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γδ T cells Derived from Peripheral Blood and Cerebrospinal Fluid of Multiple Sclerosis Patients: Cytotoxic Mechanisms, Inhibitory Natural Killer cell Receptors, and Protein Analysis

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Abstract

Multiple sclerosis (MS) is characterized by inflammation of the central nervous system, destruction of the oligodendrocyte/myelin unit, demyelination, and axonal loss. MS is believed to result from an autoimmune reaction mediated by αβ T cells against the myelin/oligodendrocyte unit. There is, however, increasing evidence that γδ T cells are also responsible for the damage observed in the MS brain: γδ T cells are found in increased numbers in the peripheral blood (PB) and cerebrospinal fluid (CSF) of MS patients, accumulate in MS lesions, and lyse oligodendrocytes *in vitro*. The cytotoxic mechanism(s) utilized by γδ T cells and the control of these mechanisms are incompletely understood, although it has been reported that γδ T cell mediated cytotoxicity can be regulated via inhibitory natural killer cell receptors (iNKR).

The work described in this thesis was undertaken to gain a more complete understanding of the cytotoxic mechanism(s) utilized by γδ T cells, to investigate the control of γδ T cell mediated cytotoxicity by iNKR, and to search for differences in cytotoxic phenotype and function between PB and CSF derived γδ T cells.

My results have demonstrated that γδ T cell mediated cytotoxicity proceeds via the perforin/granzyme and Fas/FasL pathways, that granzyme B is an especially potent cytotoxic mediator for these cells, and that the exact method of attack is influenced by the target cell. As well, the cytotoxicity mediated by γδ T cells cannot be modulated solely via manipulation of the iNKR/HLA class I molecule interaction, but instead is under the control of a number of inhibitory and activating receptors. It is the balance of the signals these receptors generate that determines the action of the γδ T cell. A number of functional and phenotypic differences were observed between PB and CSF derived cells.

Two dimensional electrophoretic analyses of protein isolated from PB and CSF derived γδ T cells revealed an MS CSF specific protein "profile" consisting of 7 proteins absent from the MS CSF samples.

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List of Abbreviations

ADP	adenosine diphosphate
AIDS	acquired immunodeficiency syndrome
Apaf 1	apoptotic protease activation factor 1
APP	amyloid precursor protein
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
BA	brefeldin A
BBB	blood brain barrier
BLAST	basic local alignment search tool
BLT	N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester
CAD	caspase activated DNAse
CMA	concanamycin A
CNS	central nervous system
cR/A	complete RPMI/AIM-V mixture
cRPMI	complete RPMI
CSF	cerebrospinal fluid
DFF	DNA fragmentation factor
DISC	death-inducing signalling complex
DMSO	dimethyl sulfoxide
DTNB	5,5′-dithio-bis(2-nitrobenzoic acid)
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid,
E:T	effector to target ratio
FACS	fluorescence activated cell sorting
FasL	Fas ligand
Fc	fragment, crystalline
FcR	Fc receptor
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FS/SS	forward scatter/side scatter
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GTP	guanosine triphosphate
HC	healthy control
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HTLV-1	human T cell lymphotropic virus type 1
HSV	herpes simplex virus
IEF	isoelectric focusing

IFN	interferon
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
iNKR	inhibitory natural killer cell receptor
IPG	immobilized pH gradient
ITAM	immunoreceptor tyrosine based activation motif
ITIM	immunoreceptor tyrosine based inhibition motif
KIR	killer inhibitory receptor
mAb	monoclonal antibody
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
OMK	owl monkey kidney cells
OND	other neurological disease
NK	natural killer
NKR	natural killer cell receptor
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PFA	paraformaldehyde
PHA	phytohemagglutinin
pI	isoelectric point
PLP	proteolipid protein
PMA	phorbol myristate acetate
PMSF	phenylmethanesulfonyl fluoride
QR	quantum red
rIL-2	recombinant interleukin 2
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEA/SEB	Staphylococcal enterotoxin A/B
SLAM	signalling lymphocyte activation marker
SLE	systemic lupus erythematosus
SH2	Src homology 2
shTNF-R	soluble human tumor necrosis factor receptor
tBid	truncated Bid
TCID	tissue culture infectious dose

TCR	T cell receptor
TNF	tumor necrosis factor
TSST-1	toxic shock syndrome toxin 1

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Chapter One- Introduction

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system (CNS; 1). This disease typically occurs in young adults, the mean age of onset is about 30 years of age (2), and affects twice the number of women as men (3). It is widely believed that MS is the result of an autoimmune reaction directed against the myelin sheath or its components [such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), myelin associated protein (MAP), or proteolipid protein (PLP)] or against the myelin-producing cells (the oligodendrocytes; 4-7). The genetic work completed to date indicates that multiple sclerosis is due to multiple genes which confer susceptibility in combination with environmental factors (8-11).

The course and severity of MS are quite variable and unpredictable. The majority of patients, approximately 85%, endure a relapsing/remitting type of disease (2). Relapses may present with new symptoms, the worsening of old symptoms, or both (12). For between 40 and 65% of patients who present with a relapsing/remitting disease, a secondary progressive disease develops (2). That is, although the patient experiences relapses followed by remissions, the extent of recovery from each relapse is not complete and so with time a gradual worsening of the patient is observed.

In contrast, there is a minority of patients for whom the disease is primary progressive. These patients do not undergo the relapsing/remitting form of disease, but rather become progressively worse from the onset of disease without any remissions (13).

Some of the most commonly occurring symptoms and clinical features of MS include: optic neuritis, vertigo, vomiting, pain, scanning speech, weakness, spasticity,

bladder and bowel dysfunction, tingling or tightness of the extremities, band-like sensations about the trunk, depression, weight loss, fatigue, and intention tremor (1,12, 14). The number of symptoms experienced and the order in which they occur varies from patient to patient. The fact that there is no "pattern" of symptoms can be explained by the observation that myelinated axons anywhere in the CNS can be involved (15).

The diagnosis of MS relies mainly on clinical history and neurologic examination, as a specific laboratory test is not available. The most widely used criteria for the clinical diagnosis of MS are those proposed by Poser et al. (16) and MacDonald et al. (17).

1.2 The Pathology of the Multiple Sclerosis Lesion

The characteristic pathology of multiple sclerosis includes inflammation of the CNS, demyelination, axonal injury/loss, destruction of oligodendrocytes, and astroglial scarring (18-21). Although this is the characteristic pathology of MS, there is much variability between different stages of the disease and from patient to patient. Based on their examination of actively demyelinating lesions isolated from MS patients, Lucchinetti et al. (22) identified four patterns of demyelination. The only similarity between these four patterns is that the inflammatory infiltrates are dominated by T cells and macrophages. Pattern I and II lesions are typically centered on small veins and venules and have sharply demarcated edges with perivenous extensions. Staining for myelin proteins is absent (MBP, PLP, MAG, MOG). The loss of oligodendrocytes is variable at the lesion borders, while high numbers of oligodendrocytes are present within the lesion center. There is also a high incidence of remyelinated shadow plaques. Pattern II lesions are the only category to have prominent deposits of immunoglobulins (mainly IgG) and complement C9 neo antigen. Pattern III plaques are not centered by veins and

venules and do not have well defined borders. There is a preferential loss of MAG, while other myelin proteins (PLP, MBP, MOG) are still present within partially damaged myelin sheaths. The plaque center is devoid of oligodendrocytes and a pronounced loss of oligodendrocytes is observed at the lesion border. Shadow plaques are absent. Pattern IV plaques are sharply demarcated with radial expansion of the lesion. Oligodendrocyte death occurs in a small area of periplaque white matter and an almost complete loss of oligodendrocytes is seen within the lesion. Remyelinated shadow plaques are absent and myelin proteins are similarly distributed. These results suggest that MS is a disease with heterogeneous pathogenetic mechanisms and targets.

Demyelination leads to the formation of multifocal lesions known as plaques, which are typical of MS. These plaques are well demarcated from the surrounding tissue, can be of various sizes, and can be formed in almost any region of the CNS (1,23,24). Plaques are generally limited to white matter of the CNS (1,25). Myelin acts as an insulator for axons and as such increases the speed of conduction of an impulse along the axon from the nerve cell body to the synaptic junction (26-28). Therefore, demyelination of the axons decreases the speed of conduction and may also leave them susceptible to damage by macrophages, lymphocytes, cytokines, or antibodies (18).

For many years it was thought that the axons in multiple sclerosis brain were "relatively spared". It is now recognized that axonal injury or loss occurs in all MS plaques to some degree, is an early event in MS plaque development, and correlates with patient disability (29-36). Acute axonal damage can be detected by immuno-cytochemistry for amyloid precursor protein (APP). APP is produced in neurons and undergoes anterograde axonal transport. When an axon is transected, the transport is

interrupted, APP accumulates in the proximal axon ends, and APP-positive spheroids are formed (38-41). Since these spheroids are short-lived, lasting less than 30 days, their detection indicates acute axonal damage. Studies with brain tissue obtained from MS patients have demonstrated that axonal damage occurs early during disease and lesion development (the highest number of APP positive axonal spheroids was detected in tissue obtained from patients with disease duration of less than one year) and decreases with time (33,41). In addition, the extent of acute axonal damage correlated significantly with the number of infiltrating $CD8^+$ T cells and macrophages/microglia (i.e. inflammation) and was independent of demyelinating activity. This led to the suggestion that axonal damage occurs without visible demyelination or perhaps even precedes demyelination and that $CD8^+$ T cells and macrophages/microglia or their mediators are responsible for the injury to axons.

1.3 The Immunology of Multiple Sclerosis

The immune system is thought to be responsible for the damage incurred in multiple sclerosis. It is still not known if the destructive immune response is primarily directed against the CNS or if the damage seen in MS is the result of an immune response to some other antigen or infectious agent. There is also much debate concerning the target of demyelination. The most likely candidates are the myelin sheath, or its components, or the oligodendrocytes.

It has been suggested that demyelination is the result of "dying-back oligodendrogliopathy" (18,42). That is, it is not the oligodendrocyte itself which is damaged in MS, but rather its "luxury" function, namely myelination. Such damage

could result in the production of abnormal myelin, which may elicit an immune response, or could prevent the production and/or maintenance of myelin altogether.

Although the possibility of lesion subtypes is still being investigated, the MS lesion is typically inflammatory and contains cytokine secreting T lymphocytes ($CD4^+$ and $CD8^+$ $\alpha\beta$ T cells and an accumulation of $\gamma\delta$ T cells), activated macrophages and microglia, and a few B lymphocytes and plasma cells (21,43-47,344-346). Whether or how these cell types injure myelin and/or oligodendrocytes is still largely unknown. The sequence of events may be similar to the following scenario: Lymphocytes, activated in the periphery, penetrate the blood brain barrier (BBB) in an antigen non-specific manner (48-51). This movement across the BBB is mediated by adhesion molecules on the endothelial cells of the BBB and on the T lymphocytes. In fact, the expression of many adhesion molecules is increased on endothelial cells in MS lesions (52-55) and on activated T cells (52,54-56).

Once inside the CNS, activated T lymphocytes specific for CNS antigens are re-activated and remain in the CNS, while T lymphocytes which do not find their specific antigens return to the peripheral circulation (49,50). *In vitro* studies have demonstrated that re-activated $CD4^+$ and $CD8^+$ T cells can kill (or damage) oligodendrocytes (57-59). In addition, the re-activated T lymphocytes are able to incite a number of responses through the secretion of cytokines, particularly IFN-γ and TNF-α. These responses include: (1) the induction of inflammation (50), (2) increased expression of adhesion molecules which promotes T cell homing to the CNS (60-63), (3) recruitment and activation of lymphocytes and macrophages (51), (4) up-regulation of MHC class II molecules on microglia, and therefore increased antigen presentation (64-66), (5)

alterations in the permeability of the BBB thereby allowing the entry of B lymphocytes, macrophages, immunoglobulins, and complement (50,67), and (6) direct damage to CNS tissue (68-72), including the induction of apoptosis of oligodendrocytes (73,74).

The activation of macrophages and microglia may contribute to demyelination in a number of ways as these cells are capable of: (1) releasing cytokines, such as TNF-α, capable of damaging myelin and oligodendrocytes (70,75,76) and up-regulating the expression of adhesion molecules (62,77), (2) acting as antigen presenting cells to $CD4^+$ T lymphocytes (65,78-80), (3) damaging myelin sheaths or killing oligodendrocytes through the release of various mediators, including proteolytic enzymes, nitric oxide, and oxygen radicals (81-85), and (4) actively phagocytosing myelin (86).

1.4 γδ T Lymphocytes

While the majority of circulating $CD3^+$ T lymphocytes express the αβ T cell receptor (TCR), a small minority (0.5-10%) express the γδ TCR (87-90). γδ T cells are also present in lymph nodes, spleen, tonsils, intestine, and thymus (89-91). Although the γδ TCR gene diversity is limited (92,93), these cells are able to achieve a large TCR repertoire through multiple combinations of genes and junctional insertions and deletions (94). The V gene expression correlates somewhat with the localization of the cells, for example, 60-95% of peripheral blood (PB) γδ T cells co-express the Vγ9Vδ2 genes, with a smaller percentage expressing the Vδ1 gene (88,95,96), while in cerebrospinal fluid (CSF), the Vδ1 gene is more frequently expressed than Vδ2 (97).

1.4.1 Antigen Recognition

Unlike αβ T cells, γδ T cells do not typically express CD4 or CD8 accessory molecules (87,98-100). Antigen recognition by γδ T cells also differs from that of αβ T cells and more closely resembles antibody-antigen interaction than αβ TCR-MHC-peptide interaction (101). γδ T cells do not require antigen processing and presentation and are not restricted by classical MHC class I or II molecules (102-105). Instead γδ T cells are capable of directly recognizing antigens on pathogens and tumour cells, and self-antigens on damaged/stressed tissues (103,104,106-109). This direct recognition allows for a very rapid response. As expected, the nature of these antigens is quite different from the peptide antigens recognized by αβ T cells. γδ T cells respond to low molecular mass non-peptide antigens, including small phosphorylated microbial antigens (phosphoantigens; 110-113), alkylamines (114), and synthetic compounds such as monoalkyl phosphates (115) and aminobisphosphonates (116,117). γδ T cells are also stimulated by superantigens, such as TSST-1, SEA, and SEB (118-120).

Antigenic stimulation of γδ T cells typically results in proliferation, cytokine and chemokine production, and/or cytolytic activity. γδ T cells are capable of rapidly producing and secreting a variety of pro- and anti-inflammatory cytokines, including IL-2, -4, -5, -6, -7, -8, -10, IFN-γ, TNF-α, lymphotoxin, and GM-CSF (97,121-125). In fact γδ T cell clones simultaneously producing pro- and anti-inflammatory cytokines have been described, IL-4 and IFN-γ (126), Il-2, TNF-α, and IL-10 (127). γδ T cells also produce chemokines, such as RANTES, MIP-1α, MIP-1β, CCR1, CCR5, and lymphotactin (128,129), capable of recruiting monocytes/macrophages to sites of inflammatory reactions.

Stimulated γδ T cells also display profound MHC unrestricted cytotoxic activity against various tumour cell lines (102,117,130,131) and virus or bacteria infected cells (97,100,132-136), but not against mock-infected cells. Interestingly, the cytotoxic activity of γδ T cells is not limited to the antigen with which the cells are stimulated. For example, γδ T cells stimulated with mycobacterial antigen were able to lyse a broad range of target cells (B lymphoblastoid and T and B lymphoma lines; 121), as well as, HSV and vaccinia virus infected targets (134), γδ T cells stimulated with synthetic monoalkyl phosphate were able to lyse Daudi and K562 target cells (115), and γδ T cells stimulated with pamidronate (an aminobisphosphonate) were cytotoxic against a broad range of tumour targets, including Daudi and THP-1 (117). Further evidence of the broad cross-reactivity of γδ T cells comes from experiments carried out by Burk et al. (137), this group stimulated γδ T cell clones with several naturally occurring metabolites and were unable to isolate cells specific for only one ligand.

Although antigen recognition by γδ T cells is not MHC restricted, it is mediated through the TCR complex. The addition of anti-CD3 and/or anti-γδ TCR monoclonal antibodies (mAb) interferes with antigen stimulated cytokine production (121,138), proliferation (115,137), and cytotoxicity (97,102,132,134,136). Several other experiments have shown that transfection of the Vγ9Vδ2 genes into TCR deficient Jurkat mutants confers the antigen responsiveness of the "parent" γδ T cell to the mutant Jurkat cells (107,115,117).

There is also evidence that the natural killer cell receptors, a group of receptors which recognize HLA class I molecules on the surface of target cells, are important regulators of the γδ T cell response to target cells, including proliferation, cytokine

production, and cytotoxicity. These receptors and their role in γδ T cell function are discussed in later sections.

1.4.2 Function of γδ T Cells

While the exact role(s) of γδ T cells in immune responses is still poorly understood, evidence is mounting that these cells are important in microbial infections, autoimmunity, tumour immunology, and immunoregulation.

1.4.2.1 Microbial Infections

Many experiments indicate that γδ T cells play a role in the elimination of microbes or in the early immune response to infection. For example, γδ T cells proliferate in response to a broad spectrum of microbes, including *M. tuberculosis, M. leprae, S. aureus, Listeria monocytogenes*, group A streptococci (132), *Plasmodium falciparum* (139), *Salmonella* species (140), *Borrelia burgdorferi* (141), superantigens (139), and numerous virally infected cells (134,142). γδ T cells are also able to reduce the viability of *M. tuberculosis* (136), inhibit the asexual blood stages of *Plasmodium falciparum* (124), and kill a variety of virus and bacteria infected cells (97,100,132-136).

Increased numbers of γδ T cells have been detected in the peripheral blood of HIV (particularly $V\delta1^+$ cells; 143-145), EBV (146), *Plasmodium vivax* (147), *Leishmania donovani* (148), *Brucella melitensis* (149) and *Mycobacterium tuberculosis* (150,151) infected individuals. Depending upon the study and the infection, this increase in γδ T cells may represent a role in pathogenesis or protection. Perera et al. (147) found a positive correlation between the severity of malaria symptoms (especially gastrointestinal symptoms: nausea, vomiting, and anorexia) and the proportion of γδ T cells in the blood, suggesting that γδ T cells play a role in malaria pathogenesis. Sindhu et al. (131)

reported that γδ T cells were contributing to the depletion of bystander $CD4^+$ T cells in HIV infected individuals and in this way were expediting AIDS progression. In contrast, a strong correlation between the absence or loss of *Mycobacterium tuberculosis* reactive Vγ9Vδ2 T cells and the manifestation of active pulmonary tuberculosis was observed by Ueta et al. (152) and Li et al. (153). This loss of Vδ2 cells was not seen in patients with non-active tuberculosis, suggesting that γδ T cells contribute to the development of protective immunity to *M. tuberculosis*.

γδ T cells express molecules, such as NKRP1A, CCR1, and CCR5, which facilitate transmigration across vascular endothelium and homing to sites of inflammation (129,154) and have been shown to accumulate at the sites of infection, including the lesions of cutaneous leishmaniasis (155) and granulomatous leprosy (156), and the synovial fluid of Lyme arthritis patients (141).

This broad cross-reactivity of γδ T cells may be due to their ability to recognize a cellular ligand (perhaps a phosphoantigen) or MHC molecules which have been modified or induced by infection (134,142,157), rather than specific microbial antigens.

1.4.2.2 Tumour Immunology

γδ T cells have also been implicated in tumour immunology. A study of melanoma patients found that the absolute number and percentage of circulating γδ T cells was reduced in these patients, as was the percentage of TNF-α and IFN-γ producing γδ T cells, as compared with healthy controls (158). The authors suggested that the "numerical and functional impairment" of γδ T cells was contributing to the impaired immune response observed in these patients. Experiments with γδ T cell deficient mice found that the incidence of induced tumours was significantly increased in the mutant

mice as compared with control mice (159). Wilhelm et al. (160) suggested that the tumour regression observed in patients treated with a combination of pamidronate and low dose IL-2 was due to the activation and proliferation of γδ T cells.

Although a large number of studies had shown that γδ T cells are cytotoxic to tumour cell lines (102,117,130,131), the antigen(s) responsible for initiating the cytotoxic attack had remained unknown. It was suggested that the γδ T cells were responding to non-polymorphic target epitopes and not individual ligands (161). An interesting study by Gober et al. (162) showed that γδ T cells respond to endogenous mevalonate metabolites (phosphorylated non-peptidic molecules) in tumour cells. Treating the tumour cells with bisphosphonate drugs induced the accumulation of these metabolites and the tumour cells were shown to stimulate γδ T cells. [Mevalonate pathway dysregulation has been previously reported in malignancies and carcinomas (163,164)]. Perhaps the accumulation of these metabolites above physiological levels represents a general mechanism by which γδ T cells identify tumour cells.

1.4.2.3 Autoimmunity

Studies of several autoimmune diseases have identified differences in the absolute number and percentage of γδ T cells in the peripheral blood of patients as compared with healthy controls, suggesting a role for these cells in the autoimmune process. These values were decreased in patients with systemic lupus erythematosus (SLE; 165,166) and untreated Graves' disease (167) and increased in patients with autoimmune hepatitis, insulin-dependent diabetes treated for 3-6 months (167,168), active inflammatory bowel disease (169), and pulmonary sarcoidosis (170). A study of rheumatoid arthritis found

that treatment with disease modifying anti-rheumatic drugs slowly decreased the elevated levels of γδ T cells in peripheral blood of the patients to normal levels (171).

γδ T cells have also been found to accumulate in the sites of autoimmune inflammation, such as the portal areas of autoimmune hepatitis patients (168), the small intestinal epithelium of coeliac disease patients (172), and the perivascular areas of the skin of systemic sclerosis patients (173), as compared with controls. Further evidence supporting a role for γδ T cells in autoimmunity comes from reports of γδ T cells being responsible for the destruction of erythroid progenitors in a case of pure red cell aplasia or autoimmune anemia (174), γδ T cell mediated cytotoxicity being down-regulated in SLE patients (166), and γδ T cells augmenting the production of pathogenic anti-DNA autoantibodies in SLE (175). The observation that γδ T cells participate in antibody production not only indicates that γδ T cells are important in autoimmune pathogenesis, but that they function in the regulation of other immune cells.

1.4.2.4 Immunoregulation

The use of γδ T cell deficient animals has identified a role for these cells in microbial infection and autoimmunity. Perhaps more importantly, these models have indicated that an important function of γδ T cells is the regulation of other immune cells. For example, lupus-prone mice, deficient in γδ T cells, showed augmented hyper-gammaglobulinemia and autoantibody production, more severe renal disease, and increased mortality associated with a polyclonal expansion of αβ T cells (176). γδ T cell deficient mice infected with *Listeria monocytogenes* (infection induced orchitis) had an accelerated inflammatory response when compared with controls (177). A model of pulmonary allergic inflammation demonstrated that γδ T cells were required for the

induction of IL-4 dependent IgE and IgG1 production (178). These studies suggest that γδ T cells are capable of regulating the actions of αβ T cells, especially autoaggressive αβ T cells.

γδ T cells have also been shown to express a number of cytokines, including B cell growth factor, B cell differentiation factor, and IL-4, -5, and -10, which directly affect the proliferation, activation, and antibody production of B cells (124,127,148, 179,180). Other experiments have shown that γδ T cells are able to inhibit the cytotoxic activities of $CD8^+$ αβ T cells (181) and lyse autologous T cells (182).

1.4.2.5 Linking the Innate and Acquired Immune Systems

The extensive cross-reactivity of γδ T cells coupled with the fact that antigen recognition requires neither antigen processing nor MHC presentation allows for quick recognition of different microbes and rapid response to microbial infection (105,137). This rapid production of cytokines and chemokines can lead to the recruitment of inflammatory cells, such as neutrophils and monocytes/macrophages, and facilitate the induction of inflammation. It can also lead to the recruitment of B cells and the subsequent production of antibodies. In this manner, γδ T cells may act as a link between the innate and acquired immune systems. For example, King et al. (183) found that γδ T cell deficient mice became ill and died within 14 days of *Nocardia asteroides* infection, while control mice showed no clinical symptoms and cleared the infection within 7 days. Histopathologic examination of the lung parenchyma led to the conclusion that the γδ T cell deficient mice were unable to recruit and perhaps activate inflammatory cells and therefore could not clear the infection.

It would seem that the γδ T cell is a multi-purpose cell, involved in many arms of the immune system, from protecting against microbial pathogens and tumour cells to regulating the actions of other immune cells.

1.5 Natural Killer Cell Receptors

The "missing self" hypothesis developed by Lunggren and Karre (184) states that NK cells recognize and eliminate cells which either fail to express MHC class I molecules or express reduced levels of these molecules. This infers the existence of a receptor or group of receptors expressed by NK cells which are capable of recognizing MHC class I molecules on the surface of target cells and indicating to the NK cell whether these potential targets are self or non-self. Indeed, the functions of NK cells are controlled by the interactions of numerous receptors including the natural killer cell receptors (NKR). The NKR scan the surface of the target cell for normal expression of MHC class I molecules. If the appropriate expression is identified, an inhibitory signal is transduced within the NK cell, and the target cell is spared, if not the target cell is lysed (185,186). The NKR include both inhibitory and activating receptors and are divided into two main groups, the killer cell immunoglobulin (Ig)-like receptors (KIR) and the type II C-type lectin family. While the activating receptors have been somewhat ignored, much research has focussed on the inhibitory NKR (iNKR).

1.5.1 Killer cell Immunoglobulin-like Receptors

Members of the KIR family possess 2 or 3 extracellular Ig-like domains (187,188) and have either a long or short cytoplasmic domain which determines whether the receptor is inhibitory or activating, respectively (189). Inhibitory KIR have 1 or 2 immunoreceptor tyrosine based inhibition motifs (ITIM) in their cytoplasmic tails (189-

191). The truncated cytoplasmic tail of the activating KIR does not contain any signalling motifs. Instead, activating KIR have a positively charged amino acid in the transmembrane domain which allows these receptors to non-covalently associate with molecules, such as DAP12, that express immunoreceptor tyrosine based activating motifs (ITAM) and therefore, serve as signal transduction units (189,192).

Each member of the KIR family recognizes specific polymorphic MHC class I alleles (193), examples are given in Table 1-1.

Table 1-1 HLA class I specificity of various killer cell immunoglobulin-like receptors.

Receptor	HLA class I Specificity	References
p70	HLA B allotypes with Bw4 serological epitope	194-196
p140	HLA A3 allotype	197,198
p58.1	HLA Cw2, 4, 5, 6	174,186,199
p58.2	HLA Cw1, 3, 7, 8	

1.5.2 C-Type Lectin Superfamily

The C-type lectin superfamily includes CD94 and the NKG2 family. CD94 is an invariant type II membrane protein which does not have a cytoplasmic tail (200). The multigenic NKG2 family consists of 5 proteins designated NKG2A through E (201,202). The NKG2A and B proteins have long cytoplasmic tails with ITIM sequences and function as inhibitory receptors (201,203-205). In contrast, the NKG2C, D, and E proteins have short cytoplasmic tails and do not contain ITIM sequences. These proteins function as activating receptors by associating with DAP12 and using its ITAM (192,203,206-208). The CD94 protein itself is not capable of transducing signals, instead it forms disulfide-bonded heterodimers with NKG2A, B, C, and E (203-206,208,209). The CD94/NKG2A and B heterodimers are inhibitory, while CD94/NKG2C is activating.

