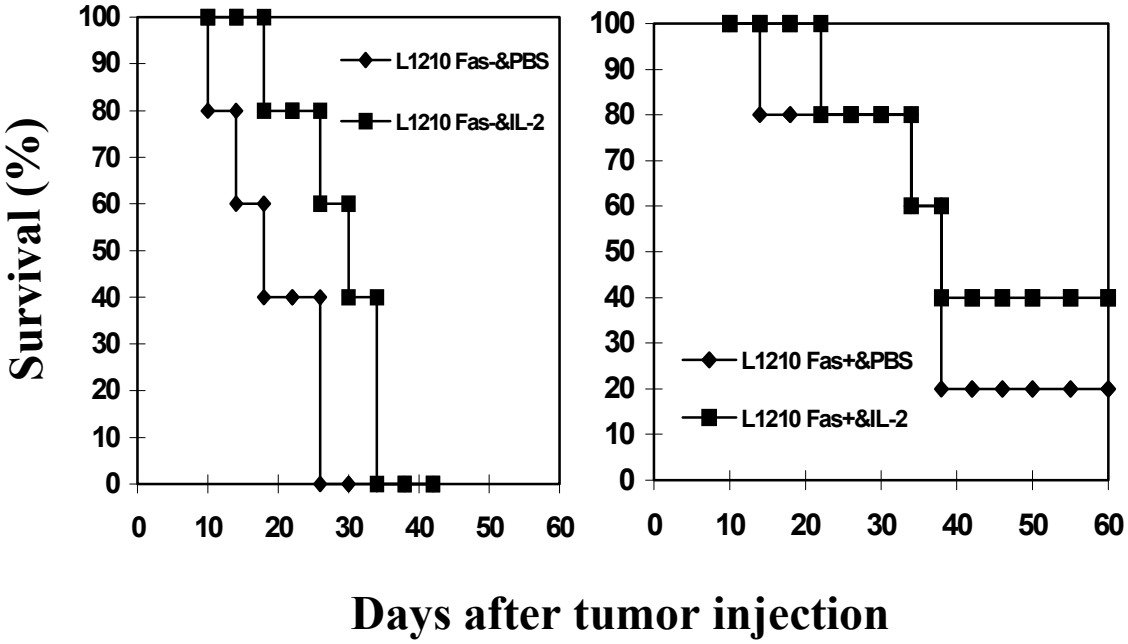


Figure 3.6: Effect of immunotherapy with IL-2 against growth of L1210 Fas^+ and L1210 Fas^- tumor cells in syngeneic host. L1210 Fas^+ and Fas^- tumor cells ($1x10^6$) were injected subcutaneously into group of 5 syngeneic (DBA/2) mice. The mice were injected with PBS (control) or with IL-2 (10,000 units/mouse, 3 times a day for one week followed by once a day for additional seven days). The mice were observed for tumor growth and survival. The experiment was terminated after 60 days.

Together, these data demonstrated that mice bearing L1210 Fas^+ tumor cells survived for longer periods than those bearing Fas^- L1210 tumor cells and furthermore, IL-2 therapy was more effective against Fas^+ than Fas^- tumor cells. It should be noted that i.p. injection of Fas^+ and Fas^- L1210 tumor cells into DBA/2 mice also led to distinct survival rates. The Fas^- L1210 tumor cell-bearing mice all died on day 12, whereas, L1210 Fas^+ tumor-bearing mice died with a MST=33.2± 7.6($p<0.01$). However, IL-2 treatment was not effective against i.p. injected tumor because IL-2 treated L1210 Fas^+ tumor-bearing mice died with a MST 31.5± 5.68 days and Fas^- tumor-bearing mice died with a MST of 18.4± 1.68 days.

To rule out the possibility that the differences in the MST of mice injected with Fas^+ and Fas^- tumor cells were due to alterations in the growth characteristics of the tumor cells, the *in vitro* growth pattern of the cells was investigated. The data shown in Fig. 3.7 indicated that L1210 Fas^+ and L1210 Fas^- tumor cells had similar growth curves when tested for 12-72 hours following *in vitro* culture.

Tumor specific Cytotoxic T lymphocytes also demonstrate preferential killing of Fas^+ but not Fas^- target cells:

Because the L1210 tumor cells could evoke CTL responses, T cells were purified from DBA/2 mice injected with Fas^+ or Fas^- L1210 tumor cells and cultured *in vitro* with respective irradiated tumor cells and tested for cytotoxicity against Fas^+ and Fas^- targets.

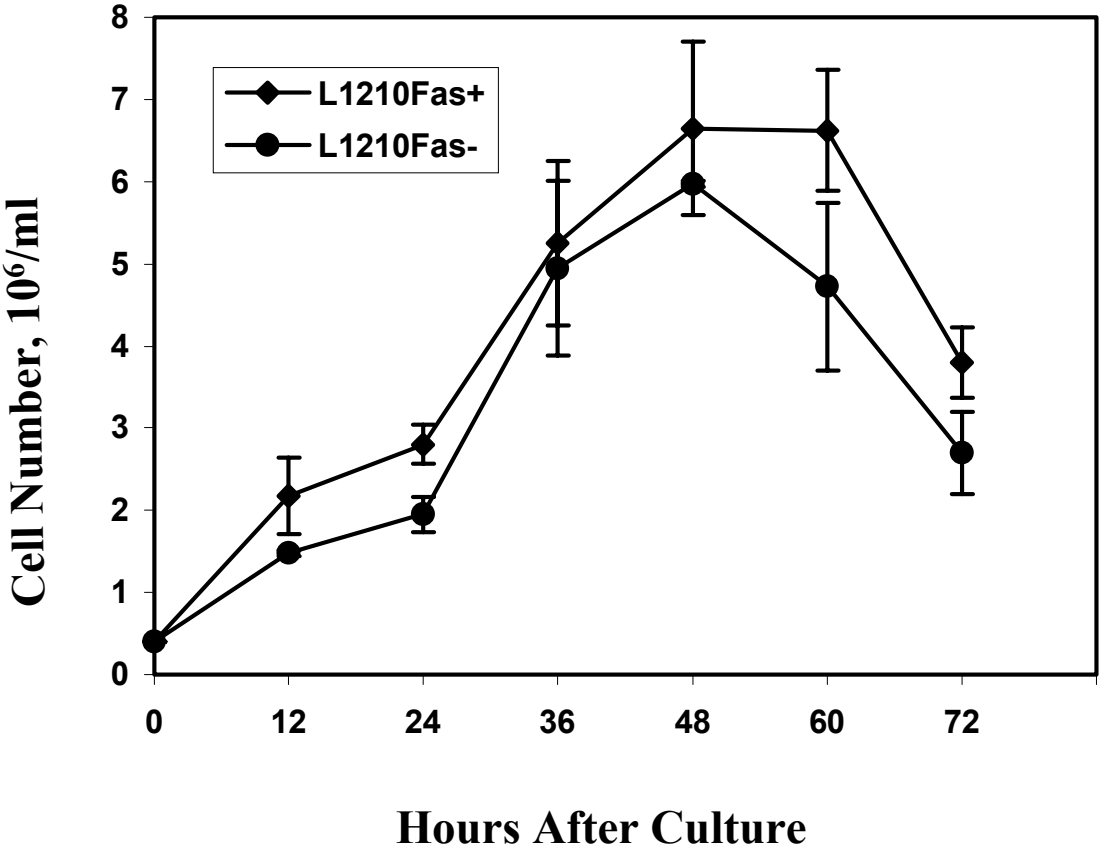
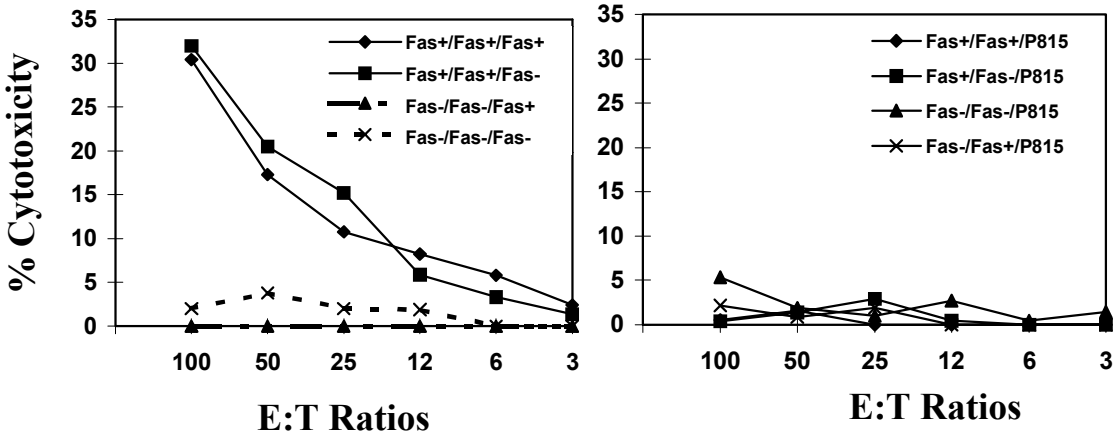


Figure 3.7: In vitro growth characteristics of L1210 Fas^+ and Fas^- tumor cells. The tumor cells were cultured in vitro as described in Methods and at various time intervals the cells were harvested and a viable count was determined. The data represent mean± S.E.M of triplicate cultures.

The data shown in Fig. 3.8 (A) suggested that CTLs from Fas^+ tumor bearers mediated stronger lytic activity against Fas^+ as well as Fas^- tumor targets, whereas, CTLs from Fas^- tumor-bearing mice, exhibited weak or no significant lytic activity. These data demonstrated that Fas^+ L1210 tumor cells triggered increased CTL activity when compared to the Fas^- L1210 tumor cells, a finding which may account for increased survival of Fas^+ tumor-bearing mice, when compared to the Fas^- tumor-bearing mice. It should be noted that the CTL obtained from Fas^+ tumor-bearing mice could kill Fas^+ or Fas^- targets to the same extent. This suggested that such CTL-mediated cytotoxicity was primarily perforin rather than FasL-mediated. To corroborate that the cytotoxicity in the above study was mediated by CTL, we tested the lytic activity against a non specific $H\text{-}2^d$ tumor cell line such as P815. The data shown in Fig. 3.8 (B) indicated that the CTL raised against L1210 tumors failed to kill P815 target cells thereby indicating the involvement of L1210 tumor-specific CTL.

Discussion:

In the current study we demonstrated that poly I:C or IL-2 triggers NK/LAK activity by enhancing both FasL- and perforin-based cytotoxicity. Fas^+ tumor targets were found to be more sensitive to poly I:C- or IL-2-induced cytotoxicity when compared the Fas^- tumor targets. Also, mice bearing Fas^+ L1210 tumor cells survived for longer periods and a significant proportion of mice injected s.c. with the tumor rejected , when compared to the mice bearing Fas^- L1210 tumor cells, which died from rapid tumor growth.


% Cytotoxicity
35
30
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100
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25
12
6
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E:T Ratios
Fas+/Fas+/Fas+
Fas+/Fas+/Fas-
Fas-/Fas-/Fas+
Fas-/Fas-/Fas-
Fas+/Fas+/P815
Fas+/Fas-/P815
Fas-/Fas-/P815
Fas-/Fas+/P815
E:T Ratios

Figure 3.8: Increased tumor-specific CTL activity in mice bearing L1210 Fas^+ but not L1210 Fas^- cells. Purified T cells from DBA/2 mice bearing L1210 Fas^+ or L1210 Fas^- tumor cells were harvested and cultured with irradiated L1210 Fas^+ and Fas^- tumor cells, respectively for 5 days. Next, the harvested cells were tested for cytotoxicity against L1210 Fas^+ or Fas^- tumor targets (panel A) or against P815 (panel B) as described in Figure 4. In the symbols, the first column represents source of T cells, second shows stimulator cells and third depicts target cells tested. For example, Fas^+ / Fas^+ / Fas^+ represents T cells from mice bearing Fas^+ tumor cells stimulated with Fas^+ tumor *cells in vitro* and tested for cytotoxicity against Fas^+ targets.