The CD94/NKG2A, B, and C heterodimers recognize HLA-E, a non-classical, non-polymorphic MHC class I molecule (210,211). The HLA-E molecule is stabilized by selectively binding peptides derived from the leader sequences of HLA-A, -B, -C, or -G alleles (210,212,213), therefore proper expression of HLA-E indicates normal expression of HLA class I molecules.

In direct contrast, NKG2D does not pair with CD94, but rather exists as an activating homodimeric receptor which recognizes the MHC class I related molecules MICA and MICB (214,215). The NKG2D receptor associates with the DAP10 molecule and uses its Src homology 2 (SH2) domain-binding site (which recruits the p85 subunit of phosphatidylinositol 3-kinase) for signal transduction (216). MICA/B is minimally expressed on normal tissues, but is up-regulated on stressed cells (108,109,217), this would suggest that the NKG2D receptor may have a unique role in the immune surveillance of stressed or damaged cells.

1.5.3 Features Common to Both KIR and C-type Lectin Family Members

Natural killer cell receptors have been detected on both NK and T cells and an individual cell can simultaneously express more than one NKR (189,218,219). As well, the NKR phenotype is not limited by the HLA class I haplotype of the individual, as these receptors are commonly expressed in the absence of the corresponding autologous HLA ligand (174,220,221).

Although the NKR have been shown to recognize the non-classical HLA-E molecule or polymorphic determinants of the MHC class I molecules, this recognition is not entirely independent of the peptide occupying the peptide binding groove of the MHC molecule. Studies have shown that the binding affinity of p70, p58.1, p58.2 (222-225)

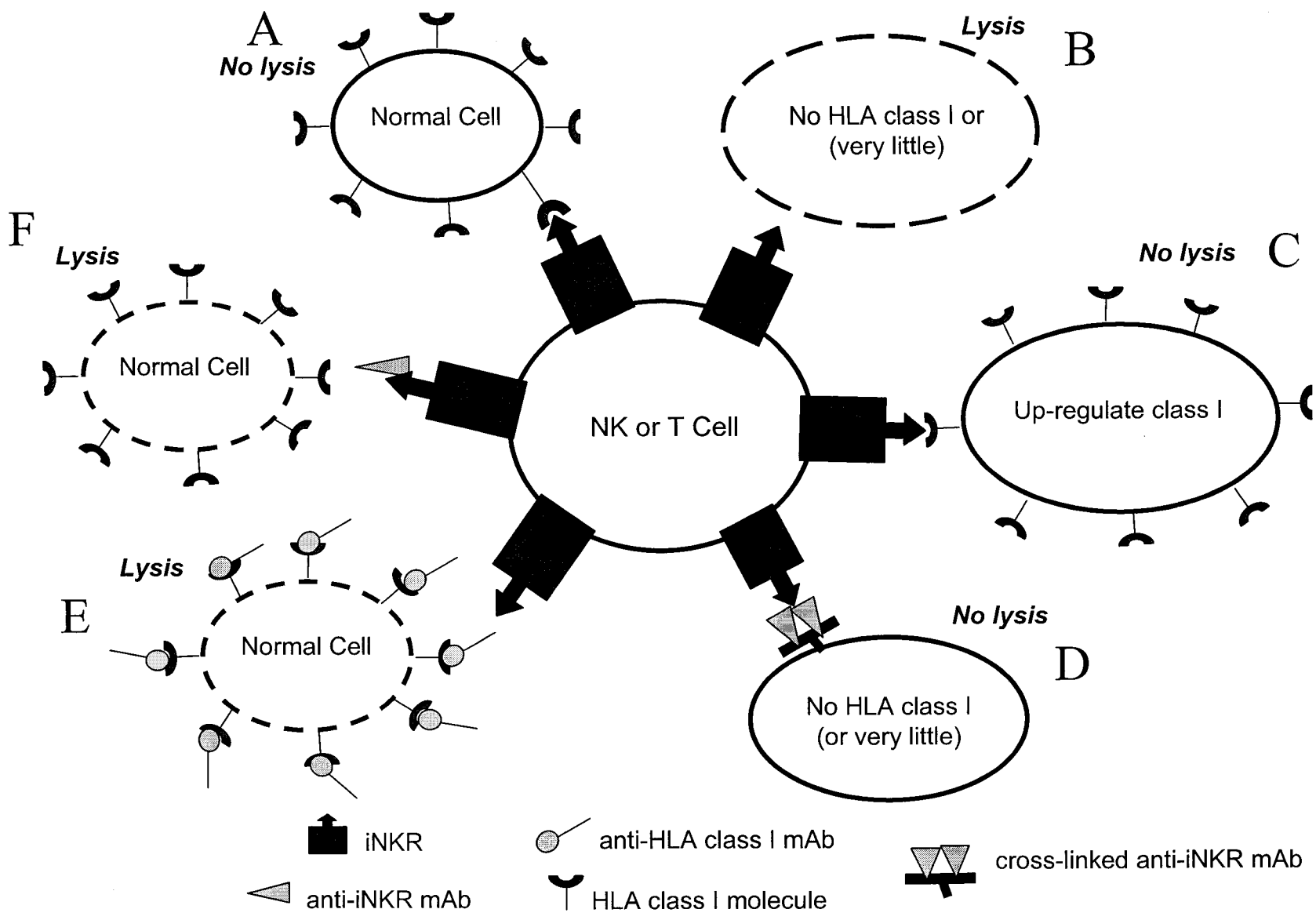
and CD94/NKG2A (210,226) for the appropriate HLA molecule is affected by the peptide presented by that molecule. Certain peptides even inhibit the binding of the NKR to HLA class I molecules.

1.5.4 Manipulation of Cytokine Production and Cytotoxicity

Engagement of inhibitory NKR modulates the functions of NK and T cells. Triggering of CD94/NKG2A, p58, or p70 has been shown to inhibit TCR mediated cytotoxicity (189,196,227,228) and either augment or inhibit TCR mediated cytokine production (208,229,230). For NK cells, triggering of CD94/NKG2A, p70, or p58 inhibits CD16 (189,204), 2B4 (231), FcR (232), and CD69 (233) mediated cytotoxicity and 2B4 (231) mediated cytokine production.

Figure 1-1 schematically depicts the methods by which NK or T cell mediated cytotoxicity can be manipulated *in vitro* via the interaction between inhibitory NKR and HLA class I molecules. (A) A "normal" cell, that is a cell with the appropriate HLA class I expression, is scanned by the inhibitory NKR of the NK or T cell. The iNKR binds the HLA class I molecules, generates an inhibitory signal, and lysis of the target cell is prevented. (B) A cell with a low level of HLA class I expression or no class I expression, for example a tumour or virus infected cell (reviewed in 234,235, respectively), is not recognized by the iNKR. In this case, an inhibitory signal is not generated, and the cell is lysed (102,236). (C) If the level of HLA class I expression is up-regulated, for example with IFN-γ (219,237,238), or induced by transfection with the appropriate HLA class I cDNA (161,174,186,210,239), the cell becomes resistant to lysis. (D) Even though the target cell has no (or very little) HLA class I expression, the cross-linking of an anti-iNKR mAb leads to the generation of an inhibitory signal and the

Figure 1-1 Schematic representation of the control of NK and T cell mediated cytotoxicity through the interaction between iNKR and HLA class I molecules. See text for details.


A
No lysis
Normal Cell
Lysis
B
No HLA class I or
(very little)
F
Lysis
Normal Cell
No lysis
C
NK or T Cell
Up-regulate class I
Lysis
E
Normal Cell
No lysis
D
No HLA class I
(or very little)
iNKR
anti-HLA class I mAb
cross-linked anti-iNKR mAb
anti-iNKR mAb
HLA class I molecule

target cell is not lysed (186,227,228,231). Cross-linking of the anti-iNKR mAb requires that the target cell be FcR^+ or that the mAb be attached to the assay wells. (E) A "normal" cell becomes susceptible to cytotoxicity if an anti-HLA class I mAb is bound to its HLA class I molecules (219,238,239). The anti-HLA class I mAb masks these molecules from the iNKR and an inhibitory signal is not generated. (F) A "normal" cell is also rendered susceptible to lysis if an anti-iNKR mAb is bound to the effector cell, but is not cross-linked (219,237). The mAb interferes with the interaction between the iNKR and the HLA class I molecule and an inhibitory signal is not generated.

1.5.5 iNKR Signal Transduction

Inhibitory NKR must be in close proximity with an ITAM dependent receptor in order to mediate their inhibitory function (240). The ITAM dependent receptors activate kinases, such as Lck, which phosphorylate the ITIM present in the iNKR (232). SHP-1 and/or SHP-2 associate with the phosphorylated ITIM (191,208,239,241), become activated, and dephosphorylate (or inhibit phosphorylation) of a number of local substrates, including 2B4 (231), ζ, ZAP-70, and PLC-γ (232). In this way iNKR ligation interrupts the NK or T cell activation signalling and therefore has the potential to inhibit proliferation, cytotoxicity, and/or cytokine production.

1.6 γδ T cells and Inhibitory Natural Killer cell Receptors

$\gamma\delta$ T cells are cytotoxic to target cells which either do not express HLA class I molecules or express low levels of these molecules (161,174,242). In fact, Rothenfusser et al. (243) were able to correlate $\gamma\delta$ T cell clone mediated cytotoxicity with the HLA class I expression of a variety of targets. $\gamma\delta$ T cells have been shown to express a number

of iNKR, including p70, p58.1, p58.2, p140, and CD94/NKG2A (122,138,161,193,244). The majority of cells express CD94 and NKG2A, while p70, p58.1, p58.2, and p140 are less frequent. In any case, the cells often express more than one iNKR.

The activation signals mediated through the γδ TCR can be down-regulated through the stimulation of inhibitory NKR. Triggering the CD94/NKG2A receptor inhibits proliferation and cytokine production in response to microbial phosphoantigens and HIV-1 infected cells (138,142,244). In addition, engagement of the CD94/NKG2A or p58.2 receptor has been shown to inhibit CD3 and γδ TCR directed lysis (142,218,242). As previously described for NK and T cells (Figure 1-1), the cytotoxicity mediated by γδ T cells is responsive to *in vitro* manipulation of the interaction between iNKR and HLA class I molecules (138,142,161,174,244).

It has been suggested that co-expression of the γδ TCR and various inhibitory NKR on the surface of γδ T cells fine tunes the anti-microbial functions of these cells and controls their self-reactive potential (138,142,193,243,245). The negative signal generated by the iNKR may provide an activation threshold which has to be exceeded before cell activation and the resulting proliferation, cytokine production, and cytotoxicity can occur (243).

1.7 Cytotoxic Mechanisms

The two dominant mechanisms of lymphocyte mediated cytotoxicity are the perforin/granzyme and the Fas/FasL pathways (246). Both pathways activate caspase cascades (reviewed in 247) and induce apoptosis in the target cell characterized by cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and

redistribution of phosphotidylserine from the inner to the outer leaflet of the plasma membrane (248, reviewed by 249).

1.7.1 The Perforin/Granzyme Pathway

The perforin/granzyme pathway involves the calcium dependent directional release of perforin, granzymes, granulysin, and other molecules from granules contained within the effector cell toward the target cell (250,251). Perforin is a pore-forming protein, while the granzymes A, B, and M are serine proteinases with tryptase (252,253), aspase (254,255), or metase activity (254,256,257), respectively.

1.7.1.1 Perforin

Perforin molecules, in the presence of calcium, bind to the phospholipid membrane of the target cell and polymerize to form membrane pores (258,259). Although perforin pores can lead to osmotic lysis of some target cells, perforin alone does not induce the type of DNA fragmentation typically observed during lymphocyte mediated cytolysis (260-262). It was therefore postulated that the perforin pores served as conduits permitting entry of the remaining granule components, particularly the granzymes, into the target cell cytoplasm. However, Browne et al. (263) found that the perforin pores were not large enough to permit the passage of granzyme B unless perforin was present in extremely high concentrations (>4000U/mL). Others have shown that granzyme B is capable of entering the target cell in a perforin independent manner and can use the mannose-6-phosphate/insulin-like growth factor receptor to gain entry into the target cell (261,264).

Although granzyme B can cross the target cell membrane via receptor-mediated endocytosis, in the absence of perforin this granzyme is confined to intracellular vesicles

and apoptosis does not occur. If the target cell is simultaneously treated with sublytic doses of perforin, granzyme B is released to the cytosol, rapidly accumulates in the nucleus, and apoptosis is initiated (261,263,265-267). Granzyme A is also capable of entering the cytoplasm of the target cell in the absence of perforin, but specific accumulation of granzyme A in the nuclei and cytotoxicity requires perforin (253,268). It has recently been suggested that the essential role of perforin is to enable the correct trafficking of other granule components, particularly the granzymes, to specific sites in the target cell, especially the cytosol and the nucleus (269).

1.7.1.2 Granzyme B

Granzyme B induces rapid caspase-dependent oligonucleosomal DNA fragmentation (253,254,267,270) by directly activating a number of caspases and apoptotic nucleases, including caspase 3 (271-273), caspase 6 (274), caspase 9 (275) and caspase-activated DNAse (CAD; 276). Activation of these caspases and of CAD results in apoptotic death (277-279). For example, caspase 3 can lead to DNA fragmentation through the activation of CAD and DNA fragmentation factor (DFF; 280-283). Granzyme B is also capable of cleaving and inactivating downstream death substrates, such as DNA dependent protein kinase catalytic subunit (DNA-PKcs) an important enzyme in DNA repair (284-286).

A second (caspase-independent) pathway of granzyme B mediated apoptosis involves the mitochondrial apoptotic pathway. Granzyme B directly cleaves Bid to produce truncated Bid (tBid). tBid translocates to the mitochondria where it disrupts the mitochondrial membrane potential, and causes the release of cytochrome c which in turn activates caspases responsible for apoptosis (262,287-291). There is also evidence that

granzyme B can directly activate DFF and induce caspase independent DNA fragmentation (292).

1.7.1.3 Granzyme A

Much less work has focused on granzyme A mediated apoptosis. It had been noted that granzyme A enhanced the DNA fragmentation mediated by granzyme B (252), and that granzyme A could mediate DNA fragmentation in the absence of granzyme B (254,293-295). However, the DNA damage mediated by granzyme A is quite different from that attributed to granzyme B. Granzyme A induces single strand breaks in the DNA, not the oligonucleosomal damage seen with granzyme B, and the process is caspase independent (253,255). Beresford et al. (253) suggested that the damage induced by granzyme A may be due to the activation of an endogenous DNAse(s), different from those activated by granzyme B, and that together granzymes A and B activate the major DNAses involved in apoptosis. It is also possible that granzyme A facilitates DNA fragmentation by degrading PHAP II and histone H1 and cleaving core histones (253,296,297), this leads to remodelling and/or unfolding of chromatin making it more accessible to exogenous nucleases.

1.7.1.4 Granulysin and Granzyme M

Another component of the cytotoxic granule is granulysin, an anti-microbial with lytic activity against a broad range of bacteria, parasites, fungi (298-300), and virus infected and tumour cells (248,301-303). It has been suggested that the mechanisms of granulysin lysis for microbial infected and tumour cells are quite distinct (304). While granulysin alone is capable of lysing tumour cells, this is not the case for monocytes/ macrophages infected with microbial pathogens. Stenger et al. (298) found that lysis of

Mycobacterium tuberculosis infected macrophages and subsequent reduction of *M. tuberculosis* viability required the addition of both granulysin and perforin. Perhaps perforin provides granulysin with access to the intracellular compartment of the cell, where it induces lesions and distortions in the microorganism's surface by inserting into the lipidic domains of the plasma membrane (298,303,305). This leads to an increase in the permeability of the bacterial membrane and osmotic lysis of the bacterium (299).

Although the exact mechanism of granulysin induced apoptosis is not fully understood, it is believed to be largely caspase independent. Insertion of granulysin into the plasma membrane causes a disruption of the membrane's integrity, this leads to increased permeability, and a rapid increase in cytoplasmic calcium concentration. The increased calcium concentration damages the mitochondria, reduces the mitochondrial membrane potential, and leads to the release of cytochrome c and apoptosis-inducing factor which initiate apoptosis (299,303,306-308).

The role of granzyme M in target cell lysis has not yet been fully elucidated, however, a recent report stated that granzyme M induces a "novel form of perforin-dependent cell death that is independent of caspases and mitochondrial disruption and occurs in the absence of discernable DNA fragmentation" (309).

The observations that the granule components have different and somewhat independent mechanisms of inducing cell death, indicates that the "...perforin/granzyme pathway is not a single pathway but rather a series of parallel pathways that depend on the particular granzyme or spectrum of granzymes expressed in a given effector lineage or activation state." (310).

1.7.2 The Fas Pathway

The Fas pathway of apoptosis involves the cross-linking of target cell Fas with Fas ligand (FasL) on the effector cell, leading to the activation of caspases and ultimately to cell death (311). Both Fas and FasL belong to the TNF family (312,313). Fas is constitutively expressed on a number of different tissues and by T and B cells, monocytes, and macrophages (314-316). FasL is expressed by activated NK and T cells (317-319) and is constitutively expressed by cells in the "immune privileged" eyes and testes (320-322).

Although FasL is typically thought of as a cell surface molecule, Bossi and Griffiths (319) discovered that newly synthesized FasL is actually stored in cytotoxic granules with perforin and granzymes. The degranulation of these granules controls the delivery of FasL to the cell surface. Since this process is calcium dependent, so is the expression of FasL on the surface of cells. This explains the observation that the up-regulation of FasL and, therefore, the induction of Fas based cytotoxicity is calcium dependent, while the binding and activation of FasL is calcium independent (251,323).

Apoptotic signalling triggered by the Fas pathway can proceed via a mitochondrial dependent or independent pathway. The Fas/FasL interaction results in the formation of a death-inducing signalling complex (DISC), procaspase 8 is recruited to the DISC and cleaved to its active form, caspase 8. Once activated, caspase 8 can trigger a cascade of proteolytic activation of other caspases, including caspase 3. In the mitochondrial independent pathway a lot of DISC is formed and a large amount of caspase 8 is activated. In the mitochondrial dependent pathway, very little DISC is formed and only small amounts of active caspase 8 are produced (324,325). This small

amount of caspase 8 cleaves Bid to tBid and subsequently leads to the release of cytochrome c from the mitochondria (326,327). Cytochrome c binds apoptotic protease activation factor 1 (Apaf-1), this complex activates caspase 9, and ultimately activates caspases 3 and 8 (325,328,329), this leads to the biochemical and morphological changes characteristic of apoptosis.

These two pathways are not mutually exclusive, even in the mitochondrial independent pathway activated caspase 8 is capable of cleaving Bid. It is the relative amount of activated caspase 8 that determines which pathway is dominant (324,325).

1.8 Cytotoxic Mechanisms Utilized by γδ T cells

The exact cytotoxic mechanism(s) utilized by γδ T cells are still under investigation. γδ T cells express Fas L, perforin, granzymes A, B, and M, and granulysin mRNA and protein (124,141,153,330-334). Mincheva-Nilsson et al. (335) observed that only a minority of γδ T cells expressed FasL on the cell surface, while almost all of the cells were positive for intracellular FasL. Further investigation by this group showed that the pre-synthesized FasL was stored in the cytolytic granules and was rapidly translocated to the cell surface upon degranulation.

Reports of γδ T cell mediated cytotoxicity are rather conflicting. It has been demonstrated that γδ T cell mediated cytotoxicity involves the release of serine esterases (97,336). Pre-treatment of the cells with strontium ions (to induce degranulation of the cells), isocoumarin compounds (to inhibit granzymes), or Mg^{2+}EGTA (a calcium chelator), or the addition of anti-perforin or anti-granzyme A mAb inhibits their cytotoxic ability (136,332,333,337-339). In addition, Mami-Chouaib et al. (332) were unable to

inhibit cytotoxicity with anti-Fas or anti-FasL mAb or with Fas-Fc fusion protein. These results indicate that γδ T cells use the perforin/granzyme pathway. In contrast, Roessner et al. (340) were able to inhibit cytotoxicity with anti-FasL blocking mAb or Fas-Fc fusion protein, but not with concanamycin A, suggesting that the Fas/FasL pathway was being used. Vincent et al. (141) demonstrated that both the perforin/granzyme and Fas/FasL pathways are utilized by γδ T cells, as the combination of Mg^{2+} EGTA and anti-Fas mAb proved to be a more potent inhibitor than Fas-Fc fusion protein or anti-Fas mAb alone. Finally, the use of Mg^{2+}EGTA, isocoumarin compounds, brefeldin A, concanamycin A, and anti-Fas mAb in various combinations led to the conclusion that the perforin-based pathway was the predominant cytotoxic mechanism used by γδ T cells, but that the cells were capable of using the Fas pathway when the perforin based pathway was eliminated (339). These conflicting results will be re-addressed in the Discussion.

1.9 γδ T cells and Multiple Sclerosis

MS is believed to be a T cell mediated autoimmune disease. Until recently, the disease process was attributed to the actions of αβ T cells, however, evidence is accumulating in support of a role for γδ T cells in the demyelinating process taking place in MS. Studies with an animal model of MS, experimental allergic (autoimmune) encephalomyelitis, have shown that the depletion of γδ T cells significantly reduced the clinical and pathological signs of disease (341,342). The authors found that this "amelioration of disease was associated with reduced expression of IL-1, IL-6, TNF-α, and lymphotoxin mRNA at the onset of disease, and a more persistent reduction in IFN-γ mRNA expression". Similar work with a mouse hepatitis virus model of demyelination

found that demyelination was almost completely abrogated in the absence of γδ T cells (343).

Numerous immunohistochemical examinations have detected accumulations of γδ T cells in MS lesions. Selmaj et al. (47,344) reported that γδ T cells accumulate in chronic active lesions, are present in large numbers in chronic silent plaques, but are infrequent in very active MS lesions (47,344). Battistini et al. (345) also identified γδ T cells in chronic active lesions. In contrast, Wucherpfennig et al. (346) found that γδ T cells accumulate in acute MS plaques but were a minor population in chronic MS lesions and normal white matter. None of these studies observed accumulations of γδ T cells in the brain tissues of controls or patients with other neurological diseases (OND). The presence of γδ T cells in active plaques has been taken as an indication that these cells are important in the genesis of the MS lesion, while their presence in silent lesions is thought to indicate their role in the persistence of the pathologic process. Both Wucherpfennig et al. (346) and Battistini et al. (345) determined that the γδ T cells in the plaques were oligoclonal and suggested that they had been stimulated in the active plaques by a specific CNS ligand.

Many studies have found an increase in the number of γδ T cells present in the peripheral blood and CSF of patients with MS as compared with healthy controls and patients with other neurological diseases (99,347). The MS CSF γδ T cells are oligoclonal, while γδ present in the PB of MS patients and the PB and CSF of control patients are not oligoclonal (348). Shimonkevitz et al. (347) found that the Vδ sequences of γδ T cells isolated from PB and CSF of MS patients with recent-onset were different

and concluded that the oligoclonal CSF derived γδ T cells were not simply the result of an influx of activated PB cells into the CSF.

Investigations of NKRP1A expression by γδ T cells have demonstrated that the percentage of circulating NKRP1A$^+$ Vδ2$^+$ T cells is higher in MS patients than in healthy controls (154) and that the MS cells have a higher expression of NKRP1A than controls (129). Transmigration of NKRP1A$^+$ Vδ2$^+$ T cells, isolated from MS patients, across endothelial monolayers *in vitro* was faster and involved a larger number of cells than that observed for healthy control cells. This migration did not require chemotactic stimuli, but was inhibited by anti-NKRP1A mAb. These experiments led to the suggestion that cytokine dependent up-regulation of NKRP1A, especially with IL-12, would enable γδ T cells to migrate through the blood-brain barrier and contribute to the immunological attack against myelin.

Perhaps the most convincing evidence of a direct role for γδ T cells in MS immunopathogenesis comes from Freedman et al. (349,350), this work demonstrated that activated γδ T cells can efficiently lyse human oligodendrocytes *in vitro* and that oligodendrocytes stimulate the *in vitro* growth and expansion of γδ T cells.

While the exact role of γδ T cells in MS remains a mystery, there is little doubt that these cells are capable of generating and/or sustaining an immunological attack within the CNS. Perhaps γδ T cells cross the BBB, initiate oligodendrocyte damage, either directly or through the release of cytokines, and thereby release myelin antigens. These antigens could then be presented to αβ T cells by microglia, astrocytes, or endothelial cells present in the CNS and in this way the autoimmune reaction could continue and expand (129,349). It is also possible that the γδ T cells participate in the

autoimmune reaction by acting as a source of pro-inflammatory cytokines and chemokines necessary to activate myelin-reactive T cells and aid their transfer into the CNS (341,342).

1.10 Hypothesis

The mechanism of damage to the oligodendrocyte-myelin unit leading to the demyelination observed in MS is unknown. γδ T cells are concentrated in MS lesions and are capable of oligodendrocyte damage through a cytotoxic mechanism. My working hypothesis was that γδ T cells are instrumental in initiating or perpetuating immune-mediated demyelination or axonal loss, thereby contributing to the pathogenesis of MS. This cytotoxic damage need not be complete, as even partial damage to the oligodendrocyte-myelin unit could lead to altered maintenance of myelin and demyelination. Demyelinated axons could be further damaged by exposure to the cytotoxic milieu. In addition, myelin autoantigens could be liberated and become available for presentation to αβ T cells. Therefore, my work has focussed on the elucidation of γδ T cell mediated cytotoxic immune damage. My specific predictions were that the interaction between the inhibitory natural killer cell receptors present on the γδ T cells and the HLA class I molecules expressed by putative target cells is an important regulator of this cytotoxicity, and that γδ T cells utilize both the perforin/granzyme and Fas/FasL cytotoxic pathways depending upon the susceptibility of the target cell. I also hypothesized that the cytotoxic mechanism(s), including regulation by iNKR, utilized by γδ T cells isolated from MS patients would differ from that of cells isolated from HC or OND patients. This would indicate that γδ T cells derived from MS

patients are indeed MS disease state specific. The results of my investigations will provide information needed in the development of therapies to either protect the oligodendrocyte-myelin unit or impede the damage it sustains in MS.

1.11 Specific Aims

The specific aims of the work described in this thesis were as follows:

1. To examine the molecular interactions between the effector γδ T cells and various target cells, focusing on the iNKR and the manipulation of iNKR/HLA class I molecule interactions. The iNKR are important regulators of γδ T cell mediated cytotoxicity. The generation of an inhibitory signal by the iNKR, in response to the "appropriate" HLA class I expression on the target cell, indicates to the γδ T cell that a cytotoxic response is not required.
2. To determine the cytotoxic mechanisms utilized by γδ T cells including soluble molecules, such as perforin, granzymes A, B, and M, and granulysin, and membrane bound molecules (FasL/FasL pathway). Also to determine if these mechanisms are used simultaneously (and synergistically) and whether they are target specific.
3. To investigate whether certain molecular interactions and cytotoxic mechanisms are specific to MS or to the PB or CSF compartment. This was accomplished by comparing the interactions and mechanisms of PB and CSF derived cells and MS, non-MS, and healthy control derived cells.

Chapter Two- Materials and Methods

2.1 Patient Samples

Peripheral blood and cerebrospinal fluid samples were obtained with informed consent from patients undergoing clinical assessment at the Ottawa Hospital Neuroscience Clinic. Patients were subsequently diagnosed with MS, according to Poser's criteria (16), or other neurological diseases, including viral meningitis, subcortical ischemia, viral transverse myelitis, fibromyalgia, HTLV-1 myelopathy, and latent diabetes.

Peripheral blood samples were also obtained from hospital personnel to be used as healthy controls (HC).

2.2 Generation of γδ T cell Lines from Peripheral Blood

Sheep anti-mouse IgG1 (The Binding Site, Birmingham, England) was diluted to 5μg/ml with 1X phosphate buffered saline (PBS, pH 7.4). Ten wells of a 24 well plate (Costar®, Corning Inc., Corning, NY) were incubated with 300μl of this solution at 37°C for 1 hour. The wells were washed twice with PBS then blocked with complete RPMI 1640 media (cRPMI, 10% FBS/ 2mM L-glutamine/ 100U/ml penicillin/streptomycin, Gibco/BRL, Burlington, ON) for 5 minutes at RT. Anti-γδ TCR mAb (a generous gift of Dr. M. Brenner, Harvard University, Boston, MA) was diluted to 1/500 000 from ascites fluid with cRPMI and 300μl was added to each well. The plate was incubated at 37°C for at least 30 minutes.

Peripheral blood (30 ml) was collected in heparinized tubes and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque™ Plus (Amersham Biosciences

AB, Uppsala, Sweden) density centrifugation. The blood was equally divided into two 50ml conical tubes, equivalent volumes of PBS and Ficoll-Paque™ Plus were added, and the tubes were centrifuged at 1800rpm for 30 minutes. The "buffy coat" was collected and washed twice with PBS. PBMC were plated in the pre-coated 24 well plate at a concentration of $3.5x10^6$ per well with cRPMI. The following day 50U/ml of recombinant human IL-2 (rIL-2, a kind gift of Chiron Corp., Emeryville, CA) was added to each well. On day 5 of culture, the cells were harvested and re-plated with AIM-V (Gibco/BRL) media and 50U/ml rIL-2. On day 7, the cells were labelled with anti-CD3-PE mAb (Sigma, St. Louis, MO) and either anti-γδ TCR-FITC or anti-αβ TCR-FITC mAb (BD Biosciences, San Jose, CA) and analyzed by flow cytometry to determine the percentage of γδ T cells. If the cell line was less than 90% γδ T cells, complement mediated lysis was performed.

2.3 Complement Mediated Lysis of αβ T cells

The cells were harvested, washed with PBS, and counted. No more than $20x10^6$ cells were added to a 15ml conical tube. The tubes were centrifuged at 1200rpm for 10 minutes and the supernatants discarded. For every $3x10^6$ αβ T cells, 5μl of mouse anti-human CD4 and CD8 mAbs (Caltag Laboratories, Burlingame, CA) were added and the cells were incubated on ice for 30 minutes. Following this incubation, the cells were washed with cold PBS and centrifuged at 4°C for 10 minutes at 1200rpm. Baby rabbit complement (Cedarlane, Hornby, ON) was added (25μl for every $3x10^6$ αβ T cells) and the tubes were incubated at 37°C for 30 minutes. The cells were washed twice with warm PBS and centrifuged at 1200rpm at RT for 10 minutes. The cell pellet was

resuspended in cRPMI and the cells were plated at $3.5x10^6$ cells per well in a fresh 24 well plate with 50U/ml rIL-2. The following day the cells were analysed by flow cytometry to check the purity. If 90% purity was not obtained, complement mediated lysis was repeated. When the purity of the cells was greater than 90% γδ T cells, the cells were expanded, used in experiments, frozen to be used at a later time, or transformed with *Herpesvirus saimiri.*

2.4 Generation of γδ T cell Lines from Cerebrospinal Fluid

Four wells of a 96 well plate (Costar®) were pre-coated with anti-γδ TCR mAb, as described above. The volumes of diluted sheep anti-mouse IgG1 and anti-γδ TCR mAbs added to each well were reduced from 300μl to 100μl per well. CSF (10ml) was collected in a vacutainer tube containing no additives. The tube was centrifuged at 1200rpm for 10 minutes, the supernatant was decanted, and the cells were resuspended in 400μl cRPMI. These cells were plated (in 100μl aliquots) on a bed of 50 000 autologous irradiated feeder cells in the pre-coated 96 well plates. The following day 50U/ml rIL-2 was added. On day 5 of culture, 100μl cRPMI and 50U/ml rIL-2 were added. On day 8, the cells were transferred to a 24 well plate with $2x10^6$ irradiated feeder cells and 1μg/ml phytohemagglutinin (PHA), the following day 50U/ml rIl-2 was added. On day 12 the purity of the cells was checked by flow cytometry. If the cell line was less than 90% γδ T cells, complement mediated lysis was performed. Once the purity was greater than 90% γδ T cells, the cells were expanded, used in experiments, frozen for use at a later time, or transformed with *Herpesvirus saimiri.*

2.5 Transformation of γδ T cells with *Herpesvirus saimiri*

2.5.1 Propagation and Titration of *Herpesvirus saimiri*

Herpesvirus saimiri strain C-488, a Level III pathogen, was propagated and titered in Owl Monkey Kidney cells (OMK; ATCC, Manassas, VA). OMK cells at a low passage number were grown to confluence in 175cm^2 tissue culture flasks with cRPMI. At this time, the viral supernatant, previously generated and frozen by Dr. Robert Pon, was thawed and added to the culture. Over the next 10 to 14 days the cells were monitored for signs of viral cytopathic effects and eventually complete lysis of the OMK monolayer. Viral supernatants were obtained by centrifugation of the culture media at 400 x g for 10 minutes. These stocks were stored for not more than 4 months before new stocks were made.

The viral supernatant was titered by adding serial 10-fold dilutions into 24 well plates, in triplicate, containing OMK cells and comparing the viral cytopathic effects to OMK cells which were not incubated with virus. After 14 days in culture, the wells which showed signs of cytopathic effect were considered to be positive for *Herpesvirus saimiri*, and the dilution of virus required to infect 50% of the cells ($TCID_{50}$) was calculated according to the Spearman-Karber method (351):

$$\text{Log } TCID_{50} = \text{highest log dilution with 100\% cytopathology} + \frac{1}{2} - \left[\frac{\text{total number of wells with cytopathology}}{\text{number of wells per dilution}}\right]$$

$$TCID_{50}\ ml^{-1} = \frac{TCID_{50}}{\text{volume of innoculate (ml)}}$$

2.5.2 Generation of *Herpesvirus saimiri* Transformed γδ T cell lines

PB and CSF derived γδ T cell lines with purities greater than 90% γδ T cells were plated at a concentration of $1x10^6$ cells/ml in 24 well plates. CSF cells were cultured with cRPMI media, while PB cells were cultured with cR/A media (45% RPMI 1640, 45% AIM –V, 10% FBS, supplemented with 50U/ml penicillin and streptomycin, and 1mM L-glutamine). To each well was added 0.5μg/ml PHA, $1x10^6$ irradiated heterologous feeders/ml, and 10% (v/v) *Herpesvirus saimiri*. The following day 50U/ml rIL-2 was added. The cells were maintained in 10% virus for 7 days. After this time, the infected cells were washed with PBS and plated in 96 well U bottom plates at a concentration of 300 000 cells per well with cRPMI (PB) or cR/A (CSF) and 50U/ml rIL-2. These cultures were maintained with media changes twice per week.

2.5.3 Generation of *Herpesvirus saimiri* Transformed γδ T cell clones

Transformed γδ T cell clones were generated through limiting dilution of the transformed lines. Twenty-one days after the cell lines were incubated with *Herpesvirus saimiri* and PHA/feeders, the cells were plated in 96 well U bottom plates at concentrations of 50 and 10 cells per well with 10 000 irradiated heterologous feeders per well. Again, PB cells were plated with cRPMI, while CSF cells were cultured with cR/A, all cells received 50U/ml rIL-2. During the next few weeks, the plates were monitored for growth and individual proliferating cells were selected and split 1:2 into 96 well plates in the appropriate media with rIL-2. When a particular clone had been split 5 times, a minimum of 50 000 cells was removed for flow cytometry analysis. The cells were labelled with anti-CD3-PE and either anti-γδ TCR-FITC or anti-αβ TCR-FITC

mAbs. Clones which were identified as γδ T cells were expanded in 96 well plates with media changes twice per week and splitting as needed.

2.6 Flow Cytometry Protocols

2.6.1 Surface Staining

The cells were routinely stained for CD3, γδ TCR, αβ TCR, CD94, NKG2A, p70, p58.1, p58.2, and p140 in various combinations, with the appropriate isotype controls. (For the work described in this thesis p70, p58.1, p58.2, p140, CD94 and NKG2A will be referred to as iNKR). Table 2-1 lists the antibodies used in these analyses and their suppliers. The anti-p140 ascites fluid was a generous gift of Dr. L. Moretta, Genoa, Italy. The staining protocol can be summarized as follows: 200 000 to 300 000 cells were washed in FACS buffer (1X PBS, 2% FBS, 0.1% sodium azide), blocked with non-specific mouse IgG (0.5μg; Sigma) on ice for 10 minutes, and incubated with the antibody or antibodies of interest for 30 minutes on ice. The cells were washed with FACS buffer and resuspended in the same buffer before being analyzed on the Beckman Coulter Epics XL flow cytometer, using the Expo32 software (Beckman Coulter, Burlington, ON). At least 10 000 events were collected. [The anti-NKG2A and anti-p140 mAbs were not conjugated to a fluorochrome, therefore a secondary mAb, goat anti-mouse IgG or IgM, respectively, (Fc specific, FITC conjugated; Sigma) was used to detect these receptors].

The staining procedure was modified slightly for cells transformed with H*erpesvirus saimiri*. Following the incubation with the antibody of interest, the cells were washed twice with PBS and incubated with 2% paraformaldehyde (PFA) overnight

Table 2-1 Antibodies routinely used in flow cytometry.

Specificity	Clone	Source
CD3	UCHT-1	Sigma
γδ TCR	11F2	BD Biosciences
CD94	HP-3B1	Coulter Immunotech
NKG2A	Z199	Coulter Immunotech
p70	DX9	BD Biosciences
p58.1	EB6	Coulter Immunotech
p58.2	GL183	Coulter Immunotech
p140	Q66	Dr. L Moretta, Genoa, Italy
perforin	δG9	BD Biosciences
granzyme A	CB9	BD Biosciences
granzyme B	2C5/F5 GB7 GB11	Serotec
granzyme M	4H10	Dr. M. Smyth, Peter MacCallum Cancer Institute, Australia
granulysin	polyclonal rabbit	Dr. A. Krensky, Stanford University, CA
Fas L	NOK-1 NOK-2	BD Biosciences

at 4°C to inactivate the virus. The next morning the cells were washed twice with FACS buffer, resuspended in FACS buffer, and analysed by flow cytometry. At least 10 000 events were collected.

2.6.2 Intracellular Staining

Flow cytometry analyses were also used for the detection of intracellular molecules, including perforin, granzymes A, B, and M, and granulysin, the mAbs used in these experiments are also listed in Table 2-1. Anti-granzyme M and anti-granulysin mAbs were not commercially available. Dr. M. Smyth (Peter MacCallum Cancer Institute, Victoria, Australia) kindly donated anti-granzyme M ascites and later purified mAb. [Prior to the purified mAb being obtained and conjugated to FITC, as described below, goat anti-mouse IgG (Fc specific, FITC) was used as a secondary antibody in the detection of granzyme M in γδ T cell clones]. Dr. A. Krensky (Stanford University, California) kindly donated anti-granulysin polyclonal rabbit anti-serum and later purified mAb. Again a secondary mAb was necessary for the detection of granulysin, goat anti-rabbit IgG (Fc specific, FITC; Jackson Immunoresearch Laboratories Inc., West Grove, PA).

The cells were surface stained with anti-CD3-QR and anti-γδ TCR-PE, using the protocol detailed in section 2.6.1. Following the incubation with these mAbs, the cells were washed twice with PBS, and fixed with 2% PFA for 30 minutes at RT. The cells were then washed twice with permeabilization buffer (PBS, 0.1% saponin, 2% FCS), incubated with the appropriate "intracellular" antibody for 30 minutes, and washed again with permeabilization buffer. The cells were washed with and resuspended in FACS buffer and analysed by flow cytometry (at least 10 000 γδ TCR events were collected).

Again the staining procedure was modified slightly for the cells transformed with *Herpesvirus saimiri*. After the incubation with the "intracellular" antibody, the cells were washed twice with PBS and incubated overnight at 4°C with 2% PFA. The next morning the cells were washed twice with and resuspended in FACS buffer, then analysed by flow cytometry.

Blocking studies were performed to check the specificity of the mAbs used in intracellular staining. The cells were surface stained, fixed, and washed with permeabilization buffer as described, then incubated with unlabelled mAb (10x the volume of labelled mAb typically used) on ice for 30 minutes. After this incubation the labelled mAb was added and the cells were again incubated for 30 minutes on ice. The cells were washed with permeabilization buffer (x2), then washed with and resuspended in FACS buffer, and analyzed by flow cytometry. As a control for the polyclonal rabbit anti-granulysin antibodies, cells were incubated first with purified rabbit IgG (Sigma), then with goat anti-rabbit IgG (Fc specific, FITC).

2.6.3 Flow Cytometry Detection of FasL

The detection of FasL by flow cytometry is hampered by the fact that the amount of FasL expressed on the surface of cells is typically very low and that FasL is quickly cleaved from the surface of the cell by a metalloproteinase (315,352). The protocols which are most commonly used to detect FasL overcome these obstacles by treating the cells with ionomycin or PHA in combination with EDTA (or another metalloproteinase inhibitor) before the cells are stained for FasL. Three such protocols were used in this work and are detailed below:

1. $1x10^6$ cells/ml were incubated with 50U/ml rIL-2, 0.5μg/ml PHA and 5mM EDTA for 2 days in a 24 well plate at 37°C.
2. $1x10^6$ cells/ml were incubated in a 24 well plate with 10ng/ml PMA and 250ng/ml ionomycin (Sigma) at 37°C for 3 hours in the presence or absence of 5mM EDTA. (Adapted from 141)
3. $1x10^6$ cells/ml were incubated with 5mM EDTA in a 24 well plate for 2 hours at 37°C, followed by 10μg/ml NOK-1 or NOK-2 at RT for 5 minutes, and then 1μg/ml ionomycin for 7 minutes at RT. The contents of the wells were resuspended and aliquots were pipetted into 1.5ml tubes. (Adapted from 319)

Following the incubations described in procedures 1 and 2, the cells were incubated with unlabelled NOK-1 or NOK-2 (anti-FasL) mAb. The NOK-1 and NOK-2 mAbs were detected by incubating the cells with biotin conjugated goat anti-mouse IgG (Fc specific; Sigma), and subsequently with streptavidin-R-phycoerythrin conjugate (Sigma). The cells treated according to protocol 3 were incubated with goat anti-mouse IgG (Fc specific, FITC). Cells incubated with 50U/ml rIL-2 only were used as negative controls.

2.6.4 Alexa Fluor® 488 Monoclonal Antibody Labelling

Anti-granzyme B and anti-granzyme M mAbs were not commercially available conjugated to a fluorochrome, instead they were labelled in house with the Alexa Fluor® 488 Monoclonal Antibody Labeling kit (Molecular Probes, Eugene, OR). Purified mouse anti-human granzyme B was labelled with Alexa Fluor® 488 according to the manufacturer's instructions. Briefly, the antibody was diluted to 1mg/ml with PBS and one-tenth the volume of 1M sodium bicarbonate was added. A 100μl aliquot of this protein solution was added to a vial of "reactive dye" (supplied with the kit) and

incubated at RT for 1 hour. To purify the labelled protein, a spin column was filled with purification resin (supplied with the kit), the contents of the reaction vial were added, and the column was centrifuged at 1100 x g for 5 minutes.

The mouse anti-human granzyme M mAb was provided as a solution in Tris buffer. Since Tris buffer is not compatible with the Alexa Fluor® kit, the antibody was dialyzed against PBS using the Slide-A-Lyzer® Mini Dialysis Unit (Pierce Biotechnology, Rockford, IL). This unit consists of a disposable dialysis cup made of polypropylene and regenerated cellulose. The mAb solution was added to the cup and floated in 0.5L of PBS with stirring for 2 hours. After this time, the antibody was collected from the dialysis unit and transferred to a fresh microtube. Several attempts were necessary before the correct conditions for labelling this mAb were discovered. Optimal labelling was finally achieved by combining the contents of two vials of reactive dye and allowing the reaction to take place for 1 hour at RT, followed by incubation overnight at 4°C. The labelled protein was purified with a spin column as described above.

The degree of labelling of the purified protein was determined by measuring its absorbances at 280nm and 494nm and applying these values in the following equations:

$$\text{Protein concentration (M)} = \frac{[A_{280} - (A_{494} \times 0.11)] \times \text{dilution factor}}{203\,000}$$

$$\text{Moles dye per mole protein} = \frac{A_{494} \times \text{dilution factor}}{71\,000 \times (\text{M})}$$

2.7 IFN-β (Avonex®) and the iNKR phenotype of γδ T cells

The effect of *in vivo* and *in vitro* IFN-β treatment on the iNKR phenotype of γδ T cells was investigated by flow cytometry. The *in vivo* study was composed of 12 patients with relapsing remitting MS who were taking Avonex® (Biogen® Canada, Mississauga, ON). The dosing schedule was one 30μg injection per week. Peripheral blood samples were obtained from these patients at the following time points:

1. Two samples before the initiation of treatment.
2. 48 hours after the first injection.
3. 1 week after the first injection.
4. 1 month after the first injection.
5. 3 months after the first injection.
6. 6 months after the first injection.

γδ T cell lines were generated from these PB samples, as described in section 2.2, and analyzed by flow cytometry for CD94, NKG2A, p70, p58.1, p58.2, and p140 expression.

The *in vitro* study was carried out by incubating PB derived γδ T cells ($1x10^6$ cells/ml) in a 24 well plate with 1, 10, 100, 1000, or 2000 U/ml of IFN-β (Avonex®, a kind gift of Biogen® Canada) at 37°C. Aliquots were removed at 0, 6, 12, and 24 hours and analyzed by flow cytometry for CD94, NKG2A, p70, p58.1, p58.2, and p140 expression.

2.8 JAM Assays

The cytotoxicity of the γδ T cells was assessed using the JAM assay. The target cell lines used in these assays are listed in Table 2-2, each of these lines was purchased from ATCC and maintained with weekly media changes and splitting as required. Target

cells were passaged a maximum of 25 times before new cultures were set-up from frozen master stocks. In preparation for their use in JAM assays, the target cells were washed

Table 2-2 Target cells used in JAM assays.

Cell Line	Classification
U937	monocytic-like lymphoma
K562	chronic myelogenous leukemia
Jurkat	acute T cell leukemia
RPMI 8226	plasmacytoma
Hut 78	lymphoma
KG-1	myeloblastic leukemia
Colo 205	colrectal carcinoma

with PBS (x2), diluted to $1x10^6$ cells/ml with cRPMI, and incubated with 2.5μCi/ml [methyl-^{3}H] thymidine (Amersham Pharmacia Biotech) in a 24 well plate overnight at 37°C. The following morning the target cells were washed with PBS (x2) and diluted to 100 000 cells/ml with cRPMI. γδ T cells were harvested the day of the assay, washed with PBS (x2), and diluted with cRPMI to the appropriate dilutions, depending on the starting E:T ratio. The assays were performed in quadruplicate with serial dilutions of E:T ratios ranging from 25-0.2:1, based on a constant number of target cells (10 000 cells/well).

Equal volumes (100μl) of target and effector cells were added to 96 well U bottom plates and incubated at 37°C for 4.5 hours. After this incubation, the contents of the wells were harvested onto glass fibre filters (Canberra Packard, Meriden, CT) using the Filtermate 196 cell harvester (Canberra Packard). The filters were air dried overnight at RT and the radioactivity measured with a Matrix 9600 β-counter (Canberra Packard). The percent specific lysis was calculated using the equation given below:

$$\% \text{ specific lysis} = \frac{[\text{CPM (targets alone)} - \text{CPM(experimental)}]}{\text{CPM (targets alone)}} \times 100$$

JAM assays with *Herpesvirus saimiri* transformed γδ T cells, required an additional step before the contents of the plates could be harvested. After the 4.5 hour incubation, the virus was deactivated by adding 50μl of a detergent/chelating solution (0.001% w/v SDS, 0.01% w/v Triton X-100, 10mM EDTA in Tris-HCL, pH 8.0) to each well. The plates were incubated at RT for 30 minutes and frozen at –20°C overnight. The following day the plates were thawed and surface decontaminated before being removed from the Level III laboratory for harvesting and counting.

2.8.1 Inhibition Assays

The majority of JAM assays described in this thesis were designed to manipulate the cytotoxicity mediated by γδ T cell lines or clones through the addition of mAbs or other compounds. Untreated effector or target cells were used as negative controls, appropriate isotype controls were also included. The details of these experiments are given below:

2.8.1.1 iNKR/HLA class I Molecule Interaction

Several different methodologies were used to investigate the role of the iNKR/HLA class I molecule interaction in γδ T cell mediated cytotoxicity:

1. γδ T cell lines or clones were harvested, washed (PBS x2), and 1.5×10^6 cells were incubated with 10 or 20μg/ml anti-CD94, -p70, -p58.1, -p58.2, -p140, or –NKG2A antibody at 37°C for 30 minutes, in a final volume of 100μl. The cells were diluted with cRPMI, as required depending upon the starting E:T ratio, and used in JAM assays.

2. Anti-CD94 or anti-NKG2A mAbs were immobilized on the wells of the assay plate. One hundred µl of 5µg/ml sheep anti-mouse IgG (Fc specific) mAb (Sigma) was added to each well and incubated at 37°C for 1 hour, the wells were washed twice with PBS, then blocked for 5 minutes at RT with cRPMI, 50µl of 0.2mg/ml anti-CD94 or anti-NKG2A mAb was added and the plates were incubated at 37°C for at least 30 minutes before effector and target cells were added. Cells were also incubated in control wells coated with only sheep anti-mouse IgG.
3. Target cells were incubated with [methyl-^{3}H] thymidine and either 1000U/ml IFN-β or 60U/ml IFN-γ overnight at 37°C to increase the surface expression of HLA class I molecules. The next morning the cells were harvested, washed (PBS x2), diluted, and used in JAM assays. Aliquots of 200 000 cells were removed from each of the wells for flow cytometry analysis of the HLA class I molecules (HLA-ABC-FITC mAb, Coulter Immunotech).

2.8.1.2 Cytotoxic Molecules

Two methodologies were used to identify the cytotoxic molecules used in γδ T cell mediated killing:

1. Granzyme B inhibitor II (Ac-IETD-CHO; Calbiochem, San Diego, CA), or antibodies directed against perforin, granzymes A, B, and M, and granulysin were pipetted into the appropriate wells of the assay plate prior to the addition of target cells.
2. Target cells were incubated overnight with [methyl-^{3}H] thymidine. Fas blocking mAb (2µg/ml; clone ZB4, Coulter Immunotech) was added for the

last hour of this incubation. The cells were harvested, washed (PBS x2), and diluted to $1x10^5$/ml. An aliquot containing the required number of cells was transferred to a conical tube, 5μg/ml of Fas blocking mAb was added, and the cells were transferred into the appropriate assay wells.

2.8.1.3 Experiments Specific to γδ T cell Clones

1. γδ T cell clones ($1.25x10^6$) were pre-incubated with a 1:100, 1:1000, or 1:10 000 dilution of soluble human TNF receptor (shTNF-R; a generous gift of Dr. J. Antel, Montreal Neurological Institute, Montreal, Quebec) for 30 minutes at 37°C before being used in JAM assays or the shTNF-R was pipetted directly into the appropriate wells of the assay plate.
2. γδ T cell clones were pre-incubated with brefeldin A (BA; 20 or 40μM), concanamycin A (CMA; 1000 or 2000nM), or a combination of the two at 37°C for 2 hours before being used in JAM assays.
4. A physical separation of the γδ T cells and target cells was achieved by pipetting γδ T cell clones (125 000 in 60μl) into tissue culture inserts designed for 96 well plates (transwells). The transwells are fitted with a 0.2μm anopore membrane (Nunc, Gibco/BRL). The target cells (10 000 in 150μl) were transferred to the wells of the assay plate. The transwells were inserted into the appropriate wells of the assay plate and remained there for the 4.5 hour incubation, but were removed before the contents of the wells were harvested.
5. γδ T cell clones were incubated with dilutions of anti-γδ TCR ascites (1:500, 1:1000, 1:5000, 1:50 000, 1:500 000) at 37°C for 30 minutes, then transferred to the appropriate wells of the assay plate.

6. Anti-CD150 mAb (500ng/ml or 1μg/ml) was added to the wells of a 96 well plate immediately before the addition of the target cells.
7. Cold Hut 78 cells (2500, 5000, or 7500 cells in a final volume of 100μl) or Hut 78 supernatant (25, 50 or 100μl) were added to the wells of the assay plate. These wells also contained 125 000 γδ T cell clones and 10 000 "experimental" target cells ([methyl-^{3}H] thymidine labelled U937, RPMI 8226, or Jurkat cells).

2.9 Granule Enzyme Exocytosis Assays

The amount of serine esterase released into the supernatant by the γδ T cell lines and clones was quantified using a colorimetric esterase assay. Wells of a 96 well U bottom plate were designated for blank, background, total, and target cell or antibody stimulated exocytosis. Each assay was carried out in triplicate. The "blank" wells contained only cRPMI, all other wells contained $1x10^5$ effector cells in 50μl cRPMI. "Background" wells received 50μl cRPMI, "total" wells received 10μl of 1% Triton X-100 plus 40μl cRPMI, "target cell" wells received 8000 target cells in 50μl cRPMI, "antibody stimulated" wells received 500ng/ml anti-CD150 (SLAM) or isotype control. The plates were incubated at 37°C for 4 hours, centrifuged at 4°C for 5 minutes at 1200 rpm, and 50μl of culture supernatant was transferred from each well to a 12 x 75mm tube. To each tube was added 950μl of BLT substrate solution containing 0.2mM BLT (N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester), 0.22mM DTNB [5,5′-dithio-bis(2-nitrobenzoic acid)], and 0.01% Triton X-100 in PBS. The tubes were incubated in a 37°C water bath for 20 minutes then immediately placed in an ice bath. Phenylmethane-

sulfonyl fluoride (PMSF, 10μl of a 0.1M solution in DMSO) was added to each tube, followed by 1ml of PBS. Absorbances were measured in a spectrophotometer at 412nm.

For γδ T cell clones transformed with *Herpesvirus saimiri* it was necessary to decontaminate the culture supernatants before their removal from the Level III laboratory. This was accomplished by adding 10μl of 1% Triton X-100 to the culture supernatants, incubating for 20 minutes at RT, and freezing at –20°C overnight. The following day, the supernatants were thawed and the tubes were surface decontaminated before being removed from the lab. To each tube was added 940μl of BLT substrate solution containing 0.2mM BLT and 0.22mM DTNB in PBS. The remainder of the protocol was unchanged.