Moreover, IL-2 administration *in vivo* was shown to trigger both FasL and perforin-based cytolytic activity and was able to increase the MST of both Fas^+ and Fas^- tumor-bearing mice, particularly upon s.c. injection. Furthermore, IL-2 therapy was more effective in s.c. injected Fas^+ tumor-bearing mice in which ~40% of the mice survived for more than 60 days when compared to Fas^- tumor-bearing mice in which 0% of the mice survived.

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

Recently, freshly isolated human and murine NK cells were found to express FasL and mediate Fas-based cytotoxicity (Rafi et al., 1998; Oshimi et al., 1996). However, in the

current study we noted that *gld/gld* mice had normal levels of spontaneous NK activity against YAC-1 targets. Also, the NK cells failed to mediate spontaneous cytotoxicity against NK-resistant L1210 Fas^+ transfectants despite the fact that such cells expressed high levels of Fas. The reason for the differences in previous and current studies, is not clear. One possibility is that in the earlier study (Rafi et al., 1998; Oshimi et al., 1996), the targets used were different from the ones used in the current study. Thus, the use of FasL by NK cells mediating spontaneous cytotoxicity, may depend on the nature of target cells and not merely on the expression of Fas on target cells. Also, in the earlier study (Oshimi et al., 1996), the authors used a 12 hour fluorescent dye assay to study cytotoxicity. During such long-term cytotoxicity, molecules such as TNF have been shown to be involved, unlike short term assays in which the cytotoxicity is mediated exclusively by FasL and perforin (Lee et al., 1996).

In the current study, comparison between Fas^+ and Fas^- L1210 transfectants yielded interesting results. The Fas^+L1210 transfectants were more sensitive to poly I:C- and IL-2-activated NK/LAK cell-mediated cytotoxicity when compared to Fas^- L1210 target cells. These data together suggested that Fas expression on target cells can increase the susceptibility of the tumor targets to induced NK/LAK activity. The fact that perforin-deficient mice completely failed to mediate lysis of Fas^- L1210 targets and that they could mediate lysis of Fas^+ L1210 cells to a lower level than the *gld/gld* or wild type mice suggested that perforin may play an important role in induced NK/LAK-activity. It should also be noted that despite the strong expression of Fas on the L1210 tumor cells, FasL-based cytotoxicity as seen in perforin-knockout mice was lower than that seen in wild-type mice. Similarly, the perforin-based lytic activity seen in *gld/gld* mice was also

less than that seen in wild-type mice. Thus, the presence of both perforin- and FasL-based cytotoxicity, was clearly an advantage to efficiently kill Fas^+ tumor targets.

To further corroborate and translate the *in vitro* results to tumor rejection *in vivo*, we compared the ability of Fas^+ and Fas^- L1210 transfectants to grow and induce tumors in syngeneic mice and tested whether IL-2 administration would inhibit the growth of these tumor cells. In these studies, it was striking that mice injected with Fas^+ tumor cells survived for longer periods than those receiving Fas^- tumor cells. Furthermore, a significant proportion of mice injected s.c. with Fas^+ tumor cells could reject the tumor and this was further enhanced following IL-2 administration. It should be noted that the IL-2 treatment was effective against s.c. but not i.p. injected tumor. This may be because i.p. injected tumor may metastasize faster and kill the host earlier as evident from a shorter MST, thereby preventing an effective anti-tumor immunity to develop and act on the tumor cells. In contrast, the s.c. tumors may metastasize slower as seen from longer MST, because of which IL-2 treatment may be more effective. In the current study it was also noted that Fas^+ tumor-bearing mice had higher levels of tumor-specific CTL activity when compared to the Fas^- tumor-bearing mice. Also, T cells from Fas^- tumor-bearing mice failed to mediate cytolytic activity against Fas^+ targets. These data suggested that the expression of Fas on tumor cells may make them more immunogenic. Together, the current study demonstrates that IL-2 therapy may be more effective against Fas^+ tumors. Furthermore, transfection of the Fas gene into Fas^- tumor cells may offer a novel approach to trigger anti-tumor immunity.

Chapter 4: Growth of FASL-bearing tumor cells in syngeneic murine host induces apoptosis and toxicity in Fas^+ organs: The role of the soluble Fas ligand

Abstract:

In the current study we investigated whether the growth of FasL-bearing tumor cells would induce apoptosis and toxicity in organs that express high level of Fas. Sera from C57BL/6 +/+ (wild-type) mice injected with syngeneic $FasL^+$ tumors, LSA or EL-4, showed significantly higher level of soluble FasL than the non-tumor bearing mice. Furthermore, the soluble FasL was functional, inasmuch as, the sera from tumor-bearing mice were able to induce apoptosis in Fas^+ but not Fas^- targets. Histopathological studies and in situ TUNEL assay to detect apoptosis were carried out in C57BL/6 +/+ (Fas^+) or C57BL/6 *lpr/lpr* (Fas^-) mice injected with syngeneic LSA and EL-4 tumor cells. The morphology of the liver and thymus from tumor bearing C57BL/6 +/+ mice showed marked damage and tissue destruction. In contrast, the liver and thymus from tumor-bearing C57BL/6 *lpr/lpr* mice showed minimal damage. Furthermore, the tumor-bearing C57BL/6 +/+ but not C57BL/6 *lpr/lpr* mice, exhibited significant apoptosis in the liver and thymus. Together, these data suggested that *in vivo* growth of FasL-bearing tumor cells, can induce significant apoptosis and toxicity in Fas^+ tissues of the host. Such toxicity may be mediated by the soluble FasL produced by tumor cells.

Introduction:

FasL is a 40-k-Da type II transmembrane protein belonging to the TNF family (Nagata, 1995; Suda et al., 1993). The ligation of Fas, a cell surface protein belonging to the TNF receptor family, transduces an apoptotic signal leading to the cell death (Nagata, 1995). FasL is abundantly expressed in the testis and to a lesser extent in the spleen, thymus, lung and small intestine (Suda et al., 1995). Fas is expressed at high levels in the thymus, lung, heart and liver and at lower concentration in the spleen, lymph nodes and small intestine. Recent studies have suggested that several tumor cell lines constitutively express Fas ligand including the colon carcinomas (O'Connell et al., 1996), melanomas (Hahne et al., 1996), hepatacellular carcinomas (Strand et al., 1996) and astrocytoma (Saas et al., 1997). Such studies have suggest that FasL may be used by the tumor cells to evade the actions of the immune system (Walker et al., 1997).

Previous studies from our lab demonstrated that a FasL-expressing tumor cell line killed the tumor specific cytotoxic T lymphocytes (CTL) which expressed Fas (Zeytun et al., 1997). These data suggested that FasL-bearing tumor cells may use FasL as a mechanism of immune evasion. Furthermore, this observation may explain why adoptive transfer of tumor specific T cells into tumor bearing mice fails to cure a high percentage of the mice (Nagarkatti et al., 1988; Hammond-McKibben et al., 1995).

Despite the above studies, it is not clear whether the growth of FasL-bearing tumor cells would induce toxicity in the organs that express high levels of Fas. FasL is known to be expressed in both membrane-bound and soluble form. Matrix metallaproteinase

cleaves the membrane bound FasL to produce the soluble form (Tanaka et al., 1997). The soluble form of FasL is less cytotoxic than the membrane-bound (Schneider et al., 1998). However, increased level of soluble FasL have been described in a number of clinical situations and have been shown to cause apoptosis and toxicity in the host (Sato et al., 1996; Tanaka et al., 1996; Toyozaki et al., 1998). We reasoned that at the peak of tumor growth in the host, there may be sufficient soluble form of FasL produced by the tumor cells that may cause immunotoxicity. The current study demonstrates that growth of FasL-bearing tumor cells induces apoptosis in the thymus and liver of wild-type mice but not Fas-deficient lpr mice. Furthermore, the sera of tumor bearing mice exhibited significant levels of the functional FasL. These data demonstrated that toxicity seen in some cancer patients may results from the FasL produced by the tumors.

Materials and Methods:

Mice:

Adult female C57BL/6 +/+ (wild-type) mice were purchased from Jackson laboratories and maintained in our animal facility. C57BL/6 (*lpr/lpr*) and C57BL/6 (*gld/gld*) mice were bred in our animal facilities as described (Hammond et al., 1993). All strains of mice were used at 4 weeks of age.

Tumor cell lines:

T cell lymphoma lines EL-4 and LSA are both sygeneic to the C57BL/6 strain and express high level of FasL (Zeytun et al., 1997). L1210 Fas^+ and Fas^- cell lines represent

murine lymphoma lines derived from DBA/2 mice transfected with sense and antisense of Fas cDNA, respectively (Rouvier et al., 1993). The tumor cells were passaged in vivo or grown in culture in RPMI (GIBCO, Grand Island, NY) supplemented with 2 mmol/L glutamine, 40 ug/ml Gentamicin, 50 umol 2-mercaptoethanol, 10 mmol/L HEPES, and 10 % fetal bovine serum (Summit Biological, Forth Collins, CO). The cells were grown at a concentration of $2x10^5$ cells/ml of medium. Supernatant from *in vitro* grown tumor cells were collected after 24 hours of cultures by centrifuging to remove any cells or cellular depris to detect FasL.

Injection of tumor cells into mice:

LSA or EL-4 tumor cells ($1x10^6$) were injected into 4-week old C57BL/6 (+/+) or C57BL/6 (*lpr/lpr*) in 100 ul PBS via i.p route. Mice injected with PBS served a control. Each group consisted of 5 mice. On day 7 (for LSA) or 10 (for EL-4) post tumor-growth, the mice were sacrificed and serum was collected for detection of soluble FasL. In addition, thymus and liver from these mice were removed and fixed in 10% of neutral formalin solution.

Antibodies:

Monoclonal (clone, Kay-10, mouse IgG2a) and polyclonal anti-FasL (AB-1) were purchased from Pharmingen (San Diego, CA) and Oncogene (Cambridge, MA), respectively. Alkaline Phosphatase conjugated anti-rabbit IgG was obtained from Jackson Immunoresearch (West Grove, PA). The synthetic FasL peptide was purchased from Oncogene (Cambridge, MA).

Detection of soluble FasL:

To detect the soluble form of FasL, we used double sandwich enzyme linked immunoabsorbant assay (ELISA) developed in our lab. Elisa plates were coated with 5 ug/ml Kay-10 anti-FasL mAbs (Pharmingen, San Diego, CA) overnight at 4^{o}C and non-specific-binding was blocked with 3% BSA. Freshly collected mouse sera (50 ul) or 100 ul of supernatant from in vitro culture of tumor cells were added to the wells. Following 4 hour incubation, plates were washed with PBS and incubated with 1ug/ml of polyclonal anti-mouse FasL (Ab-1) (Oncogene, Cambridge, MA) for 2 hour at room temperature. This was followed by 1 hour incubation with Alkaline Phosphatase-conjugated anti-rabbit antibody (Jackson, West Grove, PA) and p-nitrophenol, and absorbency was measured at 410 nm. As a positive control, we used synthetic FasL peptide (Oncogene, Cambridge, MA) recognized by anti-FasL (Ab-1) antibody.

Detection of Apoptosis:

One million thymocytes from C57BL/6 +/+ (Fas^{+}) or C57BL/6 *lpr/lpr* (Fas^{-}) were incubated in 96 well plates with 50 ul of freshly obtained sera from tumor-bearing or control mice. Following 24 hour incubation, DNA strand breaks were detected by TdT-mediated nick end-labelling, referred to as TUNEL assay (Boehringer Mannnheim, Indianapolis, IN), as described (Kamath et al., 1997). Briefly, after incubation, thymocytes were harvested and washed with PBS twice. Next, the cells were fixed with 4% p-formaldehyde for 30 min at room temperature. The cells were washed with PBS , permeabilized on ice for 2 min, and incubated with FITC-dUTP for 1 hour at 37^{o}C.

Fluorescence of the cells was measured by flow cytometry as previously described (Kamath et al., 1997). The analysis was performed by a Coulter Epics V flow cytometer (Miami, FL). Five thousand cells were analyzed per sample.