The percentage values for target cell induced (or antibody stimulated) secretion of esterase were calculated according to the following formula:

$$\% \text{ secretion} = (E-B)/(T-B) \times 100$$

E = mean absorbance for target cell or antibody stimulated cell supernatants
B = mean absorbance of background supernatants
T = mean absorbance of Triton X-100 treated wells

2.10 2-Dimensional Electrophoresis

2.10.1 Protein Isolation

The samples used for protein isolation consisted of 5 γδ T cell lines derived from CSF and PB of MS patients, 5 derived from CSF and PB of OND patients, and 4 derived from PB of healthy controls. These cell lines were thawed and cultured in 24 well plates at a concentration of $3.5x10^6$ cells/well with cRPMI and 50U/ml rIl-2. On the fourth day of culture, cRPMI and 50U/ml rIL-2 were added. The following day the cells were harvested, washed with PBS (x3), and resuspended in 1ml PBS. An aliquot containing $3.5x10^6$ cells was transferred to a 1.5ml microfuge tube, centrifuged at 1200rpm for 5 minutes, and the supernatant discarded. Ready Prep Sequential Extraction Reagent #3 (Bio Rad Laboratories, Hercules, CA) was reconstituted with H_2O as directed by the manufacturer. This extraction reagent will be referred to as rehydration buffer for the remainder of this thesis. For every 1ml of rehydration buffer, 10µl of 200mM tributyl phosphine (Bio Rad Laboratories) was added, 500µl of this solution was added to each microfuge tube. The tubes were sonicated for 5 minutes at RT, then centrifuged at 14 000rpm for 5 minutes. The supernatant, containing the protein, was carefully removed to a fresh microfuge tube. An aliquot was removed for quantification of the isolated protein and the remaining samples were stored at –80°C.

2.10.2 Quantification of Protein

The concentration of the isolated protein was determined using the colorimetric RC DC Protein Assay kit (Bio Rad Laboratories) according to the manufacturer's instructions. Briefly, 25µl of samples and standards (serial dilutions of Protein Assay Standard II Lyophilized Bovine Serum Albumin, Bio Rad Laboratories) was added to

microfuge tubes and 125µl of RC Reagents I and II were added. The tubes were centrifuged at 15 000 x g for 4 minutes, and the supernatants completely drained from the tubes. Next 127µl of Reagent A' (a mixture of DC Reagents S and A) was added and the tubes were incubated at RT for 5 minutes. Finally, 1ml of DC Reagent B was added to each tube and the tubes were incubated at RT for 15 minutes. Aliquots (200µl x 3) were transferred to a 96 well plate and absorbances were read at 650nm in a Thermo Labsystems Multiskan Ascent (Kodak Imaging Systems, Rochester, NY) with Ascent version 2.6 software. A standard curve was constructed from the serial dilutions of the standard and used to calculate the concentrations of the isolated protein samples.

2.10.3 Rehydration and Isoelectric Focusing of ReadyStrip™ IPG Strips

Day 1

Seventeen cm ReadyStrip™ IPG strips (Bio Rad Laboratories) with a linear pH gradient range of 3 to 10 were utilized for isoelectric focusing (IEF). The protein samples were thawed and 50µg of protein, in a final volume of 300µl, was pipetted into one lane of the Immobiline® DryStrip Reswelling Tray (Amersham Pharmacia Biotech, San Francisco, CA). The cover sheet was removed from the IPG strip to expose the gel and the strip was placed gel-side down in the tray being careful not to trap air bubbles beneath the strip. The strips were overlaid with 3ml of mineral oil, and left at RT overnight.

The next morning electrode wicks were moistened with H_2O and placed over the electrodes in the IEF focusing tray (Bio Rad Laboratories). The IPG strips were removed from the reswelling tray, excess mineral oil was drained, and the strips were placed gel-

side down in the IEF focusing tray. Again, the strips were overlaid with 3ml of mineral oil. The focusing parameters are given below:

S1	250V	15 min	rapid increase
S2	5000V	4hr	linear
S3	5000V	85 000Vh	linear
S4	5000V	indefinitely	

Total: 98 000 Vh 24 hours

2.10.4 Reduction and Alkylation

Day 2

Following IEF, the strips were placed gel side up in a rehydration/equilibration tray and incubated for 15 minutes with shaking at 37°C in a reduction buffer containing 0.05M Tris HCL pH 6.8, 8M urea, 34% glycerol, 2% w/v dithiothreitol, and 0.3% w/v lauryl sulfate. The strips were transferred to a fresh tray and incubated for 15 minutes with shaking at 37°C in an alkylation buffer containing 0.05M Tris HCL pH 6.8, 8M urea, 34% glycerol, 2.5% w/v iodoacetamide, and 0.3% w/v lauryl sulfate. The strips were now ready to be applied to the acrylamide gels.

2.10.5 SDS-PAGE and Silver Staining

Day 2

Acrylamide gels (10%) were cast in the Protean® II xi cell casting tray using 1.0mm spacers and 2-D combs (Bio Rad Laboratories). The gels were composed of 15ml 40% acrylamide, 15.25ml Lower Tris (36.34g Tris, 0.8g lauryl sulfate, pH 8.8 H_2O to 200ml), 29.25ml H_2O, and 30µl TEMED. This solution was de-gassed for 5 minutes, 300µl of 10% ammonium persulfate was added, and the solution was pipetted between

the glass plates. After the gels had polymerized, the combs were removed, the wells were rinsed and filled with H_2O, and the gels were stored overnight at 4°C.

Day 3

The gels were transferred to RT and the wells rinsed with H_2O. ReadyPrep Overlay agarose (Bio Rad Laboratories) was heated and pipetted into the wells of the gels, this acts to seal the IPG strips in the well. The IPG strips were removed from the alkylation buffer and the overhanging plastic ends were snipped off. The strips were rinsed with 1X running buffer (4.54g Tris, 21.61g glycine, 1.5g lauryl sulfate per 1.5L H_2O) and placed into the well so that the gel of the IPG strip was against the shorter plate of the gel apparatus and the acidic end of the strip was next to the well reserved for the protein marker (BenchMark™ Protein Ladder, Invitrogen). A plastic ruler was used to gently push the IPG strip to the bottom of the well and force out any air bubbles trapped beneath the strip. The BenchMark™ Protein Ladder (0.05µg; 10-220kDa; Gibco/BRL) was added to the marker well.

The gels were placed on the cooling chambers and this apparatus was transferred to the gel containers (Protean® II xi Cell). Running buffer was added to the top of the cooling chambers and to the gel container so that the bottoms of the gels were covered. The cooling chambers were connected to a water source and the pressure adjusted to a slow constant rate. The gels were run at 20mA per gel for 15 minutes, followed by 40mA per gel until the dye front had reached the bottom of the gel plates, approximately 3 hours.

The gels were removed from the glass plates and underwent a series of washing, fixing, and staining steps (with shaking) as described below:

1. Washed for 5 minutes with H_2O (x3).
2. Fixed for 60 minutes in a solution of 50% ethanol, 5% acetic acid, and H_2O.
3. Washed for 10 minutes with a solution of 50% ethanol, 50% H_2O.
4. Washed with H_2O for 10 minutes (x2).
5. Incubated with sensitizer solution (0.02% sodium thiosulphate in H_2O) for 1 minute.
6. Rinsed with H_2O for 1 minute.
7. Incubated with silver nitrate solution (0.1% silver nitrate in H_2O) for 30 minutes.
8. Rinsed for 1 minute with H_2O.
9. Quickly rinsed with a small volume of developer (0.04% formalin, 2% sodium carbonate in H_2O), repeated. This minimises non-specific staining by reacting excess silver.
10. Incubated with developer until the desired darkness was reached.
11. Rinsed with H_2O.
12. Incubated with stop solution (5% acetic acid in H_2O) for at least 5 minutes.
13. Stored in 1% acetic acid solution.

2.10.6 Analysis

Each gel was imaged using the Kodak ds Digital Science™ Image Station 440 CF with the Kodak 1D Software (Kodak, Scientific Imaging Systems, Rochester, NY). These images were compared using PDQuest 7.0v. Software (BioRad Laboratories). Spots of interest were excised and analysed by mass spectrometry (performed by Dr. John Kelly, National Research Council of Canada, Ottawa, Ontario).

2.10.7 PlusOne™ 2-D Clean-Up Kit

During the initial optimization of the 2-D electrophoresis protocol, the PlusOne™ 2-D Clean-Up Kit (Amersham Pharmacia Biotech) was tested to see if it improved the resolution or quality of the gels. The kit was used according to the manufacturer's instructions. Briefly, protein was isolated as described above and 100µl of each sample was transferred to a 1.5ml microcentrifuge tube. To each tube was added 300µl of

precipitant and the tubes were incubated on ice for 15 minutes. The tubes were centrifuged at 12 000 x g for 5 minutes, and the supernatant was removed, being careful not to disturb the pellet. The pellet was dispersed in H_2O, and 1 ml of wash buffer was added. The tubes were incubated at -20°C for 30 minutes and centrifuged at 12 000 x g for 5 minutes. The supernatant was discarded, the pellet was air dried for 5 minutes, and resuspended in 50μl of rehydration buffer.

To compare the results of 2-D electrophoresis with and without the use of this kit, two protein samples simultaneously isolated from the same γδ T cell line were used. One isolate was treated with the kit, the other was not, both samples were then subjected to 2-D electrophoresis.

2.11 Statistical Analysis

The results of the JAM assays were analysed with a one-tailed, paired, Student's *t* test. The Pearson Product-Moment Correlation Coefficients were calculated for the mean fluorescence intensity versus percent kill experiments and tested for significance with a *t* test. The frequencies of the spots for the 2-dimensional electrophoresis gels were compared with the Fisher's exact test. For all tests a p value <0.05 was considered to be significant. Unless otherwise stated, the error bars in the figures represent mean ± SD.

Chapter Three- Results

3.1 Short Term Culture of γδ T cell lines

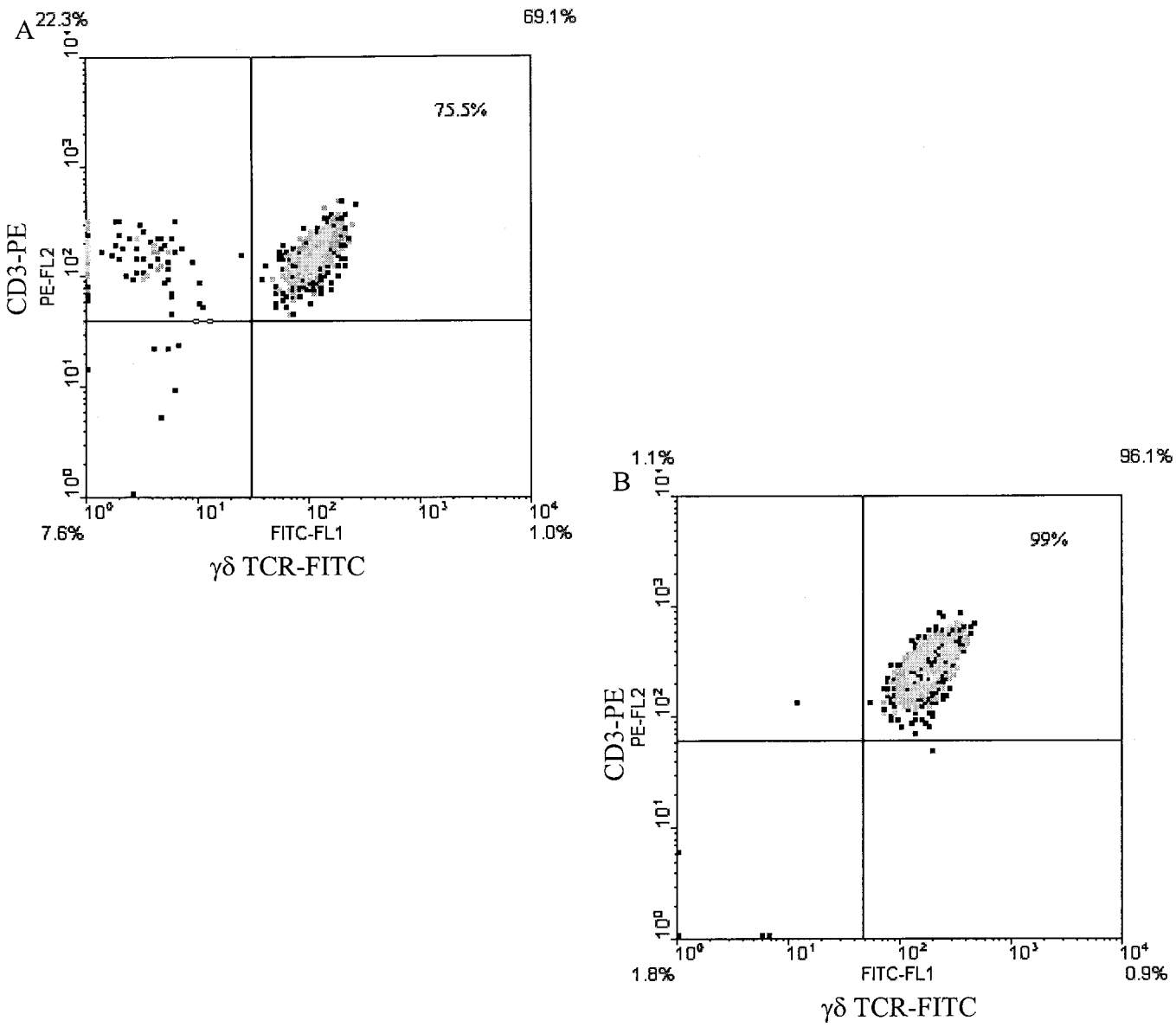
The number of γδ T cells typically present in the CSF and PB is quite small, 10ml of CSF yields 2-20 000 mononuclear cells, 10-30% of which are γδ T cells (56), 30ml of PB yields 15-20 x 10^6 PBMC, of which 1-10% are γδ T cells. Immobilized anti-γδ TCR mAb was used to stimulate the γδ T cells in a polyclonal manner and thereby generate enough cells for my experiments.

Both the PB and CSF derived γδ T cell lines were routinely examined for purity by two colour flow cytometry using anti-CD3 mAb and either anti-γδ TCR or anti-αβ TCR mAb. The percentage of γδ T cells was calculated as the ratio of γδ TCR positive cells to the total CD3 positive cells. Complement mediated lysis (with anti-CD4 and CD8 mAbs and baby rabbit complement) was used to eliminate contaminating αβ T cells. Purities greater than 90% γδ T cells were easily obtained with this technique. The flow cytometry profiles of a PB derived γδ T cell line pre-and post- complement mediated lysis are shown in Figure 3-1. When the desired purity had been obtained the cells were used in experiments, frozen for use at a later date, or transformed with *Herpesvirus saimiri.*

3.2 Long Term Culture of γδ T cell Lines and Clones

The typical lifespan of the γδ T cells was 30-45 days in culture. Expansion and purification of the cells required between 8 and 14 days, and the cells did not proliferate well after 30 days. For these reasons, the number of cells and the time frame in which they could be used was limited. In many cases a γδ T cell line could only be used for one

Figure 3-1 Typical expansion and purification of γδ T cells in culture, pre- (A) and post- (B) complement lysis. Cells were labeled with anti-γδ TCR-FITC and anti-CD3-PE mAbs and analyzed by flow cytometry. The percent γδ T cells, given in the upper right quadrant, was calculated as the proportion of γδ T cells relative to the total $CD3^+$ cells.


A
22.3%
69.1%
75.5%
7.6%
1.0%
CD3-PE
PE-FL2
FITC-FL1
γδ TCR-FITC
B
1.1%
96.1%
99%
1.8%
0.9%
CD3-PE
PE-FL2
FITC-FL1
γδ TCR-FITC

set of experiments. However, immortalizing the cells with *Herpesvirus saimiri*, and using these transformed lines to produce clones eliminated these problems. The methodologies for the viral transformation and subsequent production of clones were developed in house by Dr. R. Pon.

Several criteria were used to determine if the cells had been transformed with virus: (1) the transformed cells displayed a blastoid morphology characterized by irregular shapes and sizes, (2) the death of control cells which had been treated under identical conditions, but had not received virus, and (3) increased proliferation rates of infected cells and the continuation of these rates over several months without antigen or mitogen stimulation.

The ease with which γδ T cell clones could be generated varied with the cell line, as did the ratio of γδ to αβ clones. For example, the 11 clones generated from cell line 70 were all γδ T cells, while only 2 of the 16 clones generated from cell line 80 were γδ T cells.

3.3 Inhibitory Natural Killer cell Receptors and γδ T cells

Inhibitory natural killer cell receptors scan the surface of potential target cells for "normal" expression of HLA class I molecules. If the iNKR recognize the HLA class I expression as self, the target cell is protected from lysis. However, if the target cell does not express the "correct" HLA class I phenotype or if the level of expression is not "sufficient", then the cell is lysed. Investigating the interactions between iNKR and HLA class I molecules is important to understanding the regulation of γδ T cell mediated cytotoxicity.

3.3.1 iNKR Phenotype of γδ T cell Lines

The iNKR phenotype of each γδ T cell line was determined with two colour flow cytometry, a typical profile is depicted in Figure 3-2 (n=22). The goat anti-mouse IgM and IgG (Fc specific, FITC) controls are included to demonstrate their specificity. The majority of cells expressed CD94 and NKG2A (61.8 and 62.8%, respectively), while a much smaller percentage expressed p70 (7.5%), p58.1 (3.3%), p58.2 (20.1%), and p140 (25.6%). (CD94 was always detected as a continuum of $CD94^{lo}$ to $CD94^{hi}$, hence the smear on the flow cytometry reports. This thesis will report the percentage of $CD94^{hi}$ expressing cells as CD94). The actual percentage of cells expressing each iNKR varied between cell lines, examples are given in Table 3-1, the range of percentages was as follows: CD94 and NKG2A (40-90%), p70 (1-20%), p58.1 (1-8%), p58.2 (1-20%), or p140 (1-30%).

Table 3-1 iNKR Phenotypes of PB Derived γδ T cell Lines

	% of γδ T cells expressing iNKR					
Cell Line	CD94	p70	p58.1	p58.2	p140	NKG2A
HC1	72.6	19.7	<1	3.8	20	74.1
OND PB2	61.8	7.5	3.3	20.1	25.6	62.8
OND PB3	55.3	4.7	<1	5.8	14	58.3
MS PB2	78.6	8	1.1	3.2	7.8	78.7

Further analysis demonstrated that the iNKR phenotype not only differed between samples, but also between the cell lines derived from the PB and CSF of the same donor. The phenotypes of a representative pair (n=4) are depicted in Figures 3-2 (PB) and 3-3 (CSF). Typically, the percentage of CSF derived γδ T cells which expressed p70, p58.1, p58.2, and p140 was lower than their PB derived counterpart. For example, the percentage of cells which expressed p140 was 25.6% for the PB derived cells, but only

Figure 3-2 iNKR phenotype of representative PB derived γδ T cell line (OND PB2). Cells were labelled with anti-γδ TCR-FITC mAb and anti-CD94, -p70, -p58.1, or –p58.2 PE conjugated mAb. NKG2A and p140 were detected with anti-NKG2A or -p140 mAb plus anti-IgM or IgG-FITC mAb (Fc specific), respectively. The Fc specificity of the anti-IgM and –IgG mAbs is confirmed by the absence of a FITC signal for cells incubated with anti-IgM or –IgG mAb alone. The percentages of iNKR positive γδ T cells are indicated in the upper right hand quadrants.

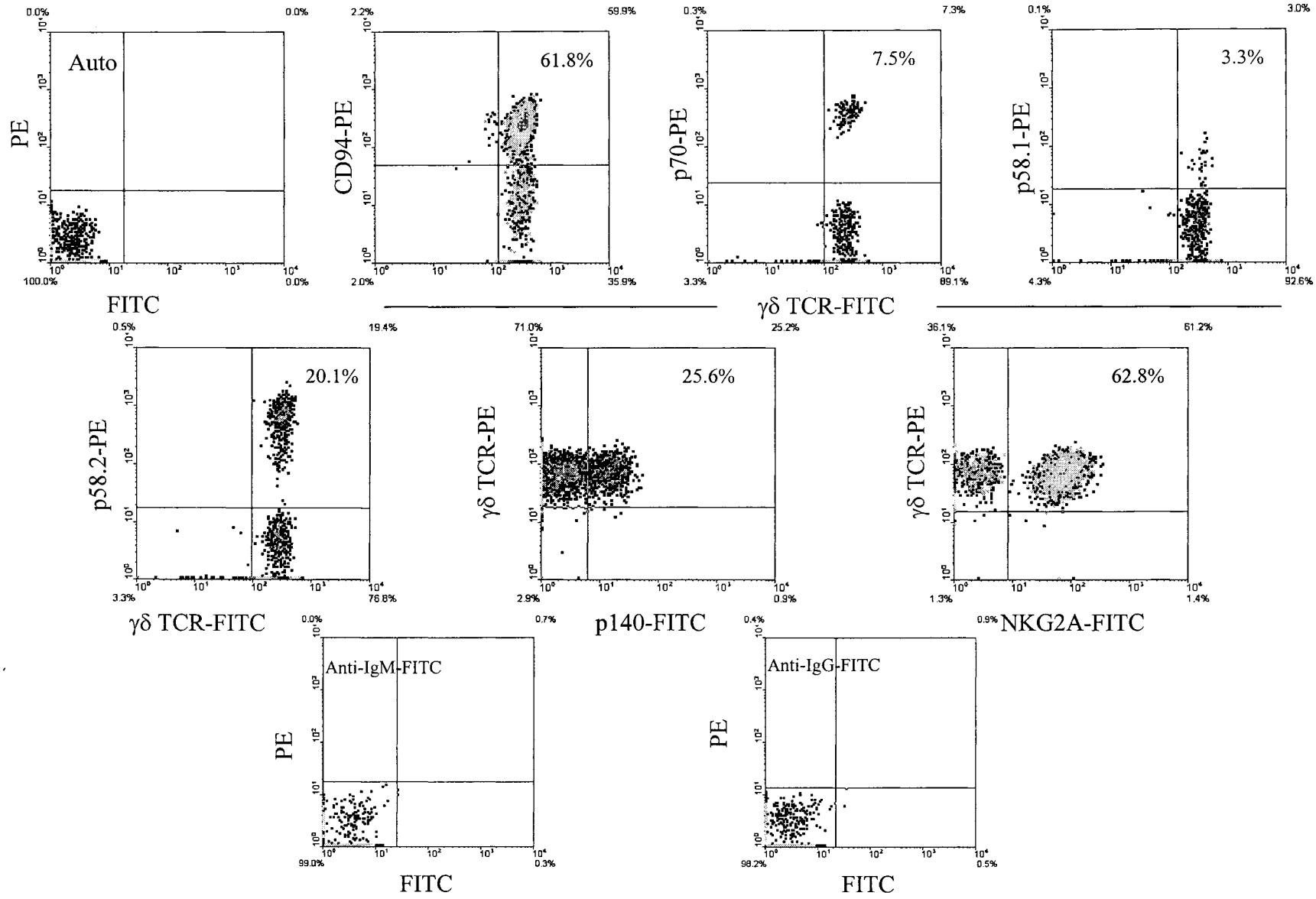
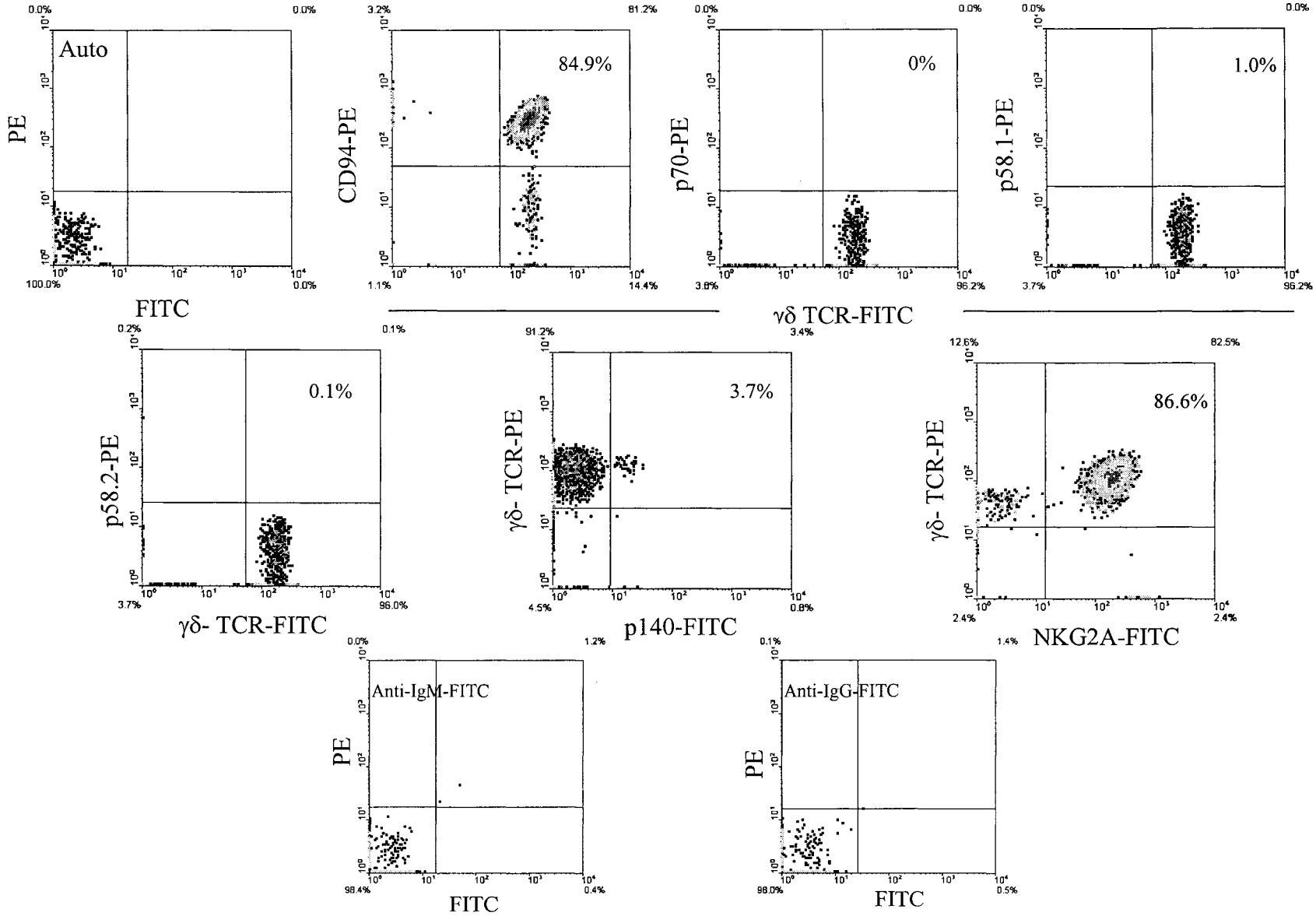

Auto
PE
FITC
CD94-PE
61.8%
p70-PE
7.5%
p58.1-PE
3.3%
γδ TCR-FITC
p58.2-PE
20.1%
γδ TCR-FITC
γδ TCR-PE
25.6%
p140-FITC
γδ TCR-PE
62.8%
NKG2A-FITC
Anti-IgM-FITC
PE
FITC
Anti-IgG-FITC
PE
FITC

Figure 3-3 iNKR phenotype of representative CSF derived γδ T cell line (OND CSF2). Together Figures 3-2 and 3-3 demonstrate the differences observed in iNKR phenotypes of PB/CSF pairs (n=4). Flow cytometry staining and analyses of the cell line pairs were performed on the same day of culture, with cell labelling as previously described. The percentages of iNKR positive γδ T cells are indicated in the upper right hand quadrants.

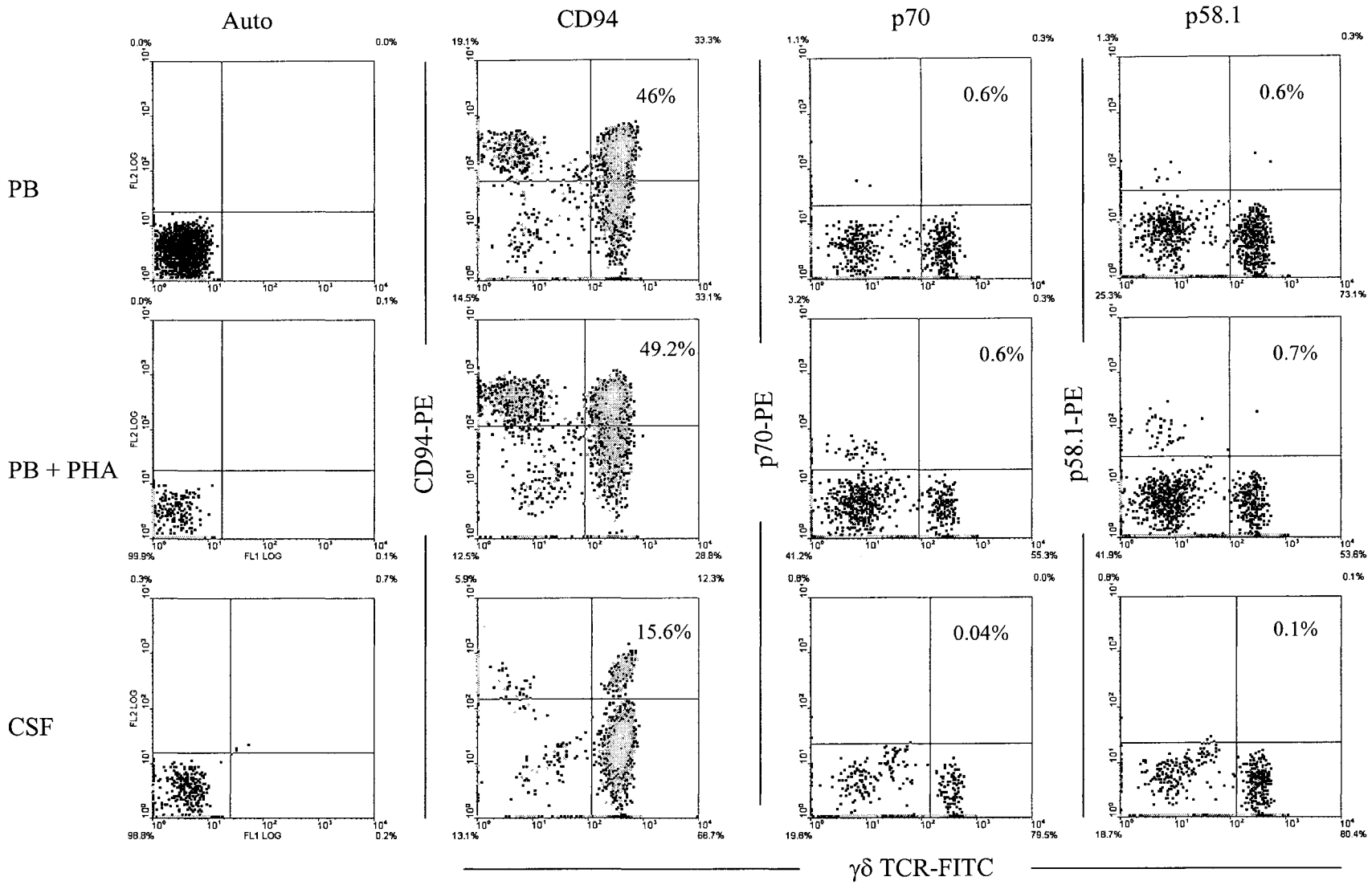

Auto
PE
FITC
CD94-PE
84.9%
p70-PE
0%
p58.1-PE
1.0%
γδ TCR-FITC
p58.2-PE
0.1%
γδ- TCR-FITC
γδ- TCR-PE
3.7%
p140-FITC
γδ- TCR-PE
86.6%
NKG2A-FITC
Anti-IgM-FITC
PE
FITC
Anti-IgG-FITC
PE
FITC

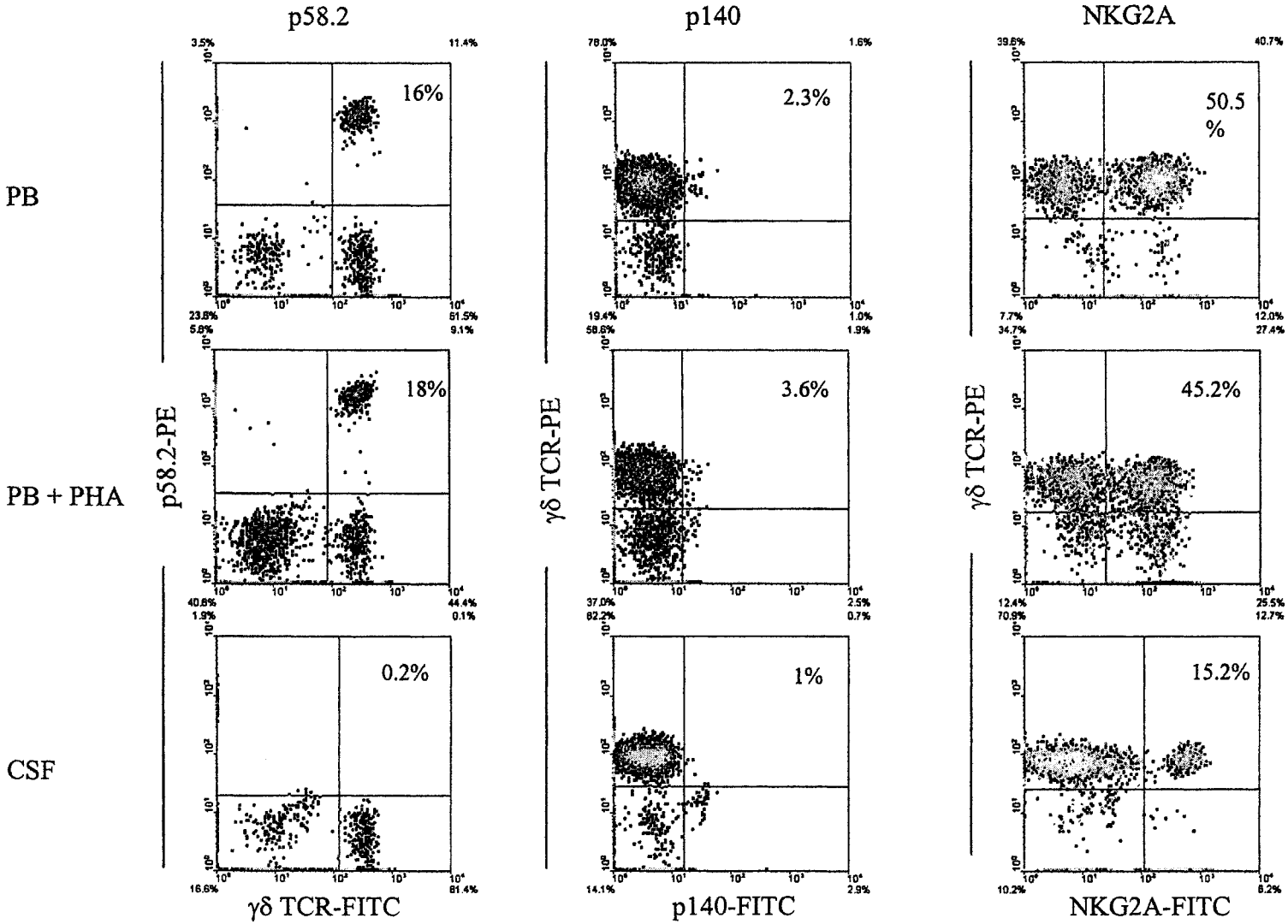
3.7% for cells derived from the CSF of the same patient. In some cases, a particular iNKR was not expressed by the CSF derived cells, for example none of the CSF derived cells expressed p70, while 7.5% of the PB derived cells were positive for this iNKR. In contrast, the percentage of cells which expressed CD94 and NKG2A was typically higher in the CSF derived samples (84.9% and 86.6%, respectively) than their PB derived counterparts (61.8% and 62.8%, respectively).

These results led to the question of whether the differences in the protocols used to generate the γδ T cell lines (especially the use of PHA for the CSF derived lines) were responsible for the different iNKR phenotypes. To test this possibility PBMC and CSF cells from PB and CSF paired samples (n=3) were isolated and cultured as per usual. On day 8 a sample of PB derived cells was transferred to a 24 well plate with PHA and irradiated feeder cells, as were the CSF derived cells. Flow cytometry analysis of the CSF, PB, and PB+PHA samples showed that the iNKR phenotypes of the PB and PB+PHA cells were not significantly different ($p>0.1$; Figure 3-4, representative). In contrast, the iNKR phenotype of the CSF derived cells was completely different from its PB and PB+PHA counterparts. For example, the percentage of cells positive for CD94 was 46% for the PB cells (49.2% PB+PHA), but only 15.6% for the CSF cells, the percent positive for p58.2 was 16% for the PB cells (18% PB+PHA), but less than 1% for the CSF cells. These results show that the dissimilarity between the iNKR phenotypes is related to the compartment from which the cells are isolated (PB versus CSF), and not an artefact due to the protocols used to generate and expand them.

The cell lines were typically used in assays as soon as their numbers and purity were sufficient, therefore, the ages of the lines used in any particular experiment could

Figure 3-4 Effect of expansion protocol on iNKR phenotypes of γδ T cell lines. PBMC were isolated and incubated with immobilized anti-γδ TCR ascites and rIL-2, as described in methods. On day 8 of culture, $3.5x10^6$ cells were transferred to wells with $2x10^6$ irradiated feeder cells and 1μg/ml PHA. The remaining PB cells received fresh media and rIL-2 only. The CSF cells were isolated by centrifugation and plated with 50 000 autologous irradiated feeder cells and immobilized anti-γδ TCR ascites. On day 8 of culture, the cells were transferred to wells with $2x10^6$ irradiated feeder cells and 1μg/ml PHA. Flow cytometry analyses of the iNKR phenotypes were performed (as previously described) between days 15 and 25. Representative results are shown, n=3 pairs. The percentages of iNKR positive γδ T cells are indicated in the upper right hand quadrants.

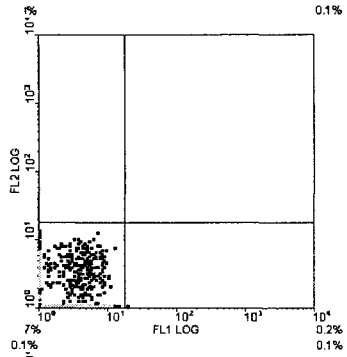

Auto
CD94
p70
p58.1
PB
PB + PHA
CSF
CD94-PE
p70-PE
p58.1-PE
γδ TCR-FITC
FL2 LOG
FL1 LOG
46%
49.2%
15.6%
0.6%
0.6%
0.04%
0.6%
0.7%
0.1%

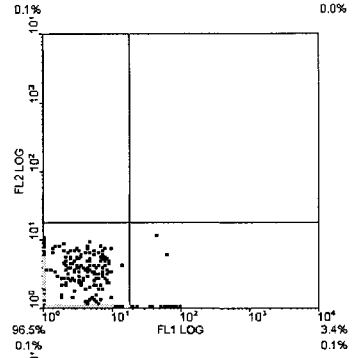

p58.2
p140
NKG2A
PB
PB + PHA
CSF
p58.2-PE
γδ TCR-PE
γδ TCR-PE
γδ TCR-FITC
p140-FITC
NKG2A-FITC
16%
2.3%
50.5 %
18%
3.6%
45.2%
0.2%
1%
15.2%

Anti-IgM FITC

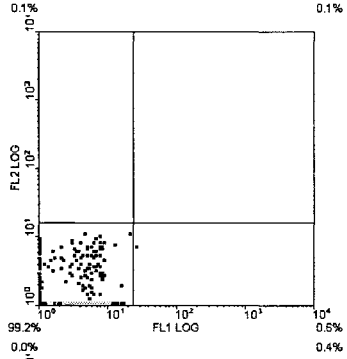
Anti-IgG-FITC

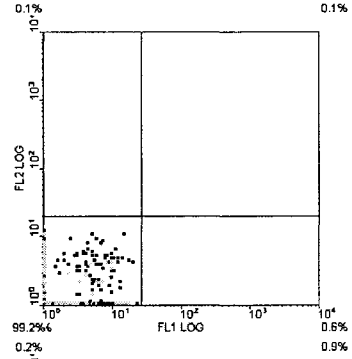
PB



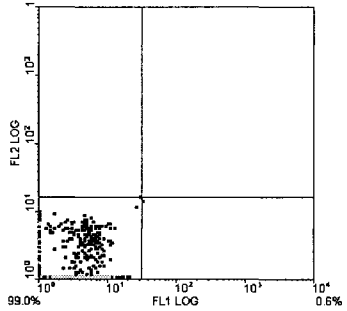


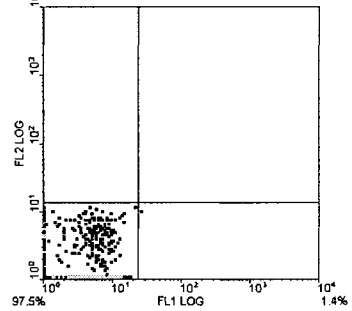
PB + PHA





CSF





differ by up to 10 days. For this reason it was important to determine whether the iNKR phenotypes were affected by time in culture. To investigate this possibility, cell lines from 9 PB samples and 1 PB/CSF pair were cultured as usual and the iNKR phenotypes were analyzed from days 7- 46 at 4-7 day intervals. As Figure 3-5A (representative) shows, there were no significant changes in the percentage of cells expressing each of the iNKR during the time points examined.

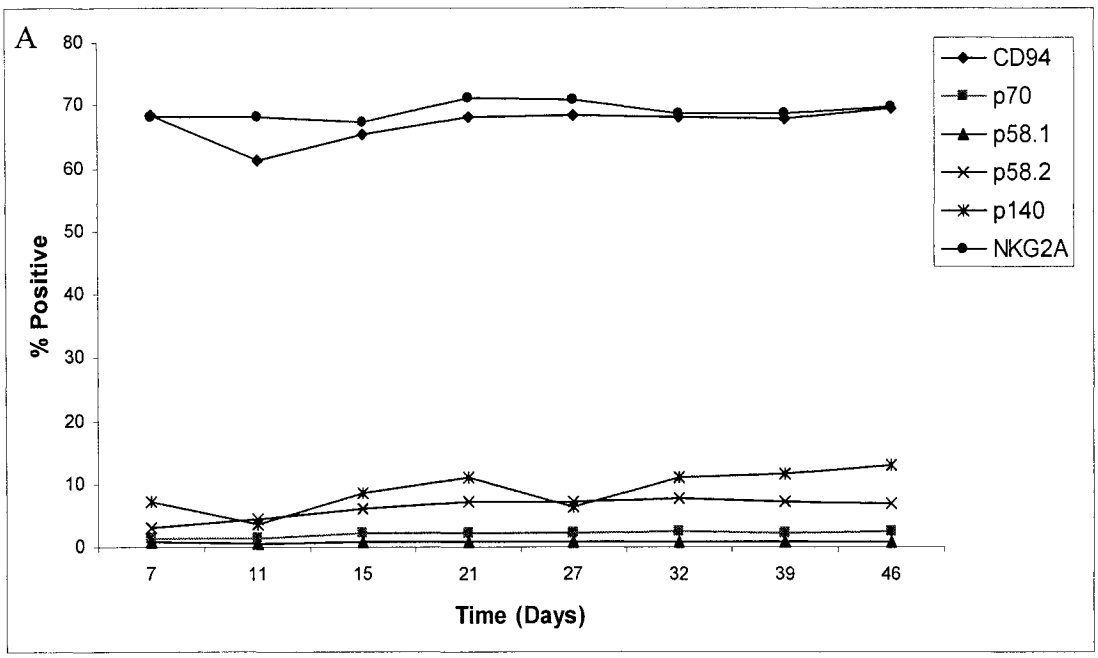
To determine if there was an iNKR phenotype specific to MS or other neurological disease patients, γδ T cell lines derived from the PB of 6 HC, 8 MS, and 8 OND samples were analyzed by flow cytometry, the mean percentage (± SEM) for each group are depicted in Figure 3-5B. No significant difference in iNKR phenotype was observed between the groups.

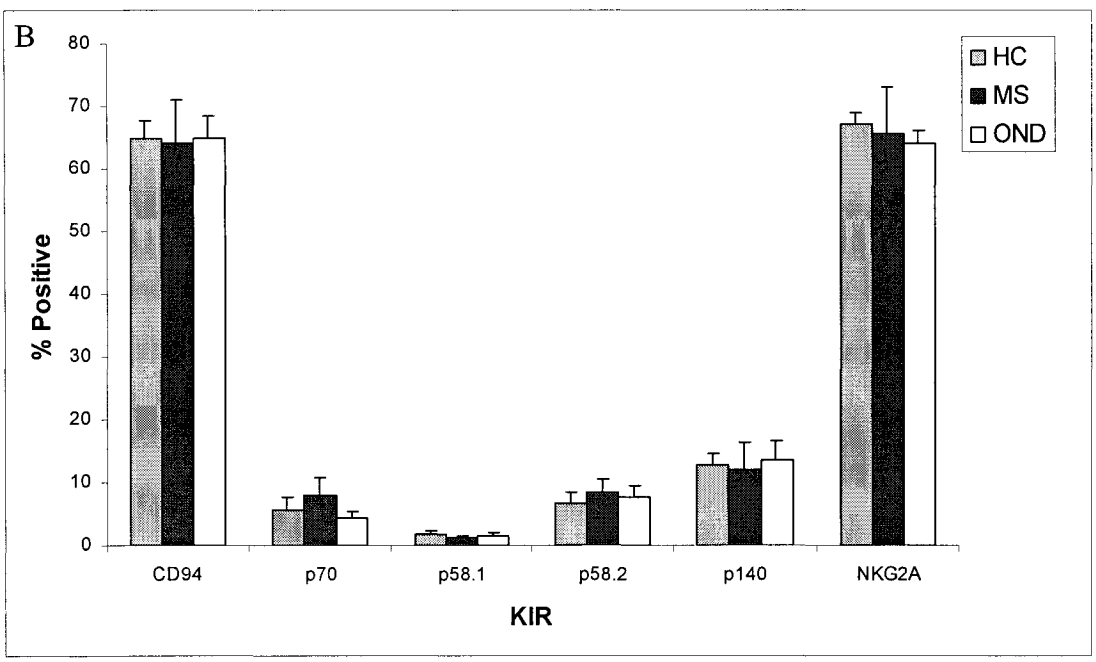
3.3.2 iNKR Expression and γδ T cell Mediated Cytotoxicity

To investigate the role of iNKR in γδ T cell mediated cytotoxicity and thereby gain insight into how this cytotoxicity could be regulated, γδ T cell lines cells were incubated with anti-iNKR mAbs before being used in JAM assays. The mAbs were carefully matched to the iNKR phenotypes of the effector cells and the HLA class I phenotypes of the target cells.

Preliminary experiments were carried out with 6 γδ T cell lines (3PB/3CSF) with U937 target cells, 2 CSF derived γδ T cell lines with K562 targets, and 1 CSF derived γδ T cell line with RPMI 8226 target cells. It was hypothesized that the anti-iNKR mAbs would bind to their respective iNKR on the effector cells, this would prevent the HLA class I molecules from binding to their appropriate iNKR, and the cytotoxicity of the target cells would increase. Figure 3-6A demonstrates that neither anti-CD94 nor anti-

Figure 3-5 (A) iNKR phenotype of γδ T cell line analyzed during time in culture. Flow cytometry analyses were performed from days 7-46 of culture at 4-7 day intervals. Cells were labelled with anti-γδ TCR-FITC mAb and anti-CD94, -p70, -p58.1, or –p58.2 PE conjugated mAb. NKG2A and p140 were detected with anti-NKG2A or -p140 mAb plus anti-IgM or IgG-FITC mAb (FC specific), respectively. The percentage of iNKR positive γδ T cells is indicated in the upper right hand quadrants. Representative results are depicted, n=11. (B) Comparison of iNKR phenotypes of γδ T cell lines derived from PB of healthy controls (n=6), patients with multiple sclerosis (n=8), and patients with other neurological diseases (n=8). Bars represent mean percentage of positive cells ±SEM.


A
80
70
60
50
40
30
20
10
0
% Positive
7
11
15
21
27
32
39
46
Time (Days)
CD94
p70
p58.1
p58.2
p140
NKG2A

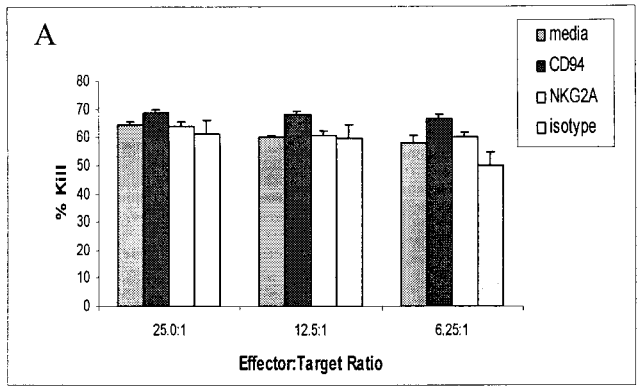

B
80
70
60
50
40
30
20
10
0
% Positive
CD94
p70
p58.1
p58.2
p140
NKG2A
KIR
HC
MS
OND

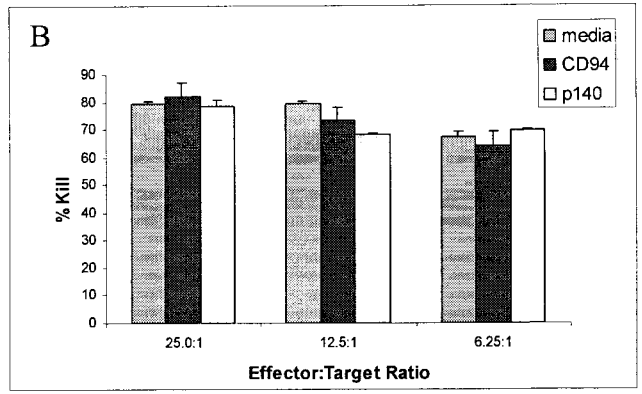
NKG2A mAb led to a significant change in the cytotoxicity of RPMI 8226 target cells. The second part of the hypothesis was that the anti-iNKR mAbs would bind to their respective iNKR on the effector cells and to the FcR of U937 or K562 target cells. This cross-linking of the anti-iNKR mAbs would lead to the generation of an inhibitory signal and the percent kill of the target cells would decrease. As Figure 3-6B (representative) shows, the cytotoxicity of U937 was not inhibited by anti-CD94 or anti-p140 mAbs. In contrast, Figure 3-6C shows that the cytotoxicity of K562 was significantly decreased by anti-p70, p58.1, or p58.2 mAb (media 33.7%; p70, 16.8%; p58.1, 2.5%; p58.2, 8.6%), isotype controls are not shown. Of the 9 experiments performed this was the only one to demonstrate significant changes in cytotoxicity. This result also served as confirmation that the mAbs were capable of cross-linking with the FcR and inhibiting the cytotoxicity mediated by the γδ T cells.

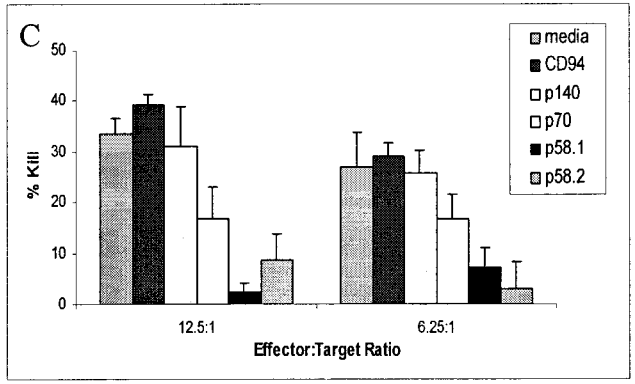
It was possible that the anti-iNKR mAbs were not cross-linking with the FcR of the U937 target cells, or that this cross-linking was not sufficient to generate an inhibitory signal. To control for this variable, anti-CD94 or anti-NKG2A mAb (or appropriate isotype control) was directly or secondarily immobilized on the assay plate. The theory was that the anti-iNKR mAbs would bind their appropriate iNKR on the γδ T cells and as the mAbs are already cross-linked (to the assay plate) an inhibitory signal would be generated within the effector cell. This means that the percent kill of the target cells should decrease regardless of their FcR status (Dr. L. Battistini, personal communication).

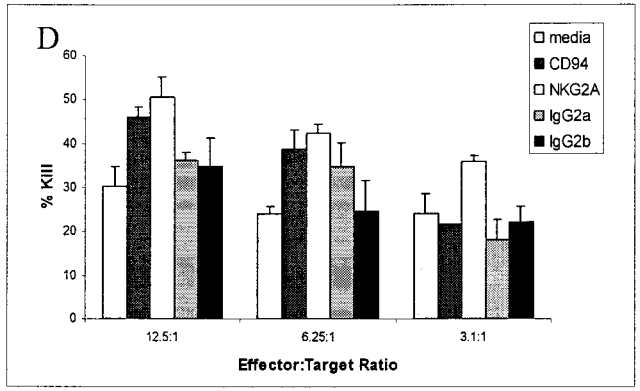
Figure 3-6D shows the results of one of the two experiments which attempted to decrease the cytotoxicity of U937 by directly cross-linking anti-CD94 or anti-NKG2A

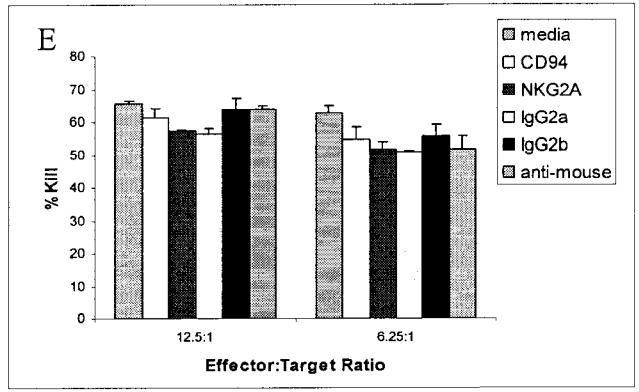
Figure 3-6 Effect of anti-iNKR mAbs on γδ T cell mediated cytotoxicity in 4.5h JAM assay. (A, B, and C) γδ T cells were incubated with 10 or 20μg/ml anti-iNKR mAbs for 30 minutes then added to assay wells with 10 000 [methyl-^{3}H] thymidine labeled RPMI 8226 n=1 (A), U937 n=6 (B), or K562 n=2 (C) target cells. (D and E) Anti-iNKR mAb was attached to wells of the assay plate prior to the addition of effector and [methyl-^{3}H] thymidine labeled target cells. (D) γδ T cells and U937 target cells were added to wells coated with anti-CD94, anti-NKG2A mAb, or isotype controls. (E) γδ T cells and RPMI 8226 target cells were added to wells coated with sheep anti-mouse IgG (FC specific) and anti-CD94, anti-NKG2A mAb, or isotype control (wells coated with sheep mAb only were also used as controls). Results shown are mean % kill ± SD of quadruplicate wells.