For in situ apoptosis detection, liver and thymus from control and tumor-bearing mice were aseptically removed and fixed in 10% neutral formalin solution. Five μm paraffin-embedded sections were adhered to slides. Deparafinization was done by heating the sections at 60^{o}C for 25 min. Rehydration was carried out by transferring the slides through the following solutions: twice in xylem for 5 min, twice in 95% ethanol for 5 min, twice in 70% ethanol for 5 min, and 10 min in distilled water. The tissues were treated with 20 ug/ml proteinase K (Sigma, St Louis, MO) in 10 mmol Tris-HCL, pH 8 for 30 min at 37 ^{o}C and then washed in PBS twice for 10 min. Endogenous alkaline-phosphatase were inactivated by treating the slides with 10 mmol levamisole (Sigma, St Louis, MO) at room temperature for 1 hour. The sections were washed in PBS for 10 min and covered by TdT-FITC-dUTP enzyme-labelling solution and then incubated at 37 ^{o}C in a humidified incubator for 1.5 hours. The slides were rinsed for 10 min in PBS and covered with alkaline phosphatase converter solution. Following 1 hour incubation, the slides were washed twice in PBS for 10 min and BM purple substrate (BCIP/NBT) was added. Dark-purple color was visible in 15-25 min. The slides were washed, counter-stained with eosin and cover-slip was placed on mounting media. The nuclear staining was evaluated under a light microscope. Identical slides were also stained with Hematoxiylin-Eosin (H&E) to detect lymphocyte infiltration and to evaluate tissue structure.

Results

Detection of soluble Fas ligand in supernatant:

Sandwich ELISA was used to detect FasL. Figure 4.1A shows the detection of a known sample of synthetic FasL peptide. When culture supernatants from $FasL^+$ tumor cell lines, LSA and EL-4, were tested, they exhibited significant levels of FasL (Fig. 4.1B). In contrast, culture supernatant from a FasL-deficient- L1210 tumor cell line did not show significant levels of FasL. Furthermore, the culture supernatant from a $FasL^+$ CTL cell

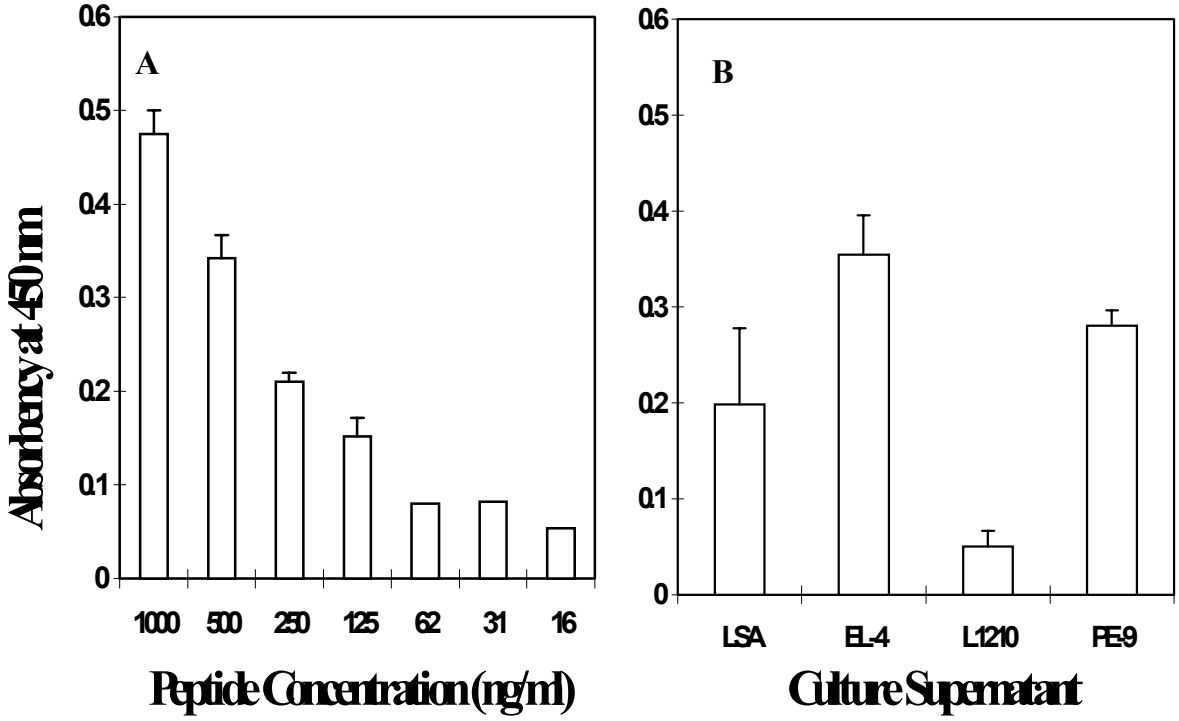

Figure 4.1: Detection of soluble FasL in the culture supernatants of tumor cells. (A) Known concentration of synthetic FasL peptides were used in an ELISA assay as a positive control. (B) Culture supernatants from LSA, EL-4, L1210 tumor cells and PE-9 line (PE-9)[9] was also found to exhibit significant levels of FasL (Figure 1B). These data suggested that the tumor cell lines LSA and EL-4 were producing soluble form of FasL. T cells were tested in the ELISA assay to detect the presence of soluble FasL.

Detection of sFasL in sera:

ELISA was also used to detect sFasL in the sera of tumor-bearing mice. To this end, C57BL/6 mice were injected with $1x10^6$ LSA or EL-4 tumor cells, i.p. and on day 7, serum was collected and pooled from 5 mice and tested for the presence of sFasL. As shown in Fig. 4.2, sera from LSA and EL-4 bearing mice showed significant levels of FasL when tested directly or following two fold dilution, when compared to the controls. It should be noted that sera from EL-4 bearing mice demonstrated higher levels of FasL, inasmuch as, FasL was detectable even after dilution of the serum 1/16 times, whereas, sera from LSA-bearing mice showed significant levels of FasL at 1/ 4 dilution, and not thereafter (data not shown). These data were consistent with the observation that EL-4 tumor cells expressed higher levels of FasL when compared to the LSA tumor cells (Zeytun et al., 1997).

Induction of apoptosis by sera from tumor-bearing mice:

We next tested whether the FasL found in the sera of tumor-bearing mice was functional and therefore could induce apoptosis *in vitro*. To this end, Fas^+ thymocytes from C57BL/6 +/+ or Fas^- thymocytes from C57BL/6 *lpr/lpr* mice were incubated with 50 µl of sera from EL-4 or LSA bearing mice for 24 hours. Next, DNA fragmentation was studied using the TUNNEL assay. As shown in Figure 4.3A, irradiated thymocytes cultured for 24 hours *in vitro* used as a positive control, showed high levels of apoptosis (broken histogram) when compared to non-irradiated thymocytes (bold histogram).

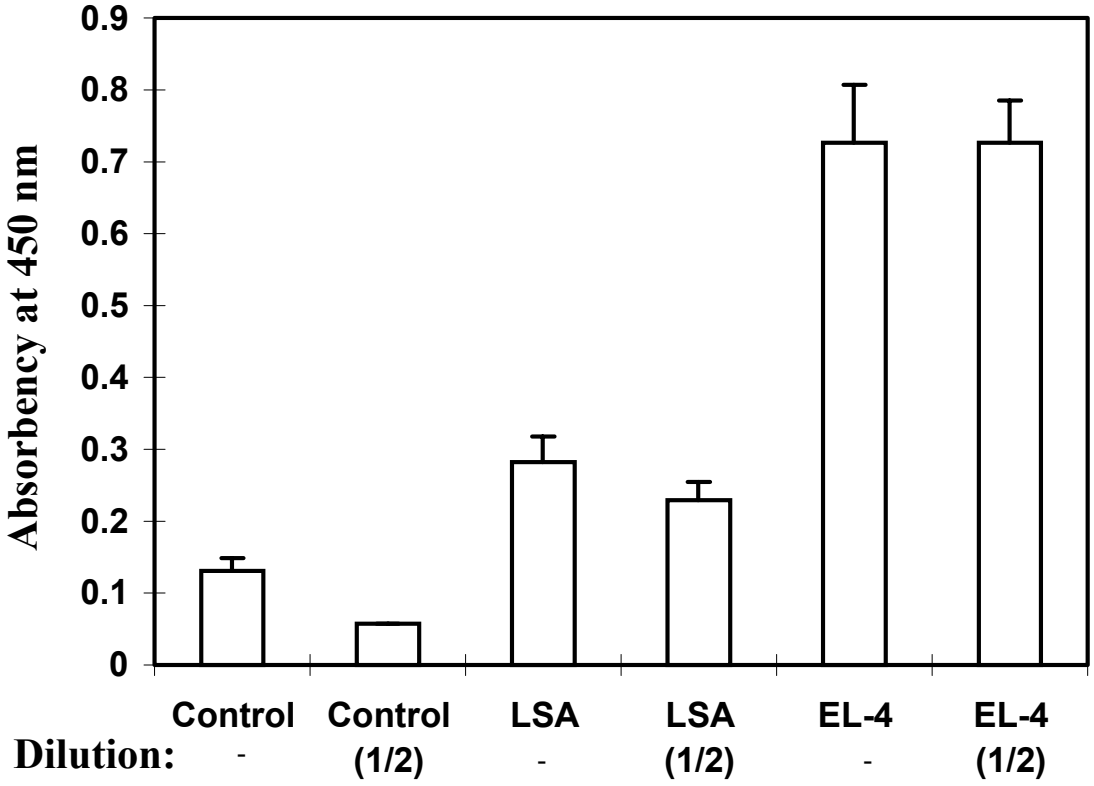

Figure 4.2: Detection of soluble FasL in the sera of C57BL/6 +/+ tumor-bearing mice. Sera from LSA or EL-4 tumor bearing mice were pooled and used directly or following 1:2 dilution. Next, using ELISA, presence of sFasL was detected. Control represents sera from C57BL/6 +/+ mice injected with PBS.

In an earlier study we noted that when normal thymocytes were cultured *in vitro* for 24 hours, they exhibited significant apoptosis (Kamath et al., 1997). In the current study, when thymocytes from C57BL/6 +/+ mice were incubated with control serum, significant level of apoptosis was detected (Fig 4.3, bold histogram in panel B). Such apoptosis was similar to that seen with thymocytes cultured with medium for 24 hours (data not shown). However, when thymocytes from C57BL/6 +/+ mice were incubated with sera from EL-4 bearing mice, significantly higher levels of apoptosis was seen when compared to cells incubated with control serum (Fig 4.3, panel B, broken histogram). The apoptosis induced by sera from EL-4 bearing mice was FasL-dependent because the same sera failed to induce marked increase in apoptosis in Fas^- thymocytes from C57BL/6 *lpr/lpr* mice(Fig 4.3C). Similar results were obtained with the sera from LSA tumor-bearing mice which induced increased apoptosis in Fas^+ (Fig 4.3D) but not Fas^- (Fig 4.3E) thymocytes.

Detection of apoptosis *in vivo*:

We further examined *in vivo*, whether FasL produced by the tumor cells was inducing apoptosis in liver and thymus. To this end, 1x 10^6 tumor cells were injected into C57BL/6 +/+ (Fas^+) or C57BL/6 *lpr/lpr* (Fas^-) mice and 7 days later, liver and thymus were removed. Apoptosis was examined in paraffin-embedded and sectioned tissues by TUNNEL assay and morphology was studied using H & E staining. Liver sections from control mice stained with H&E showed normal morphology (Figure 4.4A) and exhibited no significant apoptosis (Figure 4.4B).