A
media
CD94
NKG2A
isotype
% Kill
0
10
20
30
40
50
60
70
80
25.0:1
12.5:1
6.25:1
Effector:Target Ratio


B
media
CD94
p140
% Kill
0
10
20
30
40
50
60
70
80
90
25.0:1
12.5:1
6.25:1
Effector:Target Ratio


C
media
CD94
p140
p70
p58.1
p58.2
% Kill
0
10
20
30
40
50
12.5:1
6.25:1
Effector:Target Ratio


D
media
CD94
NKG2A
IgG2a
IgG2b
% Kill
0
10
20
30
40
50
60
12.5:1
6.25:1
3.1:1
Effector:Target Ratio


E
media
CD94
NKG2A
IgG2a
IgG2b
anti-mouse
% Kill
0
10
20
30
40
50
60
70
80
12.5:1
6.25:1
Effector:Target Ratio

mAbs to the 96 well plate. The cytotoxicity increased from 30.4% for the uncoated wells to 46.1% (CD94) and 50.5% (NKG2A) for the coated wells (E:T 12.5:1). This increase in percent kill may have been due to insufficient cross-linking of the mAbs. Instead of inducing an inhibitory signal, the mAbs actually blocked the interaction between the iNKR and the HLA class I molecules, and the percent kill increased.

In order to increase the amount of cross-linking, anti-CD94 or anti-NKG2A mAb was secondarily attached to the assay plate via goat anti-mouse IgG (Fc specific). Although decreased cytotoxicity was observed for the wells coated with anti-CD94 (Figure 3-6E; 54.5%) and anti-NKG2A (51.8%) mAb, as compared with the uncoated wells (62.7%), comparable decreases were also observed with the isotype controls and the primary antibody alone (50.9%, 55.8%, and 51.7%). This indicates that the inhibition was not specifically elicited by the anti-CD94 or -NKG2A mAb. The effector cells were responding to the presence of an antibody regardless of its specificity.

The inability to manipulate the cytotoxicity mediated by the polyclonal γδ T cell lines with anti-iNKR mAbs led to the hypothesis that although a minority of cells in each line were actually being regulated by the mAbs, their actions were being overwhelmed by the majority of cells which were not. For example, if an anti-p70 mAb was added to a cell line where only 2% of the cells expressed p70, then the majority of the cells would be unaffected by the mAb and their cytotoxic activities would continue. To investigate this possibility, it was decided to use transformed γδ T cell clonal lines. Each of these lines originated from a single *Herpesvirus saimiri* transformed γδ T cell, therefore, all of the cells in a particular line are identical. In terms of iNKR this means that all of the cells in

one line express the same iNKR repertoire and their cytotoxicity is expected to be regulated in the same manner.

3.3.3 iNKR Phenotype of Transformed γδ T cell Clones

Flow cytometry was used to determine the iNKR phenotype of 13 of our most commonly used clones. None of these clones expressed p70, p58.1, p58.2, or p140. To determine if this lack of expression was the result of the viral transformation, the iNKR phenotypes of 5 pairs of transformed and non-transformed γδ T cell lines were examined. The results obtained with 3 of these pairs are listed in Table 3-2. While the cell lines infected with virus were able to express all of the iNKR, there were sharp decreases in the percentage of cells expressing certain iNKR as compared with the non-infected parental lines. For example, for line 60 the percentage of cells expressing p140 dropped from 27% for the non-transformed line, to 2.4% for the transformed line. Similarly for line 80, p140 expression decreased from 14% for the non-transformed cells to 1.5% for the transformed cells.

Table 3-2 iNKR Phenotypes of γδ T cell Lines Transformed with *Herpesvirus saimiri* or Non-transformed Lines.

	γδ T Cell Line					
	60		70		80	
iNKR	non-transformed	transformed	non-transformed	transformed	non-transformed	transformed
CD94	29.5	56.3	61	33.4	55.3	64.2
p70	0.9	1	1.7	1.6	4.7	5
p58.1	1	1.7	0.2	0.7	0.6	0.8
p58.2	6.5	2.8	2	1.1	5.8	ND
p140	27	2.4	3.8	2.9	14	1.5
NKG2A	24	58.2	56	34	58.3	56.2

ND=not determined

These results did not demonstrate that the absence of p70, p58.1, p58.2, or p140 on the surface of the clones was a direct result of viral transformation, however, they did provide an explanation for this observation. The majority of γδ T cell lines have a very

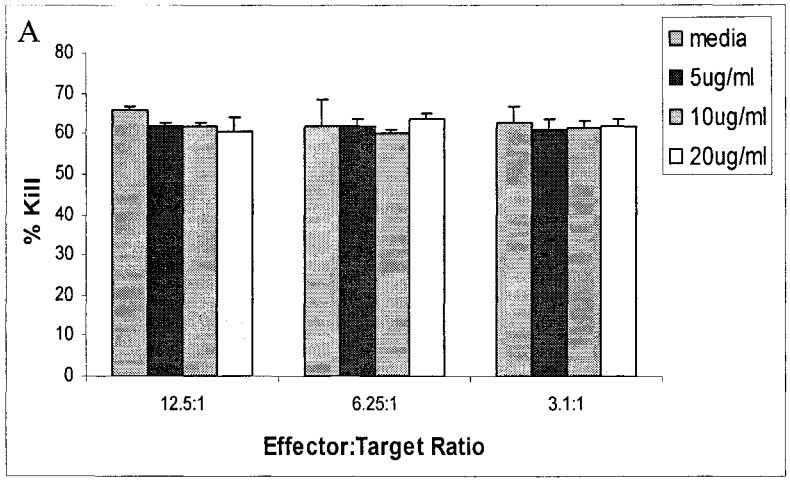
small percentage of p70, p58.1, p58.2, and p140 expressing cells. Transformation with *Herpesvirus saimiri*, may decrease this percentage. Since a small number of clones (10-30) are typically generated from each transformed γδ T cell line, the odds of selecting a p70, p58.1, p58.2, or p140 positive clone is quite low. This possibility could be explored by generating a larger number of clones from each line and analysing their iNKR phenotypes. Such analysis may also show whether the cloning efficiency is affected by the iNKR phenotype.

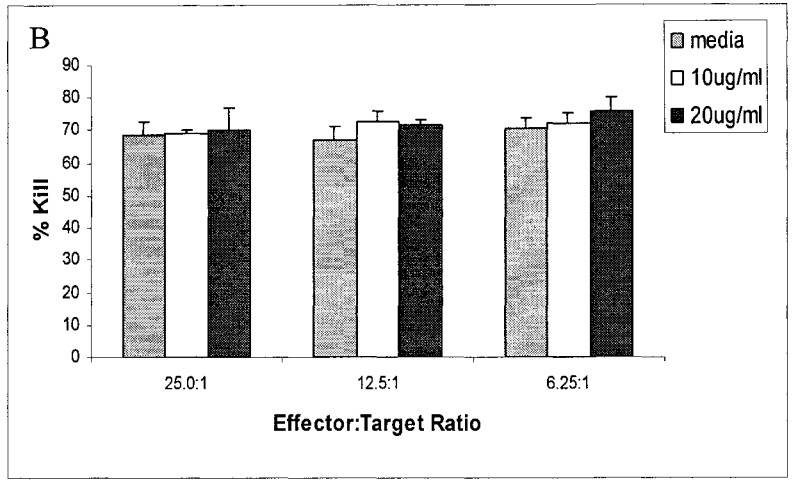
3.3.4 iNKR and Transformed γδ T cell Clone Mediated Cytotoxicity

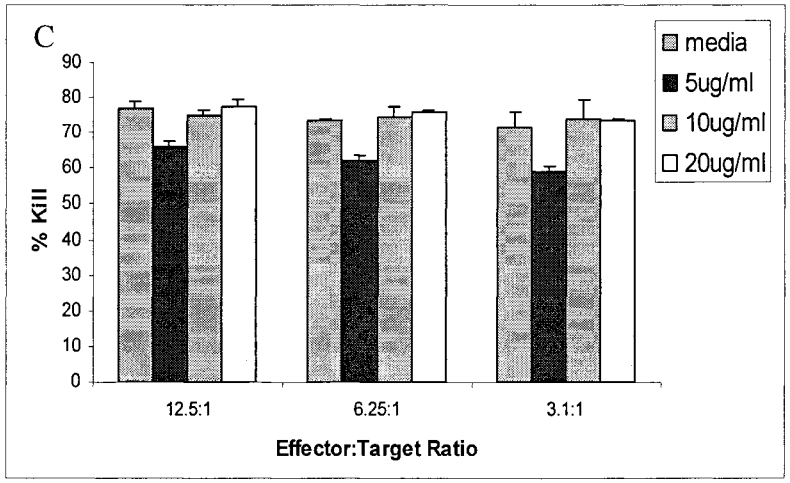
Experiments were carried out with clone 47-7 to investigate the role of iNKR in transformed γδ T cell clone mediated cytotoxicity. As Figures 3-7A and B demonstrate, the anti-CD94 mAb had no effect on the cytotoxicity of either K562 (A) or U937 (B) target cells at any of the concentrations used. Figure 3-7C shows that neither 10μg/ml nor 20μg/ml of anti-CD94 mAb changed the percent kill of RPMI 8226 targets, while 5μg/ml decreased the cytotoxicity (77.1% to 66.1%, 12.5:1). If the anti-CD94 mAb had bound to its iNKR ligands on the γδ T cells, the cytotoxicity of RPMI 8226 should have increased, as the receptors would be unable to recognize the HLA class I molecules expressed on the surface of the target cells and an inhibitory signal would not be generated. These titration experiments were repeated twice more, but the same results were obtained.

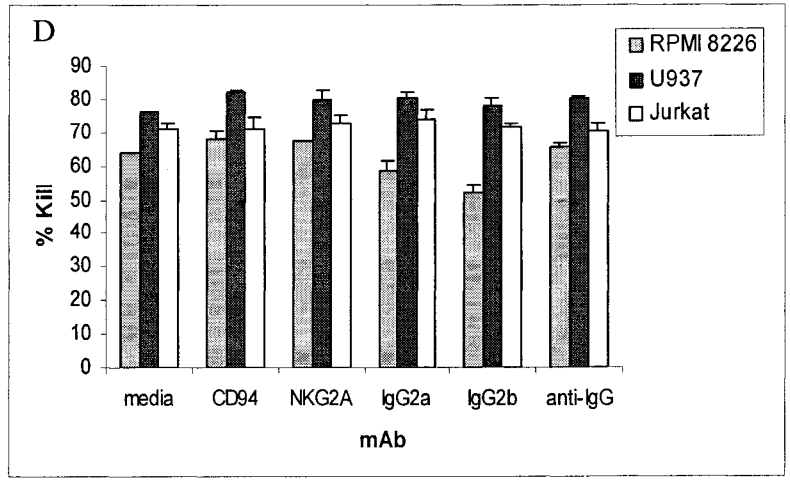
A further set of experiments to regulate the cytotoxicity mediated by γδ T cell clones involved cross-linking anti-CD94 or anti-NKG2A mAb to the assay plate via goat anti-mouse IgG (Fc specific). Figure 3-7D shows the results obtained for one of the 2

Figure 3-7 Effect of anti-CD94 mAb on cytotoxicity mediated by transformed γδ T cell clone 47-7 in 4.5h JAM assay. (A, B, and C) γδ T cells were incubated with 5, 10 or 20μg/ml anti-CD94 mAb for 30 minutes then added to assay wells with 10 000 [methyl-^{3}H] thymidine labeled K562 (A), U937 (B), or RPMI 8226 (C) target cells. (D) γδ T cells and [methyl-^{3}H] thymidine labeled RPMI 8226, U937, or Jurkat target cells were added to assay wells coated with sheep anti-mouse IgG (FC specific) and anti-CD94, anti-NKG2A mAb, or isotype control (wells coated with sheep mAb only were also used as controls). Results shown are mean % kill ± SD of quadruplicate wells.


A
media
5ug/ml
10ug/ml
20ug/ml
% Kill
0
10
20
30
40
50
60
70
80
12.5:1
6.25:1
3.1:1
Effector:Target Ratio


B
media
10ug/ml
20ug/ml
% Kill
0
10
20
30
40
50
60
70
80
90
25.0:1
12.5:1
6.25:1
Effector:Target Ratio


C
media
5ug/ml
10ug/ml
20ug/ml
% Kill
0
10
20
30
40
50
60
70
80
90
12.5:1
6.25:1
3.1:1
Effector:Target Ratio


D
RPMI 8226
U937
Jurkat
% Kill
0
10
20
30
40
50
60
70
80
90
media
CD94
NKG2A
IgG2a
IgG2b
anti-IgG
mAb

clones used in this experiment. Cross-linking the mAbs to the assay plate did not decrease the cytotoxicity of RPMI 8226, U937, or Jurkat target cells.

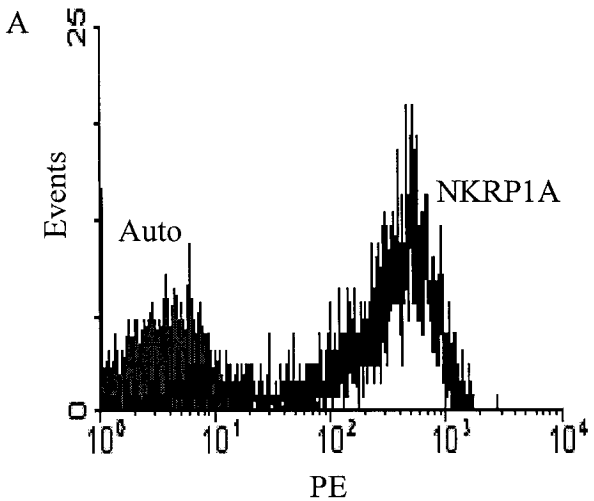
The results of the experiments with both the polyclonal γδ T cell lines and the transformed γδ T cell clones led to discussions with Drs. M. Bonneville and L. Battistini. Dr. Bonneville stated that the effect of the anti-iNKR mAbs differs from clone to clone, is highly dependent upon the presence of other inhibitory and activating NK cell receptors, and is very difficult to observe in polyclonal lines. Dr. Battistini remarked that he had observed that the presence of NKRP1A (CD161, an activating NK receptor whose activation is not HLA dependent) hinders the blocking effect of anti-CD94 and anti-NKG2A mAbs.

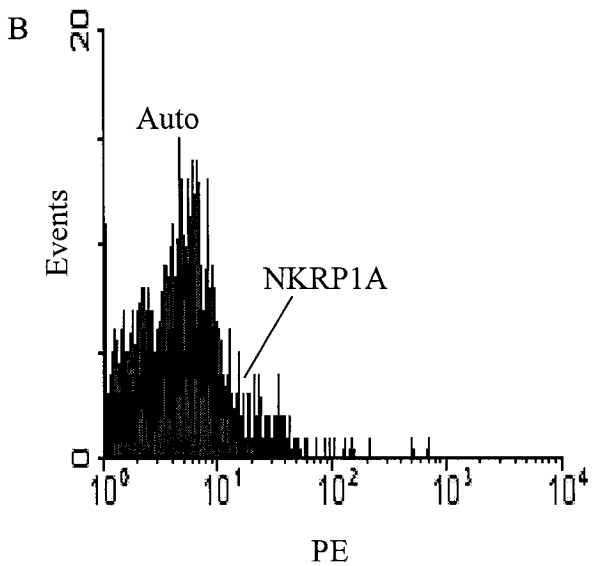
Flow cytometry analysis of 4 polyclonal γδ T cell lines and 2 transformed γδ T cell clones revealed that the majority of cells in the polyclonal lines expressed high amounts of NKRP1A (Figure 3-8A, representative). The clones also expressed NKRP1A (Figure 3-8B, representative) although at a much lower level than the polyclonal lines.

3.4 Studies with IFN-β (Avonex®)

IFN-β is one of the most widely prescribed drugs for the treatment of MS (353,354). The mechanisms underlying its therapeutic effects are not fully understood. IFN-β has been shown to modulate T cell activities and regulate the secretion of other cytokines and chemokines or their receptors (355,356). If γδ T cells are instrumental in the pathology of MS through deleterious cytotoxic actions, then it is plausible that the therapeutic effects of IFN-β may be due, in part, to an ability to manipulate iNKR expression and thereby manipulate the cytotoxicity of γδ T cells. It is also possible that

Figure 3-8 Detection of CD161 (NKRP1A) on γδ T cell lines (A; n=4) and transformed clones (B; n=2). Cells were labeled with anti-γδ TCR FITC and anti-CD161 PE mAbs and gated on the γδ FITC signal during flow cytometry.


A
25
Events
0
Auto
NKRP1A
10^0
10^1
10^2
10^3
10^4
PE


B
20
Events
0
Auto
NKRP1A
10^0
10^1
10^2
10^3
10^4
PE

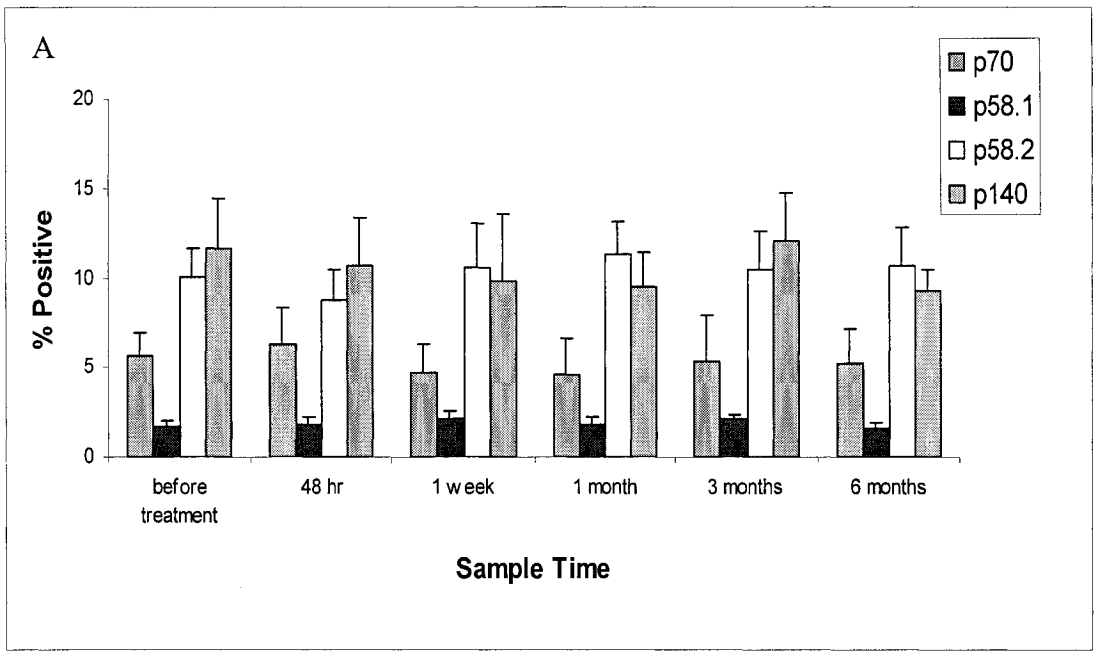
the therapeutic effect of IFN-β may be due to its ability to up-regulate the expression of HLA class I molecules on potential γδ T cell targets. The iNKR are not only sensitive to the HLA class I phenotype of the cells, but also to the level of expression of these molecules. If a target cell does not express the "appropriate" level of HLA class I molecules then it is susceptible to lysis. If the level is sufficiently increased, then the target cell is spared. In this manner, IFN-β treatment may lead to decreased γδ T cell mediated cytotoxicity.

3.4.1 Effect of IFN-β on iNKR expression *in vivo* and *in vitro*

The *in vivo* study consisted of 9 relapsing remitting MS patients who were taking Avonex®. These patients received 30μg of IFN-β intramuscularly once a week. Peripheral blood samples were taken twice before treatment began and at the following time points after the first injection: 48 hours, 1 week, 1 month, 3 months, and 6 months. (Samples were taken the day before the next scheduled injection.) γδ T cell lines were generated from these PB samples and the iNKR phenotypes were determined by flow cytometry. Figure 3-9A depicts the mean percentage (±SEM) of γδ T cells positive for p70, p58.1, p58.2, and p140 at each of the time points investigated, Figure 3-9B depicts the mean percentage (±SEM) of γδ T cells positive for CD94 and NKG2A at each time point. The percentage of cells expressing a particular iNKR did fluctuate during treatment, however, no significant differences were observed for any of the iNKR examined.

The *in vitro* study of IFN-β was investigated by incubating γδ T cells with serial dilutions of IFN-β (0, 10, 100, and 1000U/ml) and performing flow cytometry analysis at 0, 6, 12, and 24 hours. Representative results (n=2) are shown in Figure 3-10, treatment

Figure 3-9 Comparison of iNKR phenotypes of γδ T cell lines derived from PB of 9 MS patients treated with once weekly Avonex®. PB samples were taken before beginning treatment and 48 hours, 1 week, 1 month, 3 months, and 6 months after the first injection. γδ T cell lines were expanded as usual and their iNKR phenotypes determined by flow cytometry, as previously described. The results depicted here show the mean % positive cells ± SEM for p70, p58.1, p58.2, and p140 (A) or CD94 and NKG2A (B).


A
p70
p58.1
p58.2
p140
20
15
10
5
0
% Positive
before treatment
48 hr
1 week
1 month
3 months
6 months
Sample Time

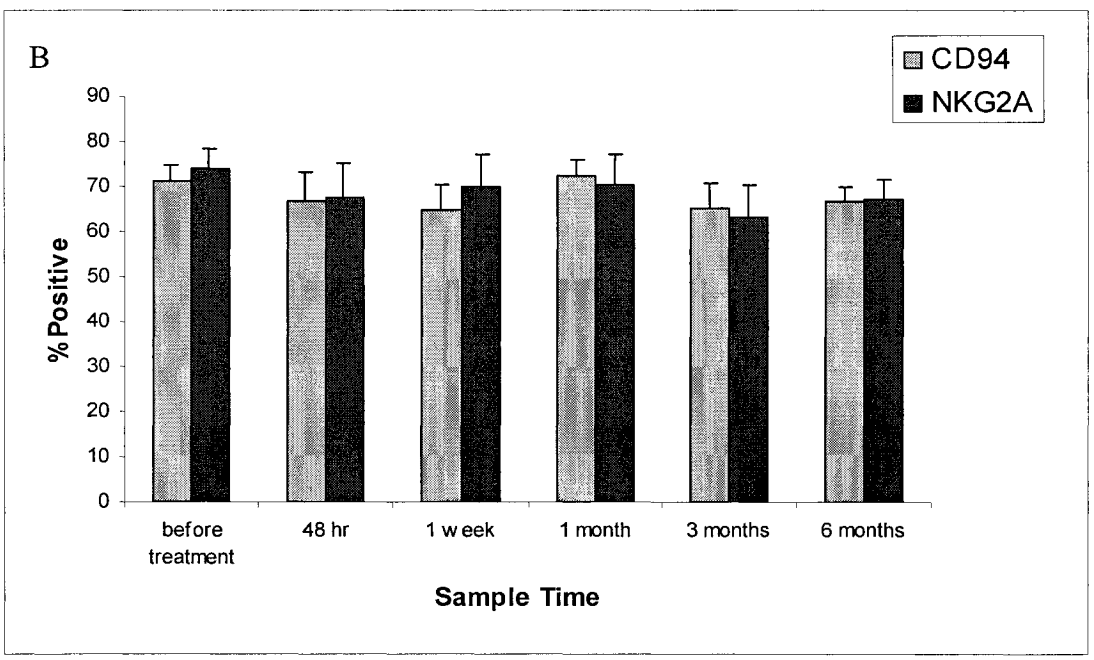
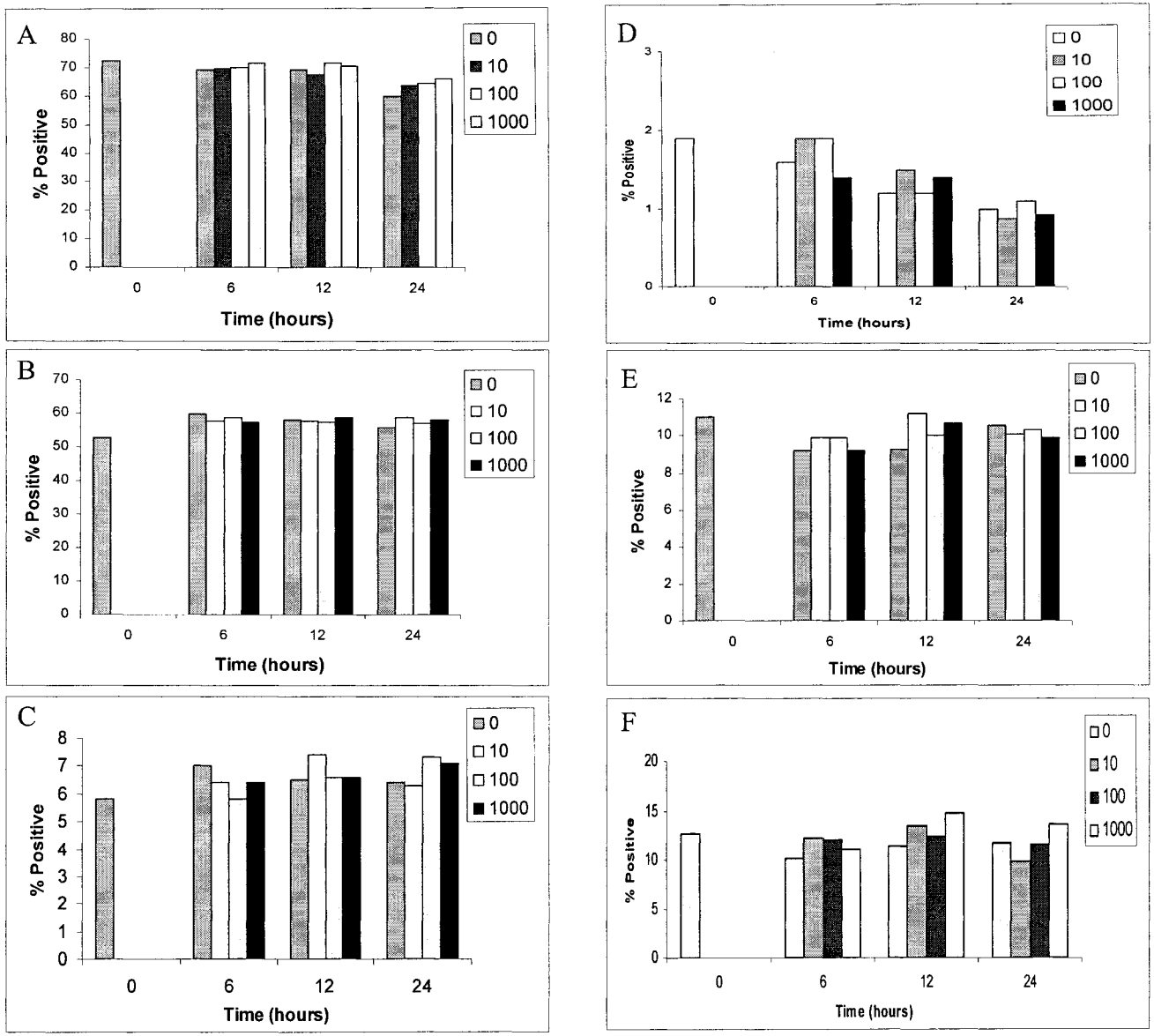

B
CD94
NKG2A
90
80
70
60
50
40
30
20
10
0
% Positive
before treatment
48 hr
1 week
1 month
3 months
6 months
Sample Time

Figure 3-10 Effect of *in vitro* treatment with IFN-β on iNKR phenotypes of γδ T cell lines. Cells were incubated with 0, 10, 100, or 1000U/ml IFN-β, aliquots were removed at 0, 6, 12, and 24 hours and flow cytometry analyses were performed to detect CD94 (A), NKG2A (B), p70 (C), p58.1 (D), p58.2 (E), and p140 (F). Representative results are shown (n=2).


A
% Positive
Time (hours)
0
6
12
24
0
10
100
1000
B
% Positive
Time (hours)
C
% Positive
Time (hours)
D
% Positive
Time (hours)
E
% Positive
Time (hours)
F
% Positive
Time (hours)

with IFN-β had no significant effect on the expression of CD94 (A), NKG2A (B), p70 (C), p58.1 (D), p58.2(E), or p140(F).

3.4.2 Increased Expression of HLA class I Molecules and Cytotoxicity

To investigate whether IFN-β induced up-regulation of HLA class I molecule expression affected γδ T cell mediated cytotoxicity, target cells were incubated with IFN-β prior to their use in JAM assays. Since IFN-γ also increases HLA class I molecule expression, target cells pre-incubated with IFN-γ were used for comparison.

The first step was to determine which of the target cell lines routinely used in the laboratory had a particularly low level of HLA class I expression that could be significantly increased with IFN-γ and β. The HLA class I expression of each of the target cell lines was analyzed by flow cytometry with anti-HLA-ABC mAb or an isotype control. Based on these results (Figure 3-11), KG-1 was selected for the up-regulation studies. Since it had a fairly low expression of HLA class I molecules, it was thought that a large increase in HLA class I expression could be easily obtained with IFNs. U937 was also chosen for use in these studies. Although U937 had a relatively high expression of HLA class I molecules, it had been used extensively in the laboratory and so was included in this study to add to the information already gathered about this line.

The concentration of IFN-γ which gave the largest increase in HLA class I expression was identified by incubating KG-1 and U937 target cells overnight with serial dilutions of IFN-γ. Flow cytometry analysis with anti-HLA-ABC mAb was performed the following day. Figure 3-12 shows the flow cytometry profiles obtained for HLA class I expression in the presence of various concentrations of IFN-γ for KG-1 (A) and

Figure 3-11 HLA class I molecule expression of Daudi, KG-1, Jurkat, Raji, RPMI 8226, U937, and Hut 78 target cells. The cells were blocked with non-specific mouse IgG, labeled with anti-HLA-ABC-FITC mAb, and analyzed by flow cytometry.

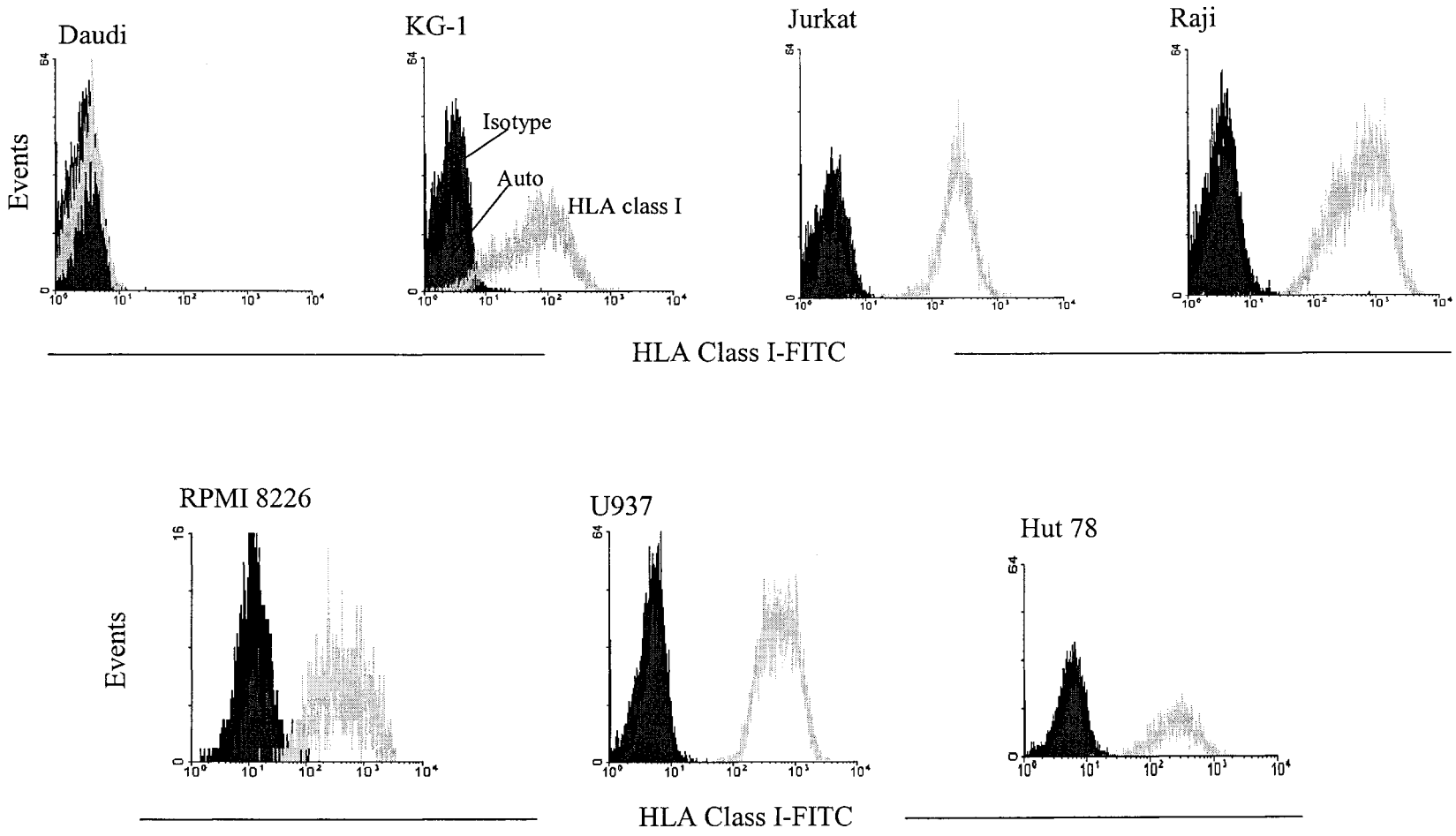
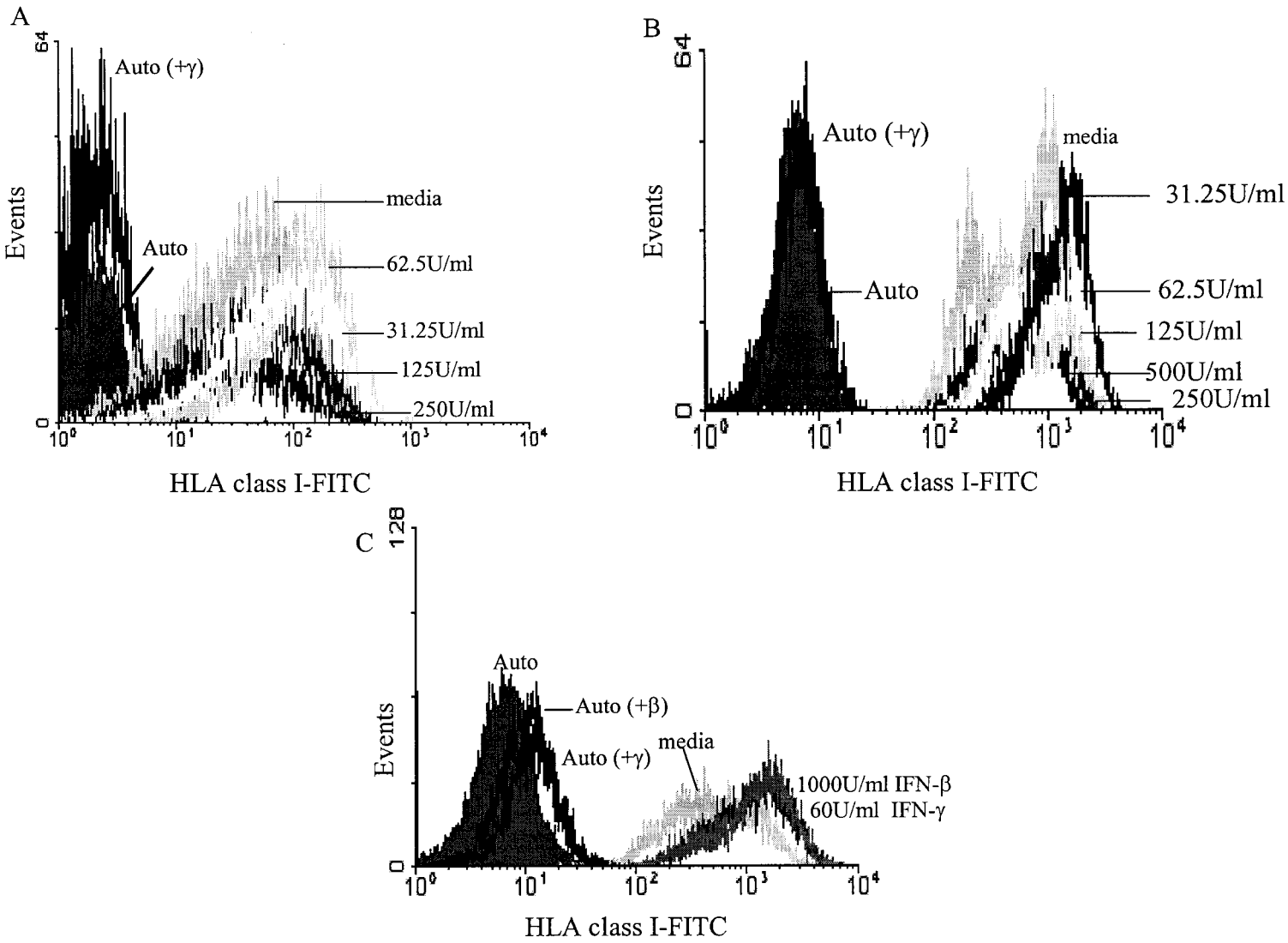

Daudi
KG-1
Jurkat
Raji
Isotype
Auto
HLA class I
Events
HLA Class I-FITC
RPMI 8226
U937
Hut 78
Events
HLA Class I-FITC

Figure 3-12 Effect of IFNs on expression of HLA class I molecules. KG-1 (A) and U937 (B) cells were incubated with titrated concentrations of IFN-γ overnight. The following day the cells were labeled with anti-HLA-ABC-FITC mAb and analyzed by flow cytometry. (C) Depicts the concentrations of IFN-γ and -β which led to the greatest increases in HLA class I molecule expression.


A
64
Auto (+γ)
Events
media
Auto
62.5U/ml
31.25U/ml
125U/ml
250U/ml
0
10^0 10^1 10^2 10^3 10^4
HLA class I-FITC
B
64
Auto (+γ)
media
31.25U/ml
Events
Auto
62.5U/ml
125U/ml
500U/ml
250U/ml
0
10^0 10^1 10^2 10^3 10^4
HLA class I-FITC
C
128
Auto
Auto (+β)
Events
media
Auto (+γ)
1000U/ml IFN-β
60U/ml IFN-γ
0
10^0 10^1 10^2 10^3 10^4
HLA class I-FITC

U937 (B). The top portion of Table 3-3 lists the mean fluorescence intensities (MFI) obtained for the results depicted in Figure 3-12A and B. (500U/ml was toxic to the KG-1 cells). Together these results indicated that the expression of HLA class I molecules on KG-1 could not be up-regulated with IFN-γ, while the expression of HLA class I on U937 was easily up-regulated in this manner. Further studies with U937 and IFN-γ identified 60U/ml as the concentration at which the maximum increase of HLA class I molecules was observed (Figure 3-12C and bottom portion of Table 3-3). Similar dilution experiments showed that a significant up-regulation in HLA class I expression was also obtained with 1000U/ml IFN-β (Figure 3-12C, note that the peaks due to incubation with 60 U/ml IFN-γ and 1000U/ml IFN-β are superimposed).

The next question to be addressed was whether an increase in HLA class I expression could protect potential target cells from γδ T cell mediated cytotoxicity. If so this may be a mechanism for the therapeutic effects of IFN-β. U937 target cells were incubated with 1000U/ml IFN-β or 60U/ml IFN-γ overnight and used in JAM assays the following morning. For each assay the target cells were analyzed by flow cytometry to ensure that the HLA class I molecules had been up-regulated. The results of these assays demonstrated that the percent kill of U937 target cells could be inhibited by IFN-γ (Figure 3-13A) or IFN-β (B) induced up-regulation of HLA class I molecules. However, these results were not easily demonstrated with all of the γδ T cell lines tested (data not shown).

3.4.3 IFN-β and the Cytotoxicity Mediated by Transformed γδ T cell Clones

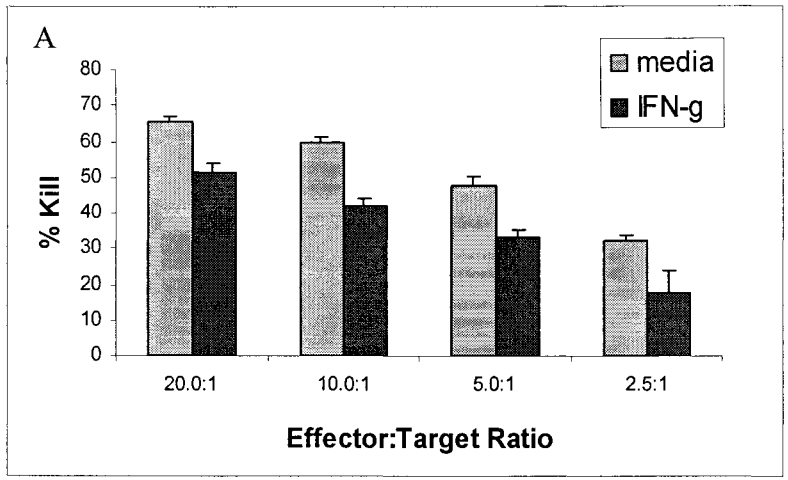
The variability observed with the polyclonal cell lines led to the decision to try the same cytotoxicity experiments with the transformed γδ T cell clones. It was

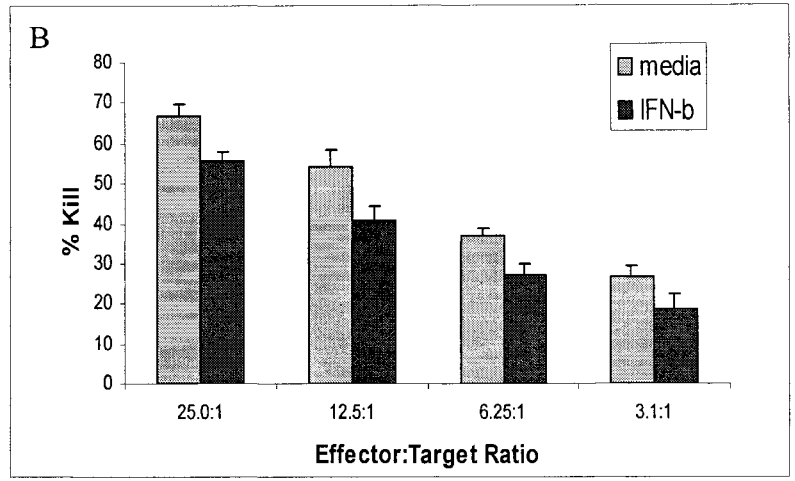
Table 3-3 Effect of IFN-γ on surface expression of HLA class I molecules by KG-1 and U937 target cells. Mean fluorescence intensity represents the relative concentration of HLA class I molecules.

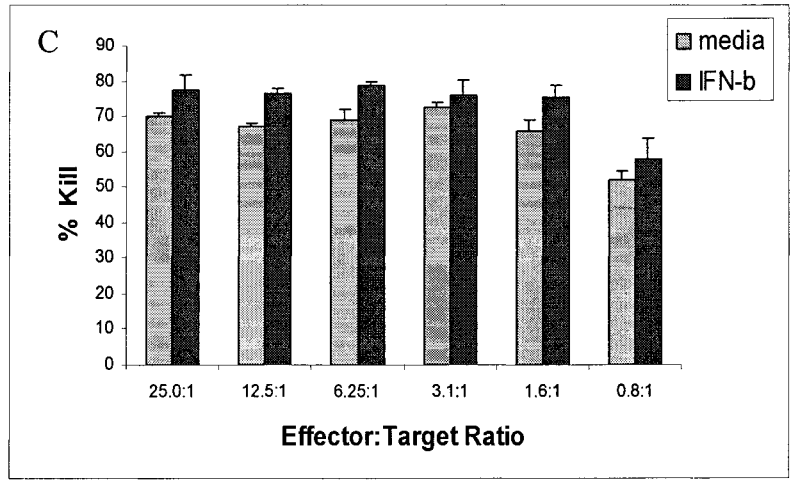
Concentration (U/ml)	Mean Fluorescence Intensity	
IFN-γ	KG-1	U937
0	4.28	57.6
31.25	8.24	135.4
62.5	10.8	88
125	4.88	74.7
250	3.16	66.8
500	N/D	79.3
0	N/D	91.5
5	N/D	125.4
10	N/D	145.4
20	N/D	153.2
30	N/D	124.3
40	N/D	125.6
50	N/D	164.1
60	N/D	172.5
70	N/D	93.5

N/D not determined

Figure 3-13 The effect of IFN induced increase in HLA class I expression on γδ T cell mediated cytotoxicity. U937 target cells were incubated overnight with [methyl-^{3}H] thymidine and either 60U/ml IFN-γ (A) or 1000U/ml IFN-β (B) then used in 4.5 hour JAM assays with γδ T cell lines. JAM assay with transformed γδ T cell clone 47-7 and [methyl-^{3}H]. thymidine labelled, IFN-β treated U937 target cells (C). Results are mean % kill ± SD of quadruplicate wells.


A
media
IFN-g
% Kill
80
70
60
50
40
30
20
10
0
20.0:1
10.0:1
5.0:1
2.5:1
Effector:Target Ratio


B
media
IFN-b
% Kill
80
70
60
50
40
30
20
10
0
25.0:1
12.5:1
6.25:1
3.1:1
Effector:Target Ratio


C
media
IFN-b
% Kill
90
80
70
60
50
40
30
20
10
0
25.0:1
12.5:1
6.25:1
3.1:1
1.6:1
0.8:1
Effector:Target Ratio

hypothesized that using a clonal line would simplify the experiment since all the cells in a particular line expressed the same iNKR phenotype. In 2 separate experiments, clone 47-7 was used in JAM assays with U937 target cells that had been pre-incubated with 1000U/ml IFN-β. (As before, the HLA class I expression was analyzed after incubation with IFN-β to ensure the expression had been up-regulated). The results of one such assay are depicted in Figure 3-13C, up-regulation of HLA class I on U937 target cells did not protect the cells from γδ T cell mediated cytotoxicity.

The results obtained from the work aimed at manipulating the cytotoxicity mediated by γδ T cell lines and transformed clones through anti-iNKR mAbs or HLA class I up-regulation could not be explained by the isolated effects of iNKR/HLA class I interactions. Therefore, concentrating my research on this specific group of molecules was not felt to be capable of yielding any further results, as the cytotoxicity of the γδ T cells was certainly controlled by a complex set of reactions with a number of different molecules.

3.5 Cytotoxic Mechanisms Utilized by γδ T cell Lines

The lack of specific reagents, particularly mAbs, to examine the cytotoxic mechanisms utilized by γδ T cell lines led to the use of more indirect methods, such as brefeldin A and concanamycin A, to investigate possible mechanisms. Based on this work it was concluded that the perforin pathway was the predominant pathway used by γδ T cells, but that the Fas/FasL pathway could be used secondarily (339).

The purpose of my new project would be to investigate the cytotoxic mechanisms of γδ T cells with the aid of more specific inhibitors and mAbs directed against various

molecules in the cytotoxic pathways. The assays would also use three target cells with different known cytotoxic susceptibilities: RPMI 8226 (perforin sensitive), U937 (perforin and Fas sensitive), and Jurkat (Fas sensitive). Analyses of the results obtained with all three targets would allow a more complete understanding of the cytotoxic pathways used by γδ T cells.

3.5.1 The Perforin/Granzyme Pathway

3.5.1.1 Background

The first step in the investigation of the perforin/granzyme pathway was the detection of these molecules within the γδ T cell lines. A number of problems had to be solved before routine flow cytometry for perforin, granzymes A, B, and M, and granulysin could begin: (1) perforin could not be detected with the intracellular staining protocol typically used for detection of intracellular cytokines, so a new protocol had to be developed, (2) the anti-granzyme B and M mAbs had to be conjugated to a fluorochrome using the Alexa Fluor® 488 Monoclonal Antibody Labeling Kit, the FITC labelled mAbs were then titrated to determine the volumes which gave the best flow cytometry results, (3) the anti-granulysin antibody was unlabelled polyclonal rabbit antibody, therefore, a secondary mAb [goat anti-rabbit-FITC (Fc specific)] was used in the detection of granulysin and the appropriate working dilutions for each of these antibodies had to be determined, (4) the Smyth lab had been unsuccessful in their attempts to use the anti-granzyme M antibody in flow cytometry, therefore, the new perforin protocol was adapted for this antibody, (5) the specificity of the mAbs had to be ensured with blocking experiments or the use of isotype controls. The results of one such blocking (isotype) experiment are shown in Figure 3-14 (n=3). The blocking

experiments for perforin, granzyme A, and granzyme B, show that the mAbs are specific. The blocking of granzyme B is not as complete as that of perforin and granzyme A. In an effort to conserve the anti-granzyme B mAb only 7x the amount of labelled mAb was used (the perforin and granzyme A blocks used 10x). The anti-granzyme M mAb was a kind gift of Dr. M. Smyth and most of this antibody was used trying to identify the correct conditions under which it would label, therefore, an isotype control was used rather than a blocking experiment. The anti-granulysin polyclonal rabbit antibody was easily detected with the secondary mAb, rabbit IgG was used as an isotype control, and a sample containing only the secondary mAb was always included in the flow analysis as a negative control.

The results shown in Figure 3-14 depict the typical flow cytometry profile obtained when the cell lines were stained for perforin (n=40), granzyme A (n=16), granzyme B (n=17), granzyme M (n=20), and granulysin (n=20).

Before experiments to investigate the role of perforin in γδ T cell mediated cytotoxicity could begin, it was important to know if the relative level of perforin stored within the granules varied with time in culture. Nine PB and 3 CSF derived γδ T cell lines were followed in culture from days 7-47 with intracellular flow cytometry analysis at 2-7 day intervals. Figure 3-15A (representative) shows that the relative amount of perforin contained within the cells (represented by the mean fluorescence intensity) increased between day 16 and day 27 of culture. Therefore, experiments were carried out with cell lines between 20 and 27 days in culture.

Another variable to be addressed was rIL-2 supplementation. JAM assays were typically carried out with cell lines (or clones) that had received rIL-2 one or two days

Figure 3-14 Detection of cytotoxic molecules stored in γδ T cells. Cells were surface stained with anti-CD3-QR and anti-γδ TCR-PE mAbs, permeabilized with a 0.1% saponin solution, and intracellularly labeled with anti-perforin or anti-granzymes A, B, or M FITC conjugated mAbs. Granulysin was detected with a combination of polyclonal rabbit anti-granulysin antibody and goat anti-rabbit IgG-FITC. The specificity of the antibodies was confirmed with isotype controls, non-specific rabbit IgG, or blocking with unlabelled mAbs. Flow cytometry analysis was performed with gating on CD3 and γδ TCR positive cells. Representative results are shown (n>15).