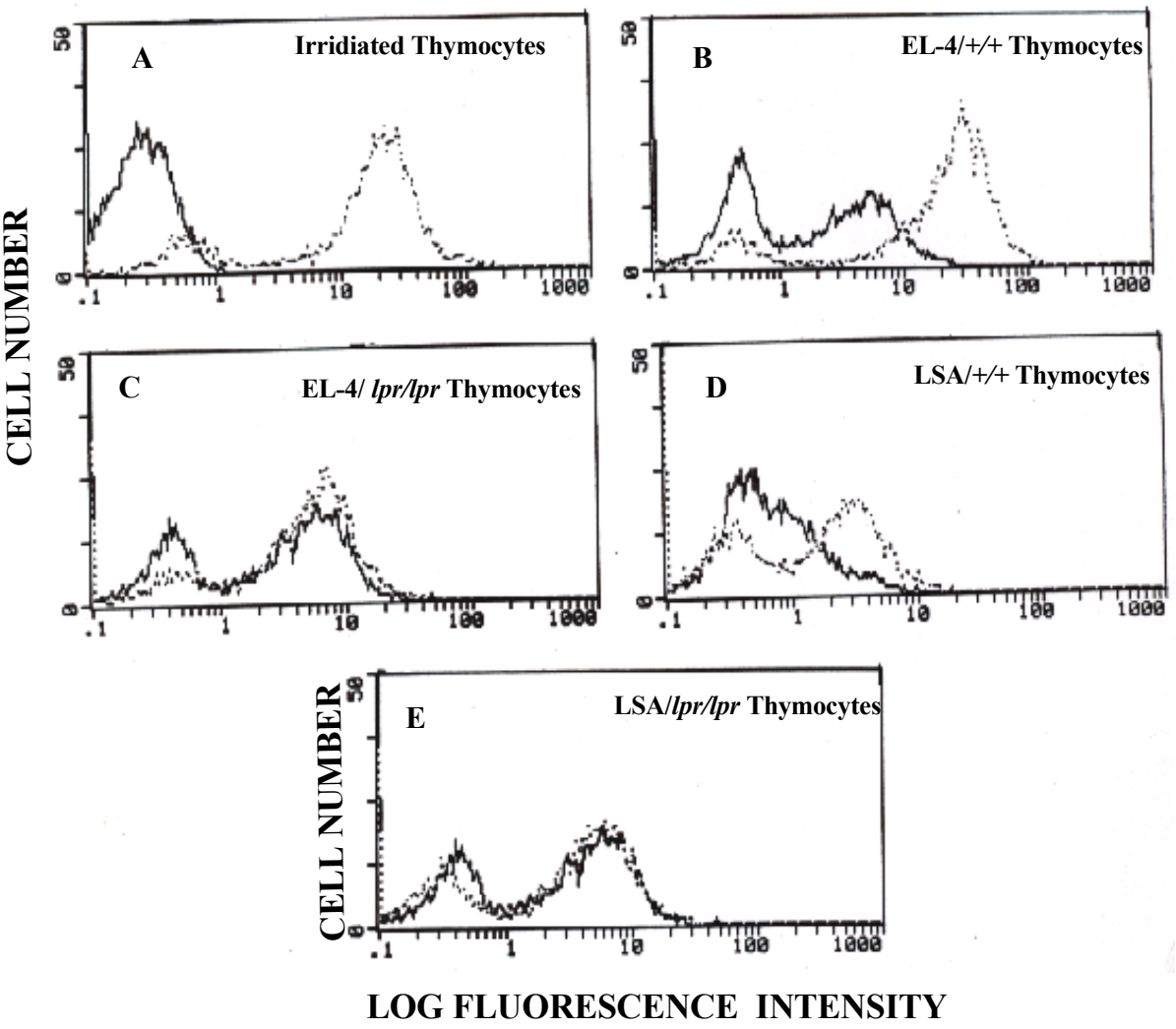
A
Irridiated Thymocytes
B
EL-4/+/+ Thymocytes
C
EL-4/ lpr/lpr Thymocytes
D
LSA/+/+ Thymocytes
E
LSA/lpr/lpr Thymocytes
CELL NUMBER
CELL NUMBER
LOG FLUORESCENCE INTENSITY

Figure 4.3: Detection of functional sFasL in the serum of tumor-bearing mice: Panel A shows freshly isolated thymocytes from C57BL/6 +/+ mice stained with TdT+FITC-dUTP (bold histogram) or stained after irradiation and culture for 24 hours (broken histogram). In panels B-E, thymocytes from C57BL/6 +/+(panel B and D), or C57BL/6 *lpr/lpr* mice (panels C and E) were cultured in the presence of sera from PBS-treated control mice (bold histogram) or from tumor-bearing mice (broken histogram). The cells were analyzed for green fluorescence using a flow cytometer. The subtraction of broken histogram from the bold histogram has been indicated as percent apoptosis in each panel.

In contrast, liver from LSA (Figure 4.4C) or EL-4 (Figure 4.4E)-tumor bearing C57BL/6 +/+ mice when examined with H&E staining, showed significant infiltration with lymphocytes and tumor cells. The tumor infiltration was predominantly restricted to the perivascular region. Also, marked structural damage to the hepatocytes was noted in these livers. When liver sections from LSA or EL-4 tumor-bearing mice were screened using TUNNEL assay (Figure 4.4 D and F, respectively), marked and extensive apoptosis was detected, as evident from dark purple staining of cells.

Similar experiments were carried out in C57BL/6 *lpr/lpr* mice. The livers from such mice injected with PBS alone (control) failed to exhibit any structural changes (Figure 4.5A) and apoptosis (Figure 4.5B). Furthermore, the liver from C57BL/6 *lpr/lpr* mice injected with LSA (Figure 4.5C) or EL-4 (Figure 4.5E) when examined after H&E staining, revealed significant infiltration with lymphocytes and tumor cells. This was similar to that seen in the C57BL/6 +/+ mice (Figure 4.4 C and E). However, the C57BL/6 *lpr/lpr* mice showed no significant damage to the hepotocytes. Furthermore, when apoptosis was studied, the C57BL/6 *lpr/lpr* mice injected with LSA (Fig 4.5 D) or EL-4 (Figure 4.5 F) failed to exhibit significant levels of apoptosis. This was incontrast to the tumor-bearing C57BL/6 +/+ mice, which had exhibited marked apoptosis in the liver (Figure 4.5 D and F). These data together suggested that LSA and EL-4 tumor cells induced apoptosis in only Fas^+ but not Fas^- liver, thereby indicating the involvement of FasL produced by tumor cells.

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Figure 4.4: Analysis of morphology and apoptosis in the liver of tumor-bearing C57BL/6 +/+ mice. Sections of liver from PBS injected control mice (panels A, B) or LSA tumor-bearing (panel C, D) or EL-4 tumor-bearing (panel E, F), mice were examined after H & E staining (panel A, C, and E) or TUNEL assay to detect apoptosis (panel B, D, and F). Small and big arrows represent infiltration of tumor cells and lymphocytes, respectively. Dark purple staining in panel D and F indicates the apoptosis.

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Figure 4.5: Analysis of morphology and apoptosis in the liver of tumor-bearing C57BL/6 *lpr/lpr* mice. Sections of liver from PBS injected control C57BL/6 *lpr/lpr* mice (A, B) or LSA tumor-bearing (panel C, D) or EL-4 tumor-bearing (panel E and F) were examined after H&E staining (panel A, C, and E) or TUNEL assay to detect apoptosis (panel B, D, and F). Small and big arrows represent infiltration of tumor cells and lymphocytes, respectively. Dark purple staining in panel D and F indicate the apoptosis.

Similar results were also obtained with other organs, including the thymus. Figure 4.6 A shows a section of the control thymus from C57BL/6 +/+ mice stained with H&E exhibiting normal morphology (Fig 4.6 A) and demonstrating no significant apoptosis (Figure 4.6 B). In contrast, the H&E staining of the thymus from C57BL/6 +/+ mice injected with LSA (Figure 4.6 C) or EL-4 (Figure 4.6 E) showed marked damage to the thymus. Furthermore, the same sections stained for apoptosis revealed marked induction of apoptosis (Figure 4.6 D for LSA and Figure 4.6 F for EL-4). In contrast to the this, the Fas-deficient C57BL/6 *lpr/lpr* mice injected with LSA (Figure 4.7 C) or EL-4 (Figure 4.7 E) showed normal morphology and failed to exhibit apoptotic cells (Figure 4.7 D and F for LSA and EL-4, respectively).

Tumor cells are the main source of Fas Ligand:

To investigate whether Fas ligand found in tumor-bearing mice, inducing apoptosis, was derived from the tumor cells or from the host, C57BL/6 *gld/gld* (FasL-defective) mice were injected with EL-4 and LSA tumor cells. Sera from tumor bearing C57BL/6 *gld/gld* mice contained significantly higher levels of Fas Ligand, compared to control mice (data not shown). The tumor-bearing C57BL/6 *gld/gld* mice exhibited significant levels of tissue destruction and more systemic apoptosis apoptosis in liver and thymus (Figure 4.8). These data suggested that the FasL found in sera and the FasL responsible for inducing apoptosis in liver and thymus, originated from the tumor cells, rather than the host.

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Figure 4.6: Analysis of morphology and apoptosis in the thymus of tumor-bearing C57BL/6 +/+ mice. Section of thymus from PBS injected control (panel A and B) or LSA tumor-bearing (panel C and D) or EL-4- tumor bearing (panel E and F) mice were examined after H&E staining (panel A, C, and E) or TUNEL assay to detect apoptosis (panel B, D, and F). More tumor cell infiltration was observed in thymus, when compared to the liver.

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Figure 4.7: Analysis of morphology and apoptosis in the thymus of tumor-bearing C57BL/6 *lpr/lpr* mice. Sections of thymus from PBS injected control (panel A and B) or LSA tumor-bearing (panel C and D) or EL-4- tumor bearing (panel E and F) mice were examined after H&E staining (panel A, C, and E) or TUNEL assay to detect apoptosis (panel B, D, and F). More tumor cell infiltration was observed in thymus, when compared to the liver.

Review Figure 4.8.tif for these slides

Figure 4.8: Analysis of apoptosis in liver of tumor-bearing C57BL/6 gld/gld (FasL-deficient) mice. Sections of liver PBS injected gld mice (panel A) or LSA tumor-bearing (panel B) or EL-4 tumor-bearing mice. (Magnification is 50X)

Discussion:

In the current study we observed that FasL-bearing tumor cells, LSA and EL-4 caused significant apoptosis in the liver and thymus of syngeneic wild-type but not Fas-deficient mice. Furthermore, significant levels of soluble FasL was detected in the sera of tumor bearing mice. Such FasL was functional inasmuch as, sera from tumor bearing mice were able to induce significant levels of apoptosis in Fas^+ but nor Fas^- targets. The current study demonstrated that growth of FasL-bearing tumors *in vivo* can induce significant toxicity in the organs expressing Fas.

Recently, several studies have demonstrated that a variety of tumor cells constitutively express Fas ligand capable of inducing apoptosis in Fas-bearing target cells (O'Connell et al., 1996; Hahne et al., 1996; Strand et al., 1996; Saas et al., 1997; Walker et al., 1997; Zeytun et al., 1997). Despite such studies, it is not clear whether the *in vivo* growth of FasL-bearing tumor can trigger toxicity in the organs and tissues expressing Fas. In the current study, using Fas^+ wild-type and Fas^- *lpr/lpr* mice, we demonstrated that the tumor-growth induces significant apoptosis and toxicity in Fas^+ but not Fas^- host. Such toxicity in the thymus and liver may partly result from metastasis of tumor cells and

direct contact with the Fas^+ cells of the host. However, this was limited because the tumor infiltration in organs was restricted to perivascular region, while apoptosis was seen through out the organ. Secondly, the soluble form of FasL produced by the tumor may also contribute towards induction of apoptosis and toxicity. This was evident from the fact that sera from the tumor-bearing mice had significant levels of FasL and more importantly such FasL was functional, inasmuch as, it induced apoptosis in Fas^+ but not Fas^- targets. Although soluble FasL may be less toxic than the membrane bound FasL (Schneider et al., 1998), at the peak of the tumor growth, we have noted that the host carries more than $2x10^8$ tumor cells. Thus, the soluble FasL may reach significant concentrations to induce toxicity. Sato et al., (1996) noted that in a case of nasal lymphoma, there was accompaniment of significant liver damage and pancytopenia which correlated with high levels of FasL. Furthermore, following chemotherapy, there was a decrease in soluble FasL in the serum and a consequent improvement in the liver damage and pancytopenia. Also, other studies have suggested that high local concentration of soluble FasL in association with other cytotoxic agents may lead to the cell death (Schneider et al., 1998). The role of soluble FasL in other disease models is also becoming increasingly apparent. For example, serum soluble FasL increased with the severity of congestive heart failure (Toyozaki et al., 1998).

In the current study we also detected FasL in the culture supernatants of LSA and EL-4 tumor cells. This is consistent with earlier reports that supernatant from COS cells transfected with full-length human FasL or mouse FasL, exhibit FasL capable of inducing apoptosis in Fas^+ target cells (Tanaka et al., 1995). Thus, chemotherapy which leads to

lysis of tumor cells may release large amount of FasL, thereby causing significant toxicity.

Apoptosis may occur in an autocrine fashion in which a cells may upregulate both Fas and FasL. Such a mechanism is known to play an important role in the liver damage seen in cirrhosis patients (Galle et al., 1995). In the current study, we addressed the possibility that the damage in the thymus and liver of tumor-bearing mice resulted from FasL produced by the host rather than the tumor cells. We observed that the liver and thymus of C57BL/6 *gld/gld* mice which exhibit a functional defect in FasL, showed significant levels of apoptosis following injection with LSA and EL-4 tumor cells. These data indicated that the apoptosis resulted from production of soluble FasL by the tumor cells rather than the host.

The toxicity induced by the $FasL^+$ tumor cells in the host may influence the survival of the host. We have observed that while wild-type mice injected with $1x10^6$ LSA tumor cells survive for ~7-8 days, the Fas-deficient C57BL/6 *lpr/lpr* mice survive for ~10-12 days. The fact that lpr/lpr mice also die ultimately of tumor-growth, suggested that immunotoxicity induced by the tumor cells may not be the sole reason as to why the host fails to reject the tumor. Alternatively, the C57BL/6 *lpr/lpr* mice may produce immunosuppressive molecules which may prevent the host from destroying the tumor (Lowrance et al., 1994). However, such molecules are produced only after they develop lymphoproliferative disease, whereas, in the current study, we used lpr mice at 4 weeks of age, during which time their immune functions are normal (Kakkanaiah et al., 1991).

In summary, the current study demonstrates that tumor derived Fas ligand in mice is functional and responsible for inducing apoptosis and damage in organs that express Fas. Such a mechanism may account for significant toxicity seen in cancer patients. Attempts to block apoptosis may provide an effective treatment to neutralize the toxicity.

Chapter 5: The role of Fas and Fas ligand in the induction and development of the chemical-induced tumors

Abstract:

In the current study, we investigated the role of the Fas, Fas ligand, perforin, and TGF-β in the development of chemically-induced tumor in *lpr* or *gld* mice. Because of the absence of the Fas-Fas ligand interactions and high levels of the TGF-β expression, *lpr* and *gld* mice developed methyl cholanthrene (MCA)-induced tumors much earlier, when compared to wild-type and perforin knockout (PKO) mice. Also, the tumor-growth in Fas or FasL deficient mice was rapid and uncotrollable. Interestingly, tumor development was delayed in perforin deficient mice. We also tested the growth of established tumor cells, LSA, in C57BL/6 +/+ (wt), C57BL/6 *lpr/lpr* (Fas-), C57BL/6 *gld/gld* (FasL-), or Perforin-deficient mice (PKO). Immunizatation of wt and PKO mice with LSA provided protection against subsequent challenge with this tumor cell. However, immunization of the *lpr* and *gld* mice at age of 8-10 weeks, failed tp protect the mice upon rechallenge. Both wt and PKO tumor are resistance to the chemical-induced tumors and growth of an established tumor cells. We reasoned that in these mice TGF-β expression was low, the expression of Fas on tumor cells was significantly higher, and very strong tumor-specific CTL response were generated. In contrary, Fas-FasL interaction is missing and TGF-β expression was high in lpr and gld mice. Therefore, we suggest that Fas-Fas ligand interaction plays an important role for providing protection from pollution-induced tumor.

Introduction:

Several human cancers, such as follicular lymphomas, carcinomas, breast, prostate, and ovarian cancers result from impairment of apoptosis (Thompson, 1995). The role played by mediators of apoptosis in the induction of chemically-induced tumors, is not clear.

Fas ligand is expressed by immune privilege sites(Green et al.,1996), different tumors (Zeytun et al., 1997; O'Connell et al., 1997) and activated T and NK cells(Suda et al., 1994). Fas Ligand-Fas interaction, which leads to induction of apoptotic signal on Fas expressing target cells, is important in the regulation of autoimmunity and cancer. Mice deficient either Fas or Fas ligand develop severe lymphoproliferative and autoimmune diseases, similar to human systemic lupus erythematosus (SLE) and B cells lymphomas at later stages of their life (Davidson et al., 1998). Similarly, in humans, multiple myeloma (Landowaski et al., 1997), extranodal diseases, and non-Hodgkin's lymphomas, type 2 autoimmune hepatitis (Pensati et al., 1997), some childhood B and T cell lineage acute lymphoblastic leukaemias (Beltinger et al., 1998), and adult T-cell leukaemia (Tamiya et al., 1998) have been shown to be associated with mutations in Fas gene.

Perforin is produced by cytolytic cells (CTLs and NK cells) and stored in their cytoplasm as lytic granules. These molecules kill the targets cells by opening pores on their membrane. Perforin knockout mice were recently generated (Walsh et al., 1994; Lowin et al., 1994), and CTL and NK cells activity against viral infections were found to be partially impaired in such mice (Walsh et al., 1994; Lowin et al., 1994). Otherwise these mice are perfectly healthy and normal.

In addition to having apoptotic molecules on the membrane and in the cytoplasm, the effectiveness of T and NK cells against cancer may depend on other immunomodulatory cytokines secreted locally or systemically, such TGF-β and IL-10. TGF-β produced by inflammatory cells and double negative T cells (in lpr and gld mice), has dual effect. In contrast to the local production leading to help tissues repair, increased amounts of TGF-β in circulation, downregulate the immune response, resulting in failere to clear bacterial infections in MRL/lpr mice (Lowrance et al., 1994).

In this study, we investigated the role of perforin, Fas, and Fas ligand in the development of MCA induced tumor. We found that because of the defect in Fas-FasL-mediated apoptosis and high expression of TGF-β and IL-10, C57BL/6 *lpr/lpr* and C57BL/6 *gld/gld* mice developed MCA-induced tumor much earlier than C57BL/6 +/+ and perforin knock-out mice. High Fas expression on tumor cells and low TGF-β secretion provide sufficient immunity to perforin-deficient mice to control MCA-induced tumor.

Materials and Methods

Mice:

C57BL/6 +/+ (wild type) female mice were obtained from the National Institute of Health (Bethasda, MD). C57BL/6 *lpr/lpr*, C57BL/6 *gld/gld*, and Perforin KO mice were bred in our animal facility. Age-matched female mice were used for all experiments.

Antibodies:

Hamster monoclonal anti-mouse Fas (Jo2), anti mouse Fas ligand (Kay-10), biotin-conjugated polyclonal anti-mouse Ig, and streptavidin-PE were purchased from Pharmingen (San Diego, CA). FITC-labelled anti-hamster IgG was obtained from Jackson Immunological Research (West Groove, PA).

Induction of Tumor by Methycholanthrene (MCA):

Eight week old C57BL/6, C57BL/6 *lpr/lpr*, C57BL/6 *gld/gld*, and PKO mice were injected with 50, 100, and 250 ug of MCA/mice in 100 ul olive oil via intramascular (i.m) or subcutaneously (s.c) route. Mice were monitored daily for appearence of tumor. In addition, the tumor incidence and the growth rate of tumor were determined by measuring of tumor size every other day. For each group and route (i.m. or s.c.) 5-15 mice were used.

Isolation of MCA-Induced Tumor:

When tumor from MCA-injected mice reached the ~0.1 cm^3, they were excised aseptically. To obtain single cells, first, the tumors were cut into small pieces and then incubated in serum-free DMEM containing trypsin-EDTA. They were washed and cultured for several weeks *in vitro* in DMEM supplemented with 10%FCS, glutamine, non-essential amino acid, sodium pyruvate, and Hepes. Cells were stained to detect Fas and Fas ligand expression on the membrane and the levels of expression were evaluated by Flow Cytometer (Coulter, Miami, FL).

Injection of mice with LSA tumor cells:

One million *in vivo* grown LSA tumor cells were suspended in 100 µl PBS and injected into C57BL/6 +/+, C57BL/6 *lpr/lpr*, C57BL/6 *gld/gld*, or PKO (Perforin-deficient) mice via intraperiteneal route. Five days later, mice were treated with a dose of 50 mg/kg body-weight of BCNU, an anti-cancer drug. Control mice received the vehicle alone. Such mice died in 7-8 days. The BCNU-treated mice which survived, were further challenged with $10x10^6$ live LSA.

Isolation of LSA specific T cells:

C57BL/6 +/+, C57BL/6 *lpr/lpr*, C57BL/6 *gld/gld*, or PKO mice were immunized same way as described above. One month after the immunization, splenic T cells were purified by nylon-wool column and further stimulated in vitro with 50.000 irradiated LSA/well in 24-well cell culture plates. Following five days incubation, dead cells were removed by histopaque-gradient centrifugation and the cytotoxicity of T cells against LSA or syngeneic, but non-specific target EL-4 tumor cells were tested by ^{51}Cr-release assay.

Detection of TGF-β and IL-10:

Total RNA was extracted from T cells. T cells were purified from the spleens of C57BL/6 +/+, C57BL/6 *gld/gld*, C57BL/6 *lpr/lpr* or PKO mice at the age of 2 months (young mice) or 5 months (old mice). Over 90% of T cells from old *gld* and *lpr* mice are double negative ($CD4^-$-$CD8^-$). Total RNA was extracted by Guanidium-thioisocyanate method. One μg RNA was translated into cDNA by using MMLV reverse trancriptase (USA-Amersham) at 37^{o}C for 1 hour. The expression levels of TGF-β and IL-10 were determined by using primers purchased from Clontech. PCR conditions used for cytokines were: 2 min at 94^{o}C followed by 35 cycles of 45 sec denaturation at 94^{o}C, 45 sec annealing at 60, and 2 min extension at 72 ^{o}C. PCR products were separated in 1.5% agarose and analyzed with gel imager. β-actin was used as an internal control.

Results

Kinetics of tumor-induction by MCA:

MCA is a well-known carcinogenic agent. To test whether +/+ (Fas^+, $FasL^+$), *gld* (Fas^+, $FasL^-$), *lpr* (Fas^-, $FasL^+$), and PKO (Fas^+, $FasL^+$, but perforin deficient) mice displayed different sensitivity, less than 2 month-old mice were injected with 100 or 250 μg/mice MCA through the intramascular route. Injection of 100 μg of MCA induced

tumor in 20% of *lpr* and *gld* mice at day 45 and 100% of *lpr* and *gld* mice developed tumors by day 75 and 90, respectively (Figure 5.1 A). However, tumors started appearing in wt and PKO mice at day 120 and 135, respectively (Figure 5.1 A). Interestingly, when

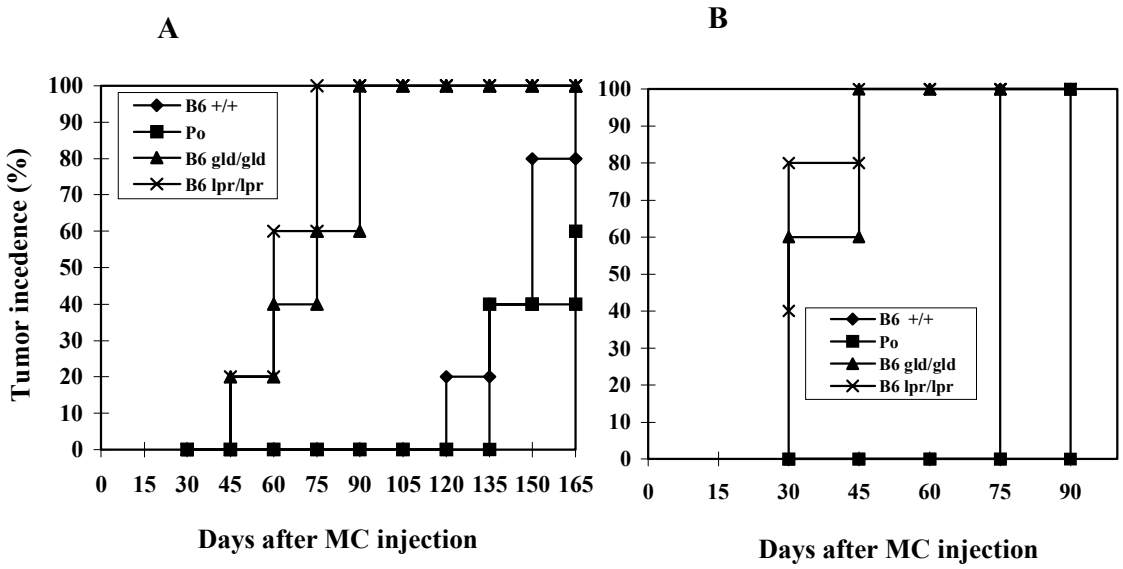

Figure 5.1: C57BL/6 +/+, C57BL/6 *lpr/lpr*, C57BL/6 *gld/gld*, or PKO mice were injected with different doses of MCA via intramascular and appearance of the tumor were observed. (A) 100 µg/mice, (B) 250µg/mice dose of MCA.

we ended experiment at day 160, 40% of perforin deficient and 20% of wt mice had not developed tumor. At higher doses (250μg/mice), 80% of lpr and gld mice developed tumors as early as 30 days after injection (Figure 5.1 B). In contrast, the tumor in wt and perforin-deficient mice started appearing 75 and 90 days after administration of MCA, respectively (Figure 5.1 B). In perforin-deficient mice, the tumor development was delayed at even higher doses of MCA, when compared to the wt mice. These data suggested that the immune system of PKO mice is fully capable of responding of induction of tumor by carcinogenesis.

We next administered 100 μg/mice MCA via different route, subcutenous (s.c) and the tumor incidence and the tumor volume were noted every other day. Similar to our observation following i.m. injection, both Fas-deficient *lpr* and FasL-defective *gld* mice (40% and 20%, respectively) showed tumors at day 38 (Figure 5.2 A). By day 80, following MCA injection, 100% of *lpr* and *gld* mice developed tumors. First signs of tumor were seen in wt and PKO mice at day 72 (Figure 5.2 A). Surprisingly, only 20% of wt and PKO mice showed tumor at day 160.

To further test whether all group of mice were capable of controlling the tumor growth, we measured tumor volume every other day. Figure 5.2 B showed that lpr and gld mice not only developed tumor much early, they could not control tumor-growth as well. In contrast, the tumor size in wt and PKO mice was significantly less than that seen *lpr* and *gld* mice.

Lpr and gld mice are sensitive the growth of an syngeneic, established tumor:

One million *in vivo* grown LSA tumor cells were injected to C57BL/6 +/+, PKO, C57BL/6 *lpr/lpr* or C57BL/6 *gld/gld* mice. These mice were treated with an anti-cancer-

drug, BCNU. The BCNU-cured mice were rechallenged with the same tumor, and 100 % of the wt and PKO mice survived with this challenge (Figure 5.3).

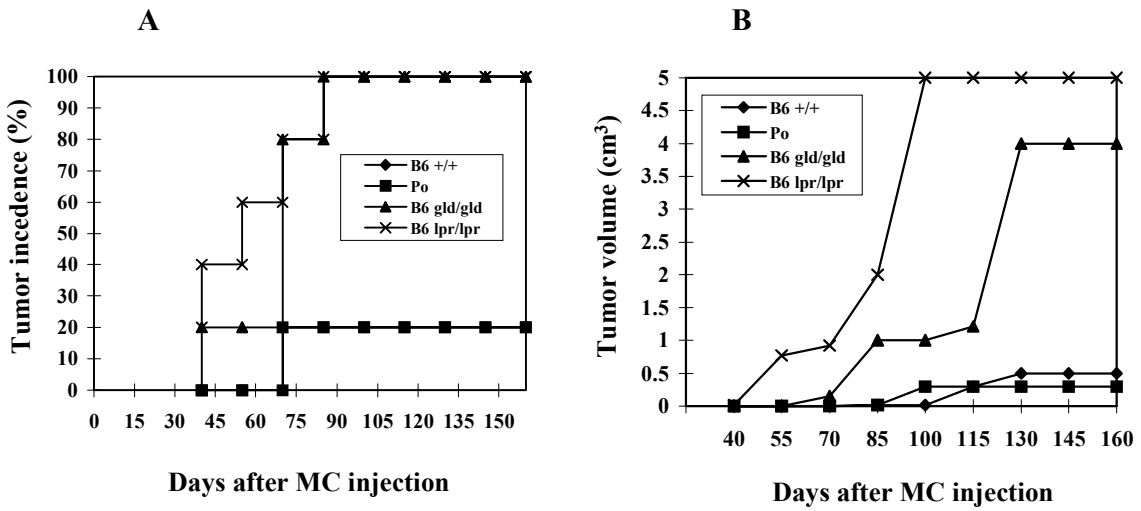

Figure 5.2: C57BL/6 +/+, C57BL/6 *lpr/lpr*, C57BL/6 *gld/gld*, or PKO mice were injected with a 100 μg/mice dose of MCA via subcutaneous. The appearance of the tumor and tumor sizes were measured. (A) Incidence of the tumor, (B) the tumor sizes (cm^3).

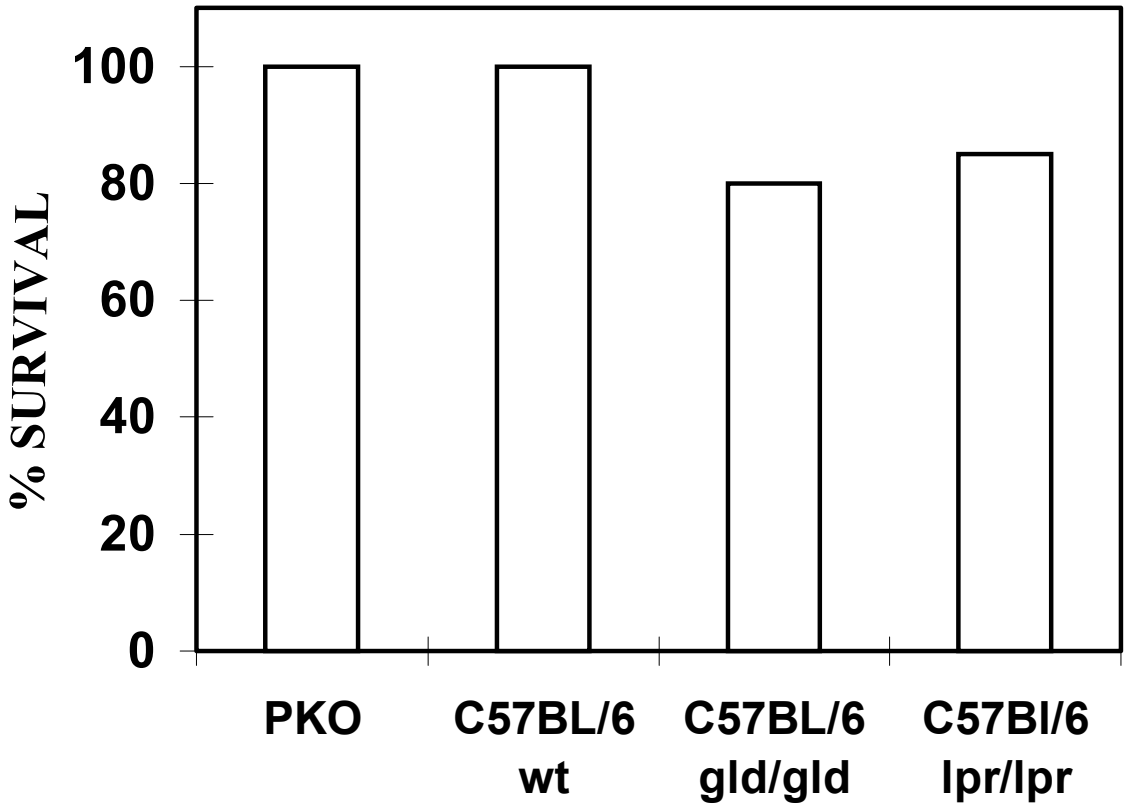

Figure 5.3: C57BL/6 +/+, C57BL/6 *lpr/lpr*, C57BL/6 *gld/gld*, or PKO mice were injected with LSA tumor cells and in day 5, were treated with BCNU. The survival rates were observed 2-3 weeks after immunization.

The young *lpr* and *gld* mice were partially protected by BNCU (Figure 5.3), but 4 month old tumor-bearing *lpr* and *gld* mice treated with BCNU died. Upon rechallenge of young mice with LSA, a very small percentage (0-20%) were found to survive (data not shown). These data suggested that young *lpr* and *gld* mice were capable of mounting anti-tumor activity, but in older mice the immune response was suppressed and therefore the mice were unable to reject the tumor.

Lpr and gld mice do not generate T cell response against tumor:

C57BL/6 +/+, C57BL/6 *lpr/lpr*, C57BL/6 *gld/gld*, or PKO mice were immunized against LSA and T cells were removed from the spleens. The specificity of T cells to the LSA was tested with cytolytic assay. The T cells from wt (Fas^+, $FasL^+$ and $perforin^+$) mice displayed highest cytoxicity to the LSA tumor cells (Figure 5.4). PKO mice which can only use Fas-Fas ligand interactions to clear the tumor, also induced significant apoptosis on the LSA targets (Figure 5.4). However, T cells from *lpr* (Fas^-) and *gld* ($FasL^-$) mice failed to show significant cytolytic activity to the same tumor (Figure 5.4). These data suggested that during the generation of T cell response against tumor cells, Fas-Fas ligand interactions play an important role. Tumor specific T cells can used both perforin and FasL, however FasL is sufficient to clear cancer cells, but not perforin.

DN T Cells Express TGF-β and IL-10:

Previous studies have shown that Fas-FasL-dependent apoptosis is impaired in *lpr* and *gld* mice, and furthermore DN T cells constitutively expressed perforin and induced apoptosis in vitro (Dennis-Hammond et al., 1993). In vivo, large number of DN T cells accumulate in LNs and spleen, due to the impaired apoptosis. Furthermore, perforin-deficient mice could control tumor growth as good as wt mice.

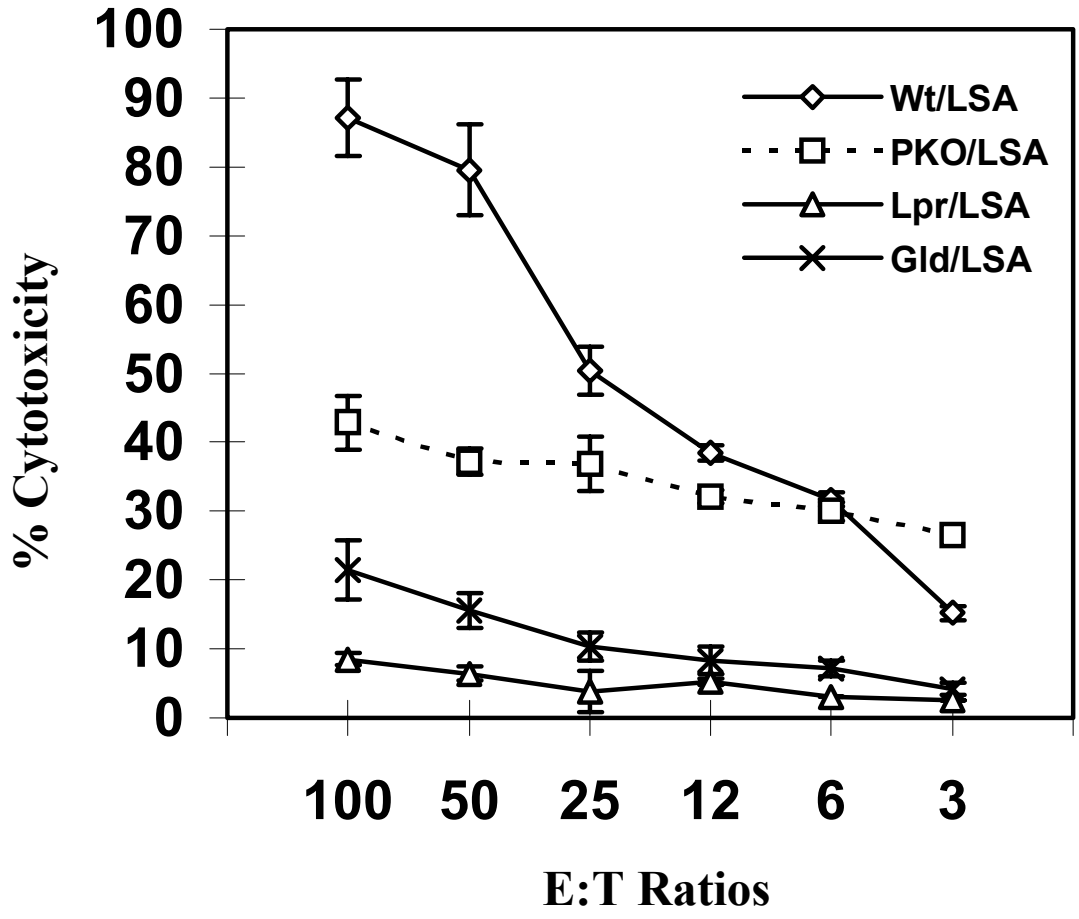

Figure 5.4: C57BL/6 +/+, C57BL/6 *lpr/lpr*, C57BL/6 *gld/gld*, or PKO mice were injected with LSA and treated with BCNU. Two-three weeks after immunization, splenic T cells were further primed with LSA *in vitro* for 5 days and the cytotoxicity of T cells againist LSA tumor cells was determined by ^{51}Cr-release assay.

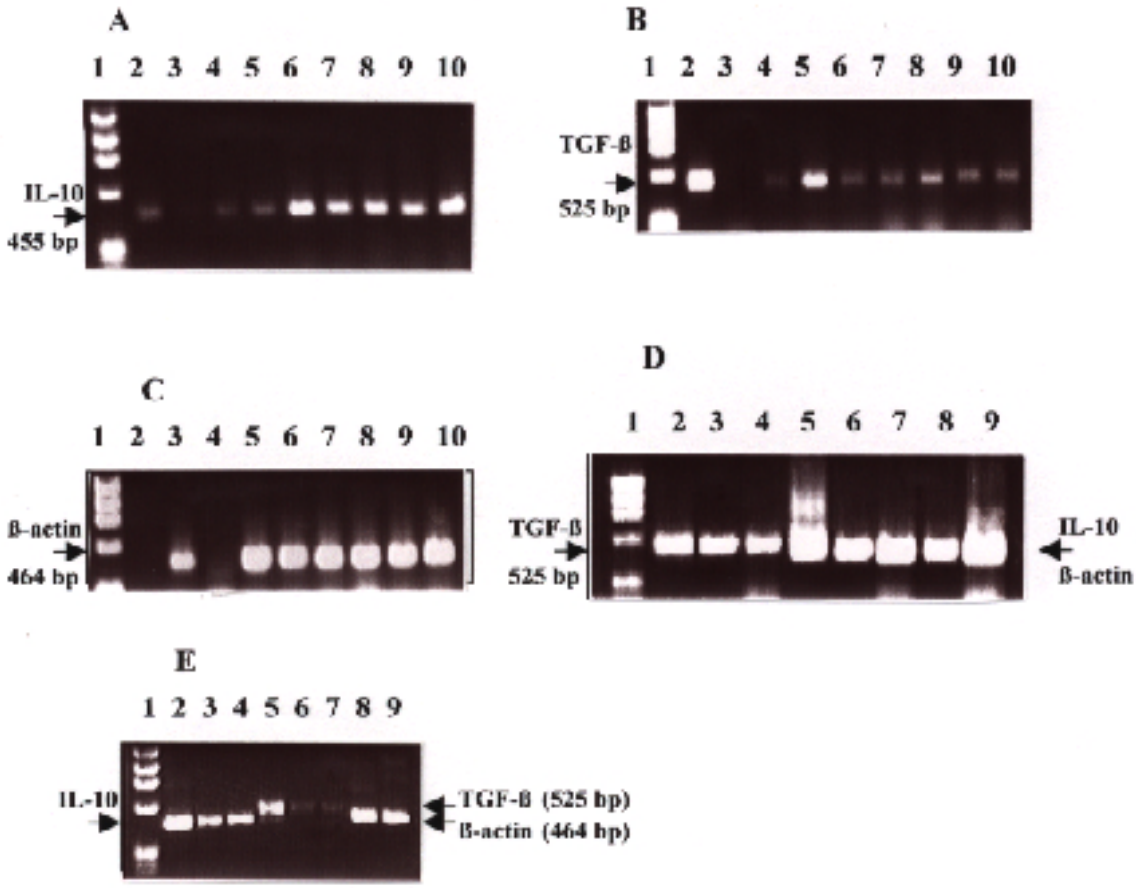

Figure 5.5: The detection of TGF-β and IL-10 expression. (A) IL-10 expression in T cell, lane 1 marker; 2, positive control; 3 young wt mice; 4, old wt mice; 5, young gld mice; 6, 7, and 8 old gld mice, and lane 9 and 10 young and old lpr mice, respectively. (B) TGF-β expression in T cells, lane 1 marker; 2, positive control; 3 and 4, old and young wt mice; 5 old gld mice, 6 and 7 young gld mice; 8 old and 9-10 young lpr mice. (C) β-actin. Lane are the same order as A or B. (D) TGF-β and IL-10 expression on double negative T cells. Lane 1 marker; 2, TGF-β positive control, 3 and 4 TGF-β gld and lpr; 5, IL-10 positive control; 6 and 7 IL-10 gld and lpr; 8 and 9, β-actin for gld and lpr DN T cells. (E) IL-10 and TGF-β expression on T cells from PKO mice. Lane 1 marker; 2 IL-10 positive control; 3 and 4, IL-10 expression by young and old PKO mice; lane 5, TGF-β positive control; lane 6 and 7, TGF-βexpression by young and old mice, and lane 8 and 9, β-actin control.

Thus, we further investigated to detect presence of some immunosuppressive molecules, such as TGF-β and IL-10. The expression levels of these molecules were studied by RT-PCR. T cells were purified from 4 groups of 2 month (young) and 5-6 month old mice. Both young and old wt mice express very low levels of TGF-β and IL-10 (Figure 5.5 A and B, lane 3 and 4). In contrast T cells from both lpr and gld mice expressed very high level of IL-10 and TGF-β. The amount of TGF-β in old mice was more pronounced in *lpr* and *gld* mice (Figure 5.5, B lane 5 and 8, when compared to the lanes 6 and 9). When we further investigated the source of immunosuppressive molecules TGF-β and IL-10, in lpr and gld mice, both molecules were constitutively expressed by splenic DN T cells (Figure 5.5 D , lanes 3, 4 and 6,7). We also looked for the expression of these molecules by T cells of PKO mice and repeated the experiment 3 times with different groups of mice. Interestingly, the TGF-β levels in young and old PKO mice was even lower than the wt mice (Figure 5.5 E, lanes 6 and 7). In two experiments we could not detect significant levels of IL-10, but when we used much older PKO mice, IL-10 levels were comparable (Figure 5.5 E, lanes 3 and 4).

Expression of Fas by MCA-induced Fibrosarcoma:

The resistance observed in PKO mice against MCA-induced tumor lead us to further investigate the expression of other apoptotic molecules. Tumor cells were removed from 4 groups of mice and adapted to *in vitro* growth. Fas and Fas ligand expression were studied by flow cytometry. Fas ligand expression was undetectable (data not shown). Tumor cells from lpr mice did not express Fas (Figure 5.6, B). However, significant levels of Fas were detected in wt and gld mice (Figure 5.6, C and D). Surprisinly, MCA-induced tumor from PKO mice displayed very high level of Fas expression, when compared to the Fas expression in *wt* and *gld* mice (Figure 5.6, E, 41.9 % compared to 25.9% wt).

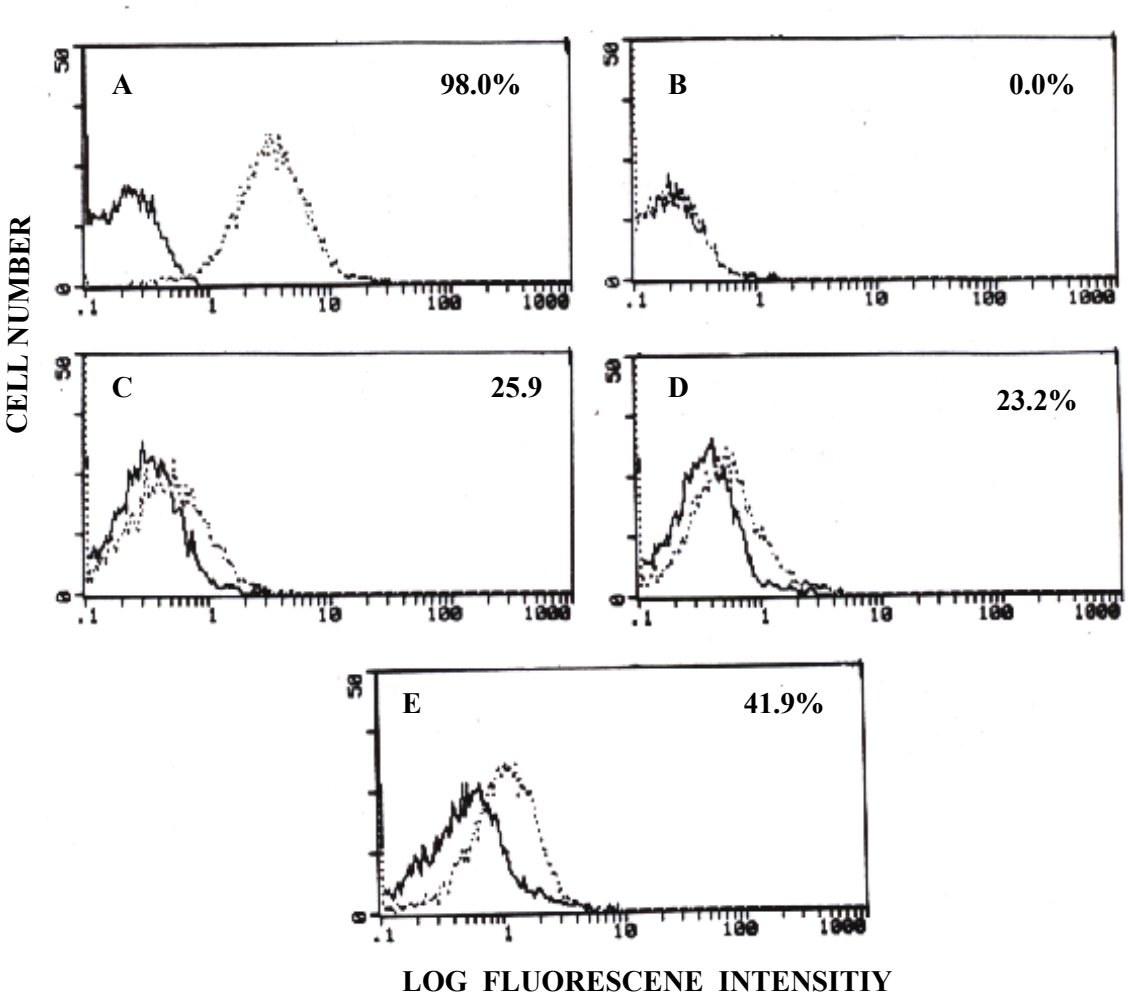
CELL NUMBER
A
98.0%
B
0.0%
C
25.9
D
23.2%
E
41.9%
LOG FLUORESCENE INTENSITIY

Figure 5.6: Detection of Fas receptor on the MCA induced tumor cells. Fibrosarcomas were isolated and adapted to in vitro growth condition. Fas expression was determined using flow cytometry. (A) Fas expression in control thymus, (B) tumor from C57BL/6 *lpr/lpr* mice, (C) tumor from C57BL/6 *gld/gld* mice, (D) tumor from C57BL/6 +/+, and (E)tumor from Perforin-deficient mice.

Discussions:

FasL-mediated apoptosis is a major cytolytic pathway used by CTLs and NK cells. The maximal activation of the CTLs, and possibly NK cells, requires reverse Fas or Fas ligand signaling. The *lpr* or *gld* mice can not use Fas-Fas ligand interactions because of defects in these molecules. In this study, we demonstrated that Fas or FasL-deficient mice were sensitive to a chemical-induced tumor, suggesting that these two molecules play an important role during the initiation of the neoplastic cells, priming of T cells and generation of tumor specific immunity. Eventually, activated tumor-specific CTLs and NK cells may clear malignant cells. C57BL/6 lpr and gld mice injected with 100 ug of MCA developed tumor much earlier than C57BL/6 wt and PKO mice in both subcutaneous and intramascular injections. Increased doses of MCA causes early appearance of tumor cells. However, development of the MCA-induced tumor in wt and PKO mice was significantly delayed. There is plenty of evidence that Fas or Fas ligand deficiency make mice more sensitive for naturally occurring tumors (Davidson et al., 1998). Some other immunosuppressive molecules, such as TGF-β and IL-10 may also accelerate tumor incidence. For example, Lowrance et al. (1995) reported that upregulation of TGF-β expression suppressed the immune response against bacterial infections in MRL/lpr mice. We used mice 4-8 weeks of age and at this stage, TGF-β expression was relatively low suggesting that increased amount of this molecule in *lpr* and *gld* mice was not the only factor to augment tumor sizes. In contrast to the report from van den Broek et al.(1997), perforin-deficient mice developed tumor as late as wt mice. Despite the deficiency of perforin, they efficiently used other cytolytic pathways. For example, Fas expression was found to be significantly higher in MCA-induced tumor cells from PKO mice, when compared to the MCA-induced tumor cells from *wt* and *gld* mice. Furthermore, PKO mice do not exhibit DN T cells and express only low levels of TGF-β. These data suggested that PKO mice may be able to reject the tumor by using Fas-FasL pathways.

Four groups of mice were also injected with $1x10^6$ LSA tumor cells and 5 days later, were treated with BCNU, an anti-cancer drug. In 8 weeks, only 60% of *gld* and *lpr* mice

survived, when compared to the 100% survival rate of wt and PKO mice. The survival rate in lpr and gld mice was closed to zero when the cured mice were rechallenged with the same tumor cells. However, the survival rate of wt and PKO mice was still 100% after rechallenging with LSA, suggesting that perforin deficient mice can efficiently use other cytolytic pathways. The lpr and gld mice failed to reject the tumor after BCNU treatment because T cells were unable to mount a cytotoxic response as seen by their inability to kill LSA tumor *in vitro* cytolytic assays. In contrast, T cells from immunized wt or PKO mice effciently killed the LSA tumor cells *in vitro* cytolytic assay, thereby suggesting that these mice could generate strong anti-tumor T cell-mediated immunity. The T cell responses were LSA-specific because same T cells failed to kill syngeneic EL-4 tumor cells. These data suggest that perforin-deficient mice carry all neccassary molecules to clear tumor cells.

In this study, we showed that deficiency Fas-FasL interactions put an individual at great risk for sensitivity for pollutant-induced cancer. Both perforin and FasL may be used to delete cancer cells, however, Fas-FasL deficiency is associated with other immunoregulatory diseases. Thus, we suggest that Fas and Fas Ligand are very important molecules for initiation and development of the cancer.

Chapter 6: Significance of the Current Research

The current study demonstrates that Fas-FasL interactions between the tumor cells and the immune cells of the host play an important role in the outcome tumor growth and host survival. Normally the cells of the immune system express FasL which they use to kill cancer cells. However, the current study demonstrates that when tumor cells express FasL, they can use this molecule to destroy the immune cells of the host. This immune-evasion may facilitate tumor-growth. Furthermore, the immunotoxicity causes by the tumor cells, may account for the immunosuppression seen in cancer patients. The soluble FasL produced by the tumor cells can also cause damage to other organs which express Fas. This may account for the severe organ toxicity seen in cancer patients. The current study also demonstrates that expression of the Fas on tumor cells may make them more immunogeneic. Thus, transfecting the Fas gene into tumor cells may constitute a novel approach to immunize cancer patients.

Our results suggested that immunotherapy against FasL-bearing tumor may pose problems and that in such instances, attempts will have to be made to block the FasL produced by the tumors. The FasL-bearing tumor cells may be less immunogeneic because they may kill the immune cells which attempt to mount a response. In such as instance, it may be possible to use a vaccine consisting of tumor cells in which FasL-epression has been blocked. Together our studies demonstrate manipulation of Fas-FasL interaction between the host derived immune cells and tumor cells may constitute a novel approach to treat cancer.

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CURRICULUM VITAE (updated on 04/25/1999)

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Educational Background:

B.S. Ondokuzmayis University, Department of Plant Sciences, 1985.
M.S. Ondokuzmayis University, Institute of Sciences, 1988.
M.S. The Ohio State University, Department of Molecular Genetics, 1993.

Research Title:

Role of perforin and Fas ligand in the activation of cytotoxic T lymphocytes and its influence on anti-tumor immunity.

Course Work: In addition to the listed courses, I took Prokaryotic and Eukaryotic Gene Expression, Microbial Genetics, Molecular Biology Labrotory, Advance Cell Biology, and Cytogenetics at the Ohio State University.

VMS 5984 Current Research in Immunology.
BIOL 4704 Immunology.
BIOL 6704 Topics in Immunology.
BIOL 6024 Topics in Molecular Cell Biology and Biotechnology.
STAT 5605 Biometry I.
BIOL 6704 Topics in Immunology.
BIOL 6704 Topics in Immunology

QCA= 3.80

Grant Submitted:

Sigma Xi grants-in-Aid of Research. Fall, 1995. Title of Study: Role of Fas in the induction of anti-tumor immunity.
Sigma Xi grants-in-Aid of Research. Spring, 1997. Title of Study: The immunotoxicity induced by tumor secreting soluble Fas ligand.
Graduate Student Assembly, Spring, 1998. Title of Study: Development of Fas specific T cells.

Grant Funded:

Sigma Xi Grants-in-Aid-of Research, $450, Spring 1996
Matched by the Biology Department, $450, Spring 1996
Sigma Xi Grants-in-Aid-of Research, $600, Spring 1997
Matched by the Biology Department, $500, Spring 1997
Graduate Student Assembly, $500, Spring 1998
Matched by the Biology Department, $500, Spring 1998

Graduate Teaching Assistantships:

Two third teaching assistantship, Spring, 1996.
Two third tuition waiver, Spring, 1996.
80% teaching assistantship, Fall, 1996.
80% tuition waiver, Fall 1996.
Full assistantships, Spring, 1997
Full assistantships, Fall 1997
Full assistantships, Spring 1998
Full assistantships, Fall1998

Professional Memberships

Virginia Acedemy of Sciences
Associate member of Sigma Xi

Student Teaching Evaluation:

Spring, 1996: 3.0
Fall, 1996: 3.2
Spring, 1997: 3.2 (Mean of the three classes. One of classes teaching evaluation is **3.6**)
Spring, 1998: 3.7 (3.6, 3.7, and 3.8)

Graduate Research Assistantships:

One third research assistantships, Spring, 1996.

Abstracts:

Zeytun, A., Nagarkatti, M., and Nagarkatti, P.S. Interaction between Fas and FasL plays an important role in the tumor specific cytotoxicity mediated by cytolytic T cells. 87th Annual Meeting of the American Association for Cancer Researchers, April 20-24, 1996.

Zeytun, A., Nagarkatti, M., and Nagarkatti, P.S. The role of perforin and Fas ligand in the induction of anti-tumor immunity. Published in the American Cancer Society, 16th Annual Cancer Researchers in Virginia Symposium booklet. March, 31, 1996.

A. Rafi, A. Zeytun, M. Nagarkatti, and P. Nagarkatti. Significance of age dependent appearance of cytolytic double-negative T cells and their ability to costitutively produce cytokines in the regulation of immune response in mice exhibiting defects in Fas and Fas ligand (FasL) expression. First International Conference On Immunology and Aging. June 16-19, 1996.

Nagarkatti, P. S., Zeytun, A., and Hassuneh, M. Apoptosis induced by cytolytic T cells and reverse apoptosis mediated by tumor cells may play a crucial role in host-tumor interactions. 4th IUBMB "The life and death of the cell" conference. Edinburgh, July 1996. Published in Biochemical Society Transactions, p. 618.

Zeytun, A., Hassuneh, M., Nagarkatti, M., and Nagarkatti, P. S. Fas-Fas ligand based interactions between tumor cells and tumor-specific CTL: A lethal two-way street. American Association of Immunologist, February 22-26, 1997, San Francisco, Puplished in symposium booklet.

Zeytun, A., Nagarkatti, M., and Nagarkatti, P.S. Immunotoxicity induced by tumor mediated sFasL. Published in the American Cancer Society, 18th Annual Cancer Researchers in Virginia Symposium booklet. March, 14, 1998.

Presentations at Meetings:

Zeytun, A., Nagarkatti, M., and Nagarkatti, P.S. Interaction between Fas and FasL plays an important role in the tumor specific cytotoxicity mediated by cytolytic T cells. Presented at 87th Annual Meeting of the American Association for Cancer Researchers, April 20-24, 1996. Washington, DC.

Zeytun, A., Nagarkatti, M., and Nagarkatti, P.S. The role of perforin and Fas ligand in the induction of anti-tumor immunity. Presented at American Cancer Society, 16th Annual Cancer Researchers in Virginia Symposium. March, 31st, 1996. Norfolk, VA.

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Zeytun, A., Hassuneh, M., Nagarkatti, M., and Nagarkatti, P. S. Fas-Fas ligand based interactions between tumor cells and tumor-specific CTL: A lethal two-way street Presented at American Cancer Society, 17th Annual Cancer Researchers in Virginia Symposium. March, 15th, 1997. Richmond, VA.

Zeytun, A., Nagarkatti, M., and Nagarkatti, P.S. Immunotoxicity induced by tumor mediated sFasL. American Cancer Society, 18th Annual Cancer Researchers in Virginia Symposium. March, 14, 1998.

Zeytun, A., Nagarkatti, M., and Nagarkatti, P.S. Immunotoxicity induced by tumor mediated sFasL. Virginia Academiy of Sciences. George Mason University, May, 29, 1998.

Seminars:

Presented a seminar entitled, "Immune response for/against bacterial and viral superantigens" to the Microbiology and Immunology division on November 20, 1996.

Papers Published:

Zeytun, A., Hassuneh, M., Nagarkatti, M., and Nagarkatti, P. S. Fas-Fas ligand based interactions between tumor cells and tumor-specific CTL: A lethal two-way street. Blood, 1997, 90 (5) September 1:1952-1959.

Asimah Q. Rafi, **Ahmet Zeytun**, Michael J. Bradley, D. Philip Sponenberg, Randolph L. Grayson, Mitzi Nagarkatti, and Prakash S. Nagarkatti. 1998. Evidence of involvement of Fas ligand and perforin in the induction of vascular leak syndrome. Journal of Immunology 1998, 161:3077.

Michael J. Bradley, **Ahmet Zeytun**, Asimah Rafi-Janajreh, Prakash S. Nagarkatti, and Mitzi Nagarkatti. 1998. Role of spontaneous and IL-2 induced NK activity in the cytotoxicity and rejection of Fas^+ and Fas^- tumor cells. Blood, 1998. 92:

Ahmet Zeytun, Mitzi Nagarkatti, and Prakash S. Nagarkatti. Growth of FasL-bearing tumor cells in syngeneic murine host induces apoptosis and toxicity in Fas^+ organs: Role of soluble form of mouse Fas Ligand. (Blood in press).

Ahmet Zeytun, Mitzi Nagarkatti, and Prakash S. Nagarkatti. The influence of Fas, Fas ligand and perforin in the induction of tumor by Methylcholanthrene. (Submitted for publication).

Ahmet Zeytun, Mitzi Nagarkatti, and Prakash S. Nagarkatti. The functional characterization of a new isoform of the mouse Fas ligand. (Submitted for puplication).

Ahmet Zeytun, Mitzi Nagarkatti, and Prakash S. Nagarkatti. The role of the Fas and Fas ligand in the growth and regression of immunogeneic vs. non-immunogeneic tumor. (Manuscript in preparation).

Work experiences:

1991-1993: Identification and cloning novel Tyrosine Phosphatase (PTP1β) in different fibroblast cell lines. RT-PCR and basic cloning techniques were used.

1993-1995: Cloning, producing, and purification of mutated form of tyroid fusion protein and the role of CAM and I-CAM in the development of brain (southern and in situ hybridization).

1995- present: I have been working on Fas, Fas ligand dependent apoptosis and cancer in Drs. Nagarkatti's labs.

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