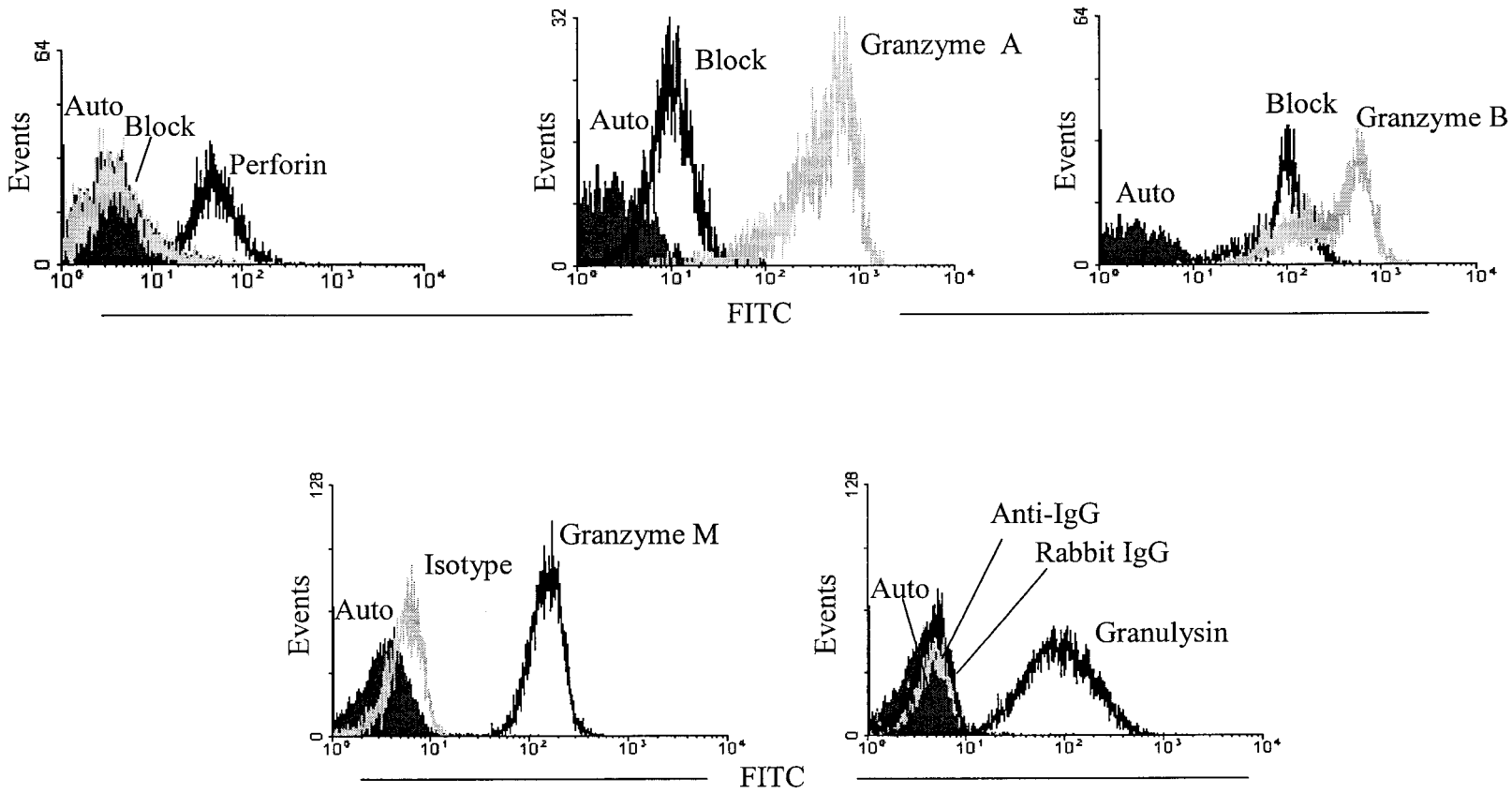
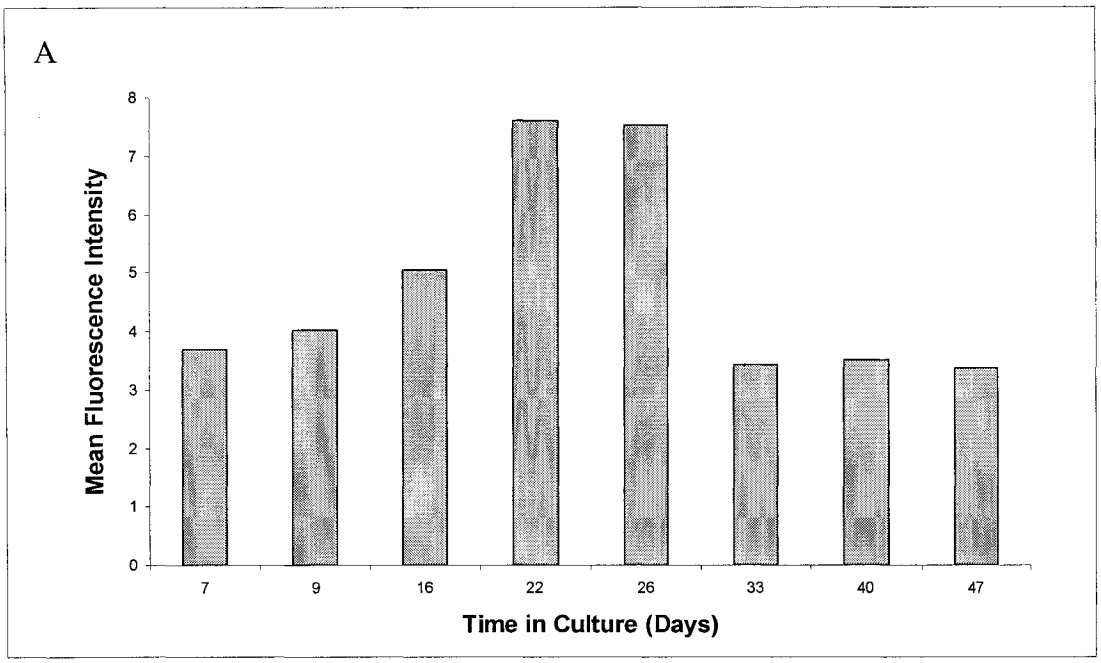
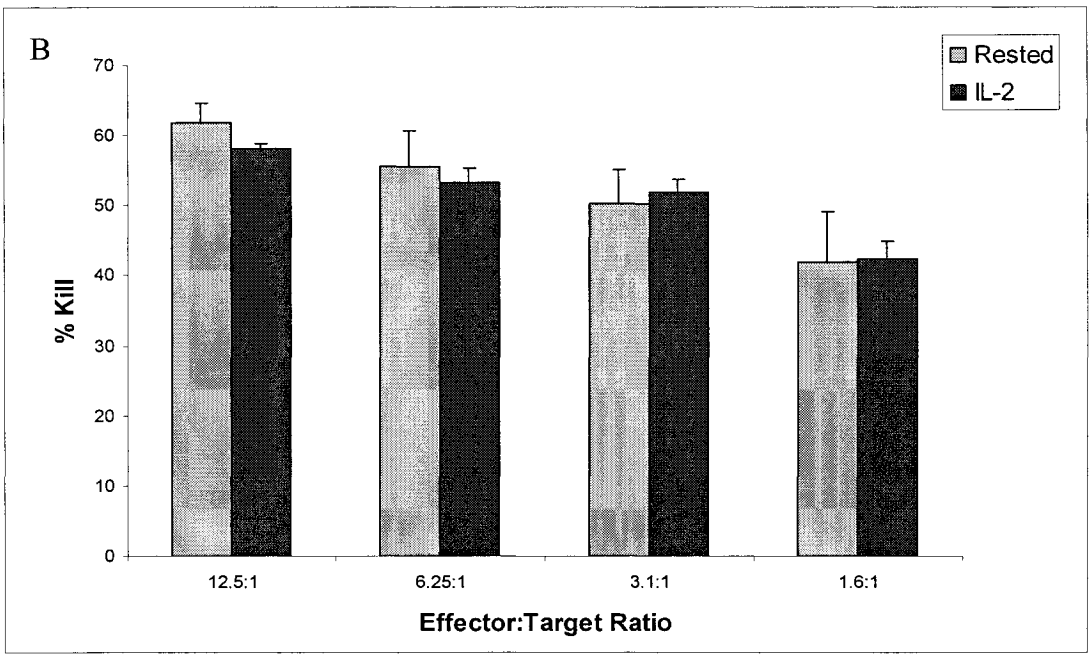

Events
Auto
Block
Perforin
Granzyme A
Granzyme B
Granzyme M
Isotype
Anti-IgG
Rabbit IgG
Granulysin
FITC

Figure 3-15 (A) Change in relative concentration of perforin stored within γδ T cells with time in culture. γδ T cell lines were generated from PB and CSF samples and perforin was detected with intracellular flow cytometry between days 7-47. The relative amount of perforin contained within the cells is represented by the mean fluorescence intensity. Results shown are representative of the 9 PB and 3 CSF derived cell lines examined. (B) The effect of rIL-2 supplementation on γδ T cell mediated cytotoxicity in 4.5 hour JAM assays. The γδ T cell lines were either supplemented with rIL-2 within 24 hours of being used in JAM assays or were "rested" (deprived of rIL-2) for 72 hours prior to the assay. Results shown are mean % kill ± SD of quadruplicate wells of representative line, n=2.


A
Mean Fluorescence Intensity
0
1
2
3
4
5
6
7
8
7
9
16
22
26
33
40
47
Time in Culture (Days)


B
Rested
IL-2
% Kill
0
10
20
30
40
50
60
70
12.5:1
6.25:1
3.1:1
1.6:1
Effector:Target Ratio

before the assay. To test whether rIL-2 supplementation had an effect on the cytotoxicity of the lines, cells were either supplemented with rIL-2 within 24 hours of the assay or were rested (no rIL-2 for 48 hours) prior to the assay. The results shown in Figure 3-15B (representative, n=2) demonstrate that the cytotoxic ability of the cells was unaffected by rIL-2 supplementation.

Preliminary cytotoxicity assays with effector to target ratios ranging from 50:1 to 0.2:1 demonstrated that the cytotoxicity mediated by γδ T cells was quite potent (>60% kill), even at a ratio of 12.5:1 (data not shown). As well, the inhibition effects of the mAbs against various cytotoxic molecules were easily observed at this ratio (data not shown). In an effort to conserve reagents and most importantly to conserve γδ T cells, I decided to use an E:T ratio of 12.5:1 for all subsequent assays investigating the cytotoxic mechanisms.

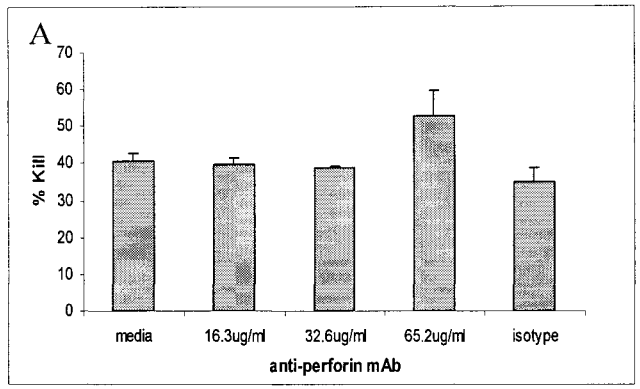
3.5.1.2 The Perforin Pathway

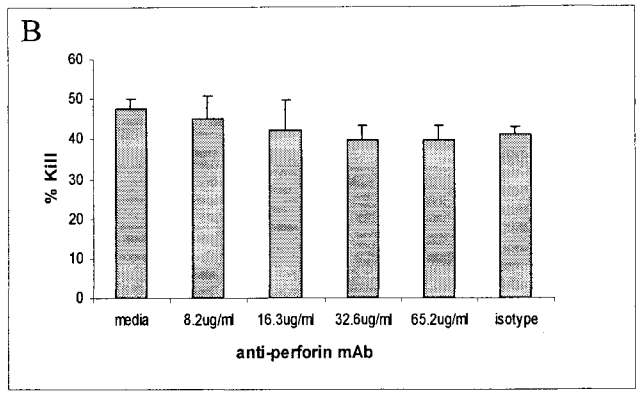
A. Perforin

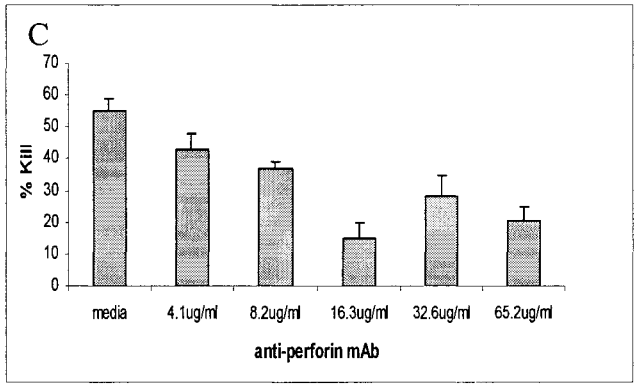
Titrations of anti-perforin mAb were carried out to determine the concentration which yielded the maximum inhibition of cytotoxicity for each target. Anti-perforin mAb did not significantly inhibit the cytolysis of U937 (Figure 3-16A) or Jurkat (Figure 3-16B) target cells at any of the concentrations tested. However, 16.3μg/ml significantly decreased the percent kill of RPMI 8226 (Figure 3-16C, 55.2% to 14.8%).

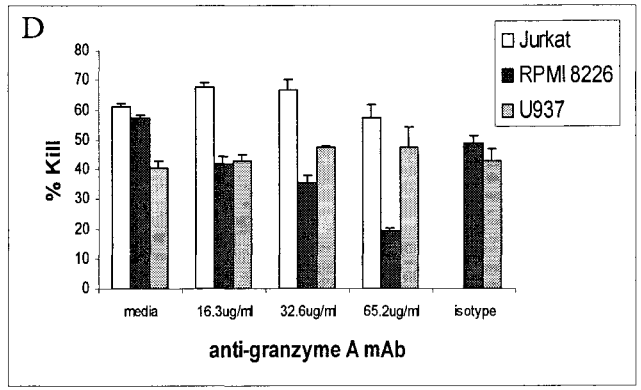
Since the cytotoxicity of U937 and Jurkat target cells was unaffected by the anti-perforin mAb, other cytotoxic mechanisms must be responsible for their lysis. In addition, the anti-perforin mAb could not account for all of the killing of RPMI 8226, so it was necessary to investigate other components of the perforin/granzyme pathway.

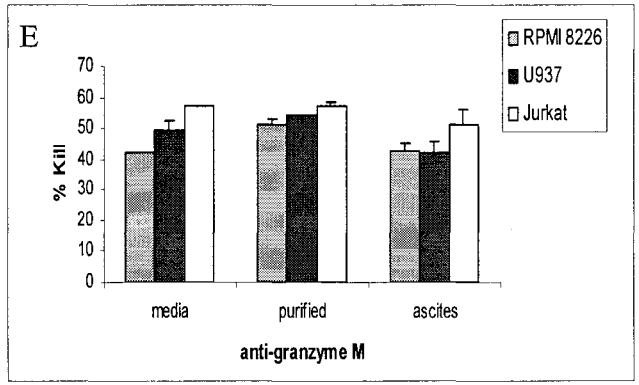
Figure 3-16 Titration of antibodies specific for cytotoxic molecules expressed by γδ T cells. JAM assays were carried out for 4.5 hours with [methyl-^{3}H] thymidine labeled targets at an E:T ratio of 12.5:1, with 10 000 target cells per well. Antibodies were added to the assay wells prior to the addition of the target cells. Anti-perforin mAb was titrated with U937 (A), Jurkat (B), and RPMI 8226 (C) target cells as was anti-granzyme A mAb (D). Anti-granzyme M mAb was added as either 43.5μg/ml (purified) or 10μl of a 1:100 dilution of ascites fluid (E). Similarly, polyclonal rabbit anti-granulysin was used at a dilution of 1:10 or 1:100, while the purified mAb was added at a concentration of 35.7μg/ml (F). Results shown are mean % kill ± SD of triplicate wells.

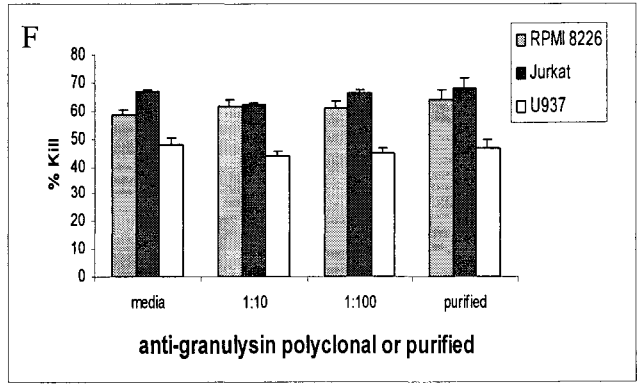

A
% Kill
media
16.3ug/ml
32.6ug/ml
65.2ug/ml
isotype
anti-perforin mAb


B
% Kill
media
8.2ug/ml
16.3ug/ml
32.6ug/ml
65.2ug/ml
isotype
anti-perforin mAb


C
% Kill
media
4.1ug/ml
8.2ug/ml
16.3ug/ml
32.6ug/ml
65.2ug/ml
anti-perforin mAb


D
Jurkat
RPMI 8226
U937
% Kill
media
16.3ug/ml
32.6ug/ml
65.2ug/ml
isotype
anti-granzyme A mAb


E
RPMI 8226
U937
Jurkat
% Kill
media
purified
ascites
anti-granzyme M


F
RPMI 8226
Jurkat
U937
% Kill
media
1:10
1:100
purified
anti-granulysin polyclonal or purified

B. Granzyme A

Titration of anti-granzyme A mAb showed a significant decrease in the cytotoxicity of RPMI 8226 (57.1% to 19.3%) at a concentration of 65.2μg/ml (Figure 3-16D). However, anti-granzyme A mAb did not decrease the cytotoxicity of U937 or Jurkat target cells.

C. Granzyme M

Anti-granzyme M mAb (ascites fluid) was tested in JAM assays undiluted (2.5μl/well) and at 1:100, 1:1000, and 1:2000 dilutions, no change in cytotoxicity was observed in any of these experiments (data not shown). Following these preliminary assays, a series of JAM assays (in total 17 γδ T cell lines were incubated with each of the 3 targets) were carried out with the 1:100 diluted anti-granzyme M ascites to determine if this mAb had a significant effect on the cytotoxicity of RPMI 8226, U937, or Jurkat target cells. Statistical analyses (one tailed, paired, *t* test) of these experiments did not identify significant inhibition (data not shown).

A small volume of purified anti-granzyme M mAb (to be labelled with FITC for the flow cytometry work) was later obtained from Dr. Smyth and tested in several JAM assays. Purified anti-granzyme M mAb (43.5μg/ml) or a 1:100 dilution of ascites were added to sample wells with U937, Jurkat, or RPMI 8226 target cells (Figure 3-16E), again there were no significant changes in the percent kill of any of the target cells. [Purified anti-granzyme M mAb (21.75μg/ml) was also used with U937 (n=2), RPMI 82326 (n=3), and Jurkat (n=2), the results were identical to those depicted in Figure 3-16E].

The small volumes of both the purified mAb and the ascites fluid available made in depth study of the role of granzyme M in γδ T cell mediated cytotoxicity impossible. The results of the preliminary experiments described here cannot be used to rule out a role for granzyme M in γδ T cell mediated cytotoxicity.

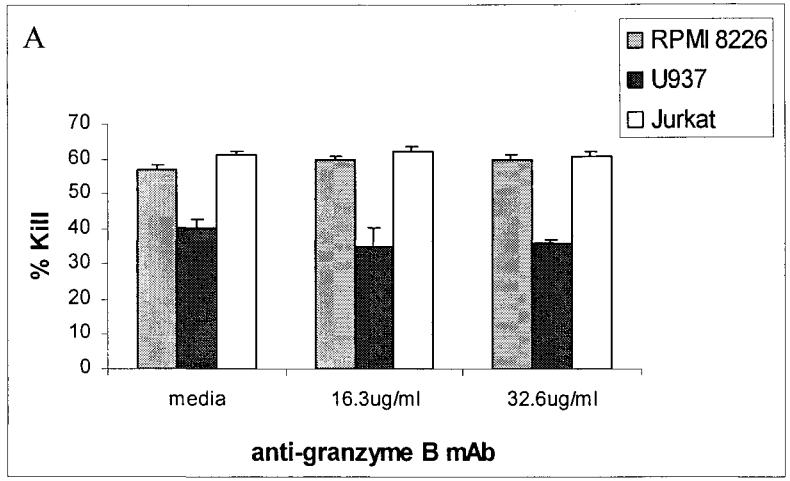
D. Granulysin

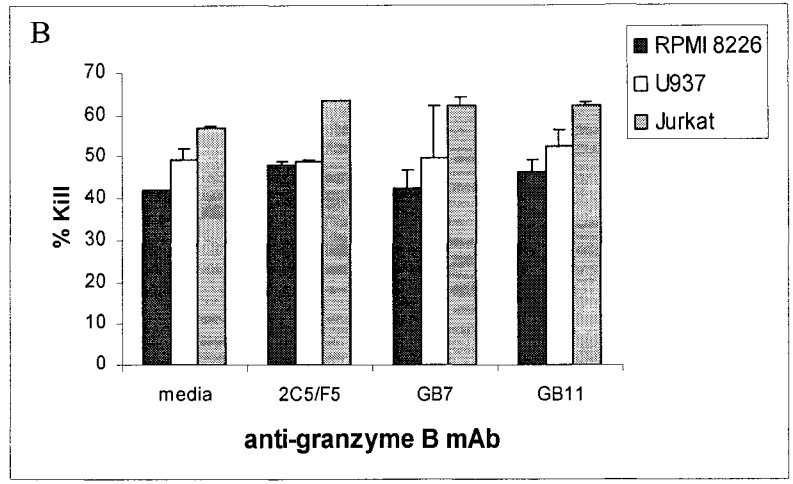
Anti-granulysin antibody was supplied as an extremely small volume of polyclonal rabbit antibody. Preliminary JAM assays tested the anti-granulysin antibody at 1:1000, 1:5000, and 1:10 000 dilutions, with rabbit IgG as a control. The percent kill of the target cells was unaffected by the antibody at each of the dilutions used (data not shown). As shown in Figure 3-16F, a 1:10 (Jurkat n=16; U937 and RPMI 8226 n=8) or 1:100 (RPMI 8226 and U937 n=16; Jurkat n=8) dilution of anti-granulysin polyclonal antibody or purified mouse anti-granulysin mAb (35.7μg/ml; Jurkat, RPMI 8226, and U937 n=4) had no effect on the cytotoxicity of either of the target cells. As for the anti-granzyme M experiments, a conclusion about the role of granulysin in γδ T cell mediated cytotoxicity cannot be made on the basis of these preliminary results.

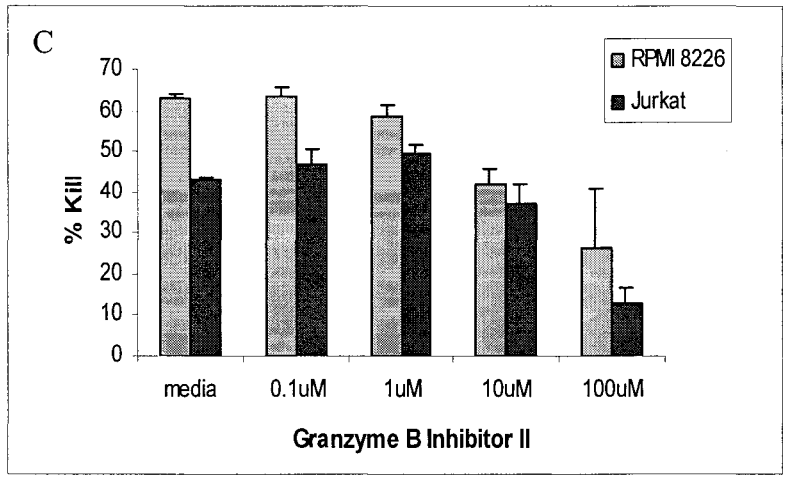
E. Granzyme B

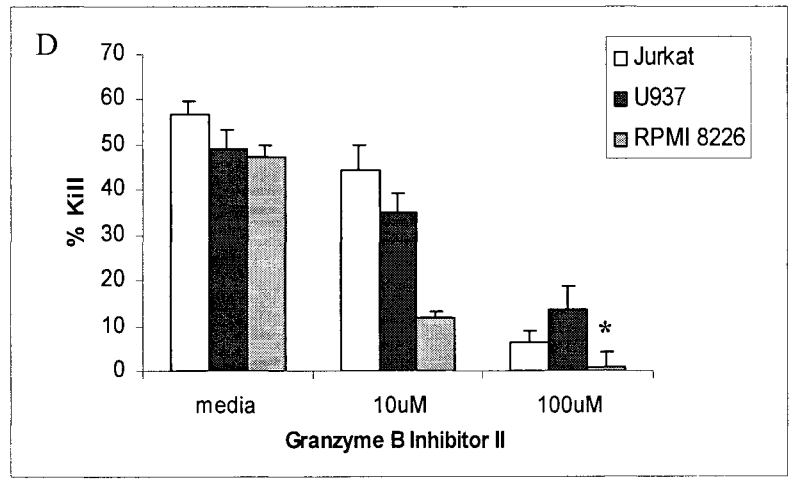
Several anti-granzyme B mAb clones were commercially available (2C5/F5, GB7, GB11), but had either not been shown to inhibit granzyme B or had not been tested in cytotoxicity assays. It was decided to try each of these clones in JAM assays. Clone 2C5/F5 was used with all 3 targets at a concentration of 16.3μg/ml or 32.6μg/ml with no change in cytotoxicity for any of the targets (Figure 3-17A, representative; U937 n=3; Jurkat n=4; RPMI 8226 n=5). A comparison of all 3 clones at a concentration of 21.7μg/ml also failed to show any change in cytotoxicity (Figure 3-17B, representative;

Figure 3-17 The role of granzyme B in γδ T cell mediated cytotoxicity. JAM assays were carried out for 4.5 hours with [methyl-^{3}H] thymidine labeled U937, Jurkat, and RPMI 8226 target cells at an E:T ratio of 12.5:1. Antibodies were added directly to the assay wells. Anti-granzyme B mAb (clone 2C5/F5) was added at a concentration of 16.3 or 32.6μg/ml (A). The three anti-granzyme B clones (2C5/F5, GB7, and GB11) were used in the assays at a concentration of 21.7μg/ml (B). Titration of granzyme B inhibitor II (C and D). (* % kill is actually 0.03%, but cannot be accurately graphed on this scale). Results are mean % kill ± SD of triplicate wells.


A
RPMI 8226
U937
Jurkat
% Kill
70
60
50
40
30
20
10
0
media
16.3ug/ml
32.6ug/ml
anti-granzyme B mAb


B
RPMI 8226
U937
Jurkat
% Kill
70
60
50
40
30
20
10
0
media
2C5/F5
GB7
GB11
anti-granzyme B mAb


C
RPMI 8226
Jurkat
% Kill
70
60
50
40
30
20
10
0
media
0.1uM
1uM
10uM
100uM
Granzyme B Inhibitor II


D
Jurkat
U937
RPMI 8226
% Kill
70
60
50
40
30
20
10
0
*
media
10uM
100uM
Granzyme B Inhibitor II

GB7 and GB11 n=4, 2C5/F5 n=10 for each target).

Although the anti-granzyme B mAbs had failed to inhibit the cytotoxicity mediated by γδ T cells, granzyme B could not yet be ruled out as a possible cytotoxic mechanism as there was no evidence that any of these mAbs were capable of inhibiting the protease activity of granzyme B. To determine if granzyme B was an important cytotoxic mediator utilized by γδ T cells, a granzyme B inhibitor (Granzyme B Inhibitor II) was employed. Titration experiments with granzyme B inhibitor II (Figure 3-17C and D) showed that a concentration of 100μM significantly decreased the cytotoxicity of Jurkat (43.3% to 13%), RPMI 8226 (62.9% to 26.3%), and U937 target cells (49.0% to 13.6%).

The results of the experiments discussed in this section demonstrate that γδ T cells use the perforin/granzyme pathway. Granzyme M and granulysin are stored by the cells and presumably have a role in γδ T cell mediated cytotoxicity, however, this cannot be confirmed from the data discussed here. Although perforin and granzyme A are important cytotoxic mediators, these results show granzyme B to be the more potent cytotoxic molecule.

3.5.2 The Fas/FasL Pathway

FasL typically has a low level of surface expression and is rapidly cleaved from the surface of the cell by metalloproteinases making detection by flow cytometry extremely difficult (315,352). A number of different protocols have been developed to allow the detection of FasL. Three such protocols have been used in the studies discussed here, however, only one of these protocols yielded positive flow cytometry results. This protocol involved incubating $1x10^6$cells/ml with 5mM EDTA for 2 hours at

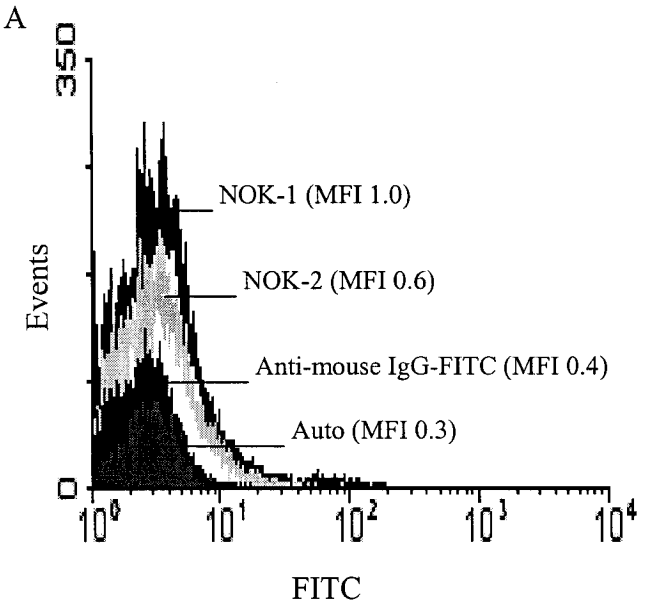
37°C, NOK-1 or NOK-2 mAb was added to a well and incubated for 5 minutes at room temperature, finally 1μg/ml ionomycin was added and incubated for 7 minutes. The cells were transferred ($5x10^5$) to a 1.5ml Eppendorf tube, fixed with 2% PFA, and washed with FACS buffer before the secondary mAb [goat anti-mouse IgG, Fc specific, FITC] was added. As a control, cells were incubated with the secondary mAb only. Figure 3-18A depicts the FasL flow cytometry profile. While neither of the mAbs resulted in a strong positive stain (NOK-1, MFI=1.0; NOK-2, MFI=0.6), the observation that the MFI for the auto and the secondary mAb were 0.3 and 0.4, respectively, indicates that the cells are weakly expressing FasL. Attempts to detect FasL intracellularly were unsuccessful (data not shown).

The next step was to use a Fas blocking mAb to investigate whether or not the Fas/FasL pathway was important in γδ T cell mediated cytotoxicity. Titration experiments with this mAb (Figure 3-18B) showed significant decreases in the percent kill of each of the target cells (at 2μg/ml; U937 50.2% to 33.9%, RPMI 8226 67.9% to 55.6%, Jurkat 65.1% to 31%). A concentration of 2μg/ml was chosen for all subsequent experiments.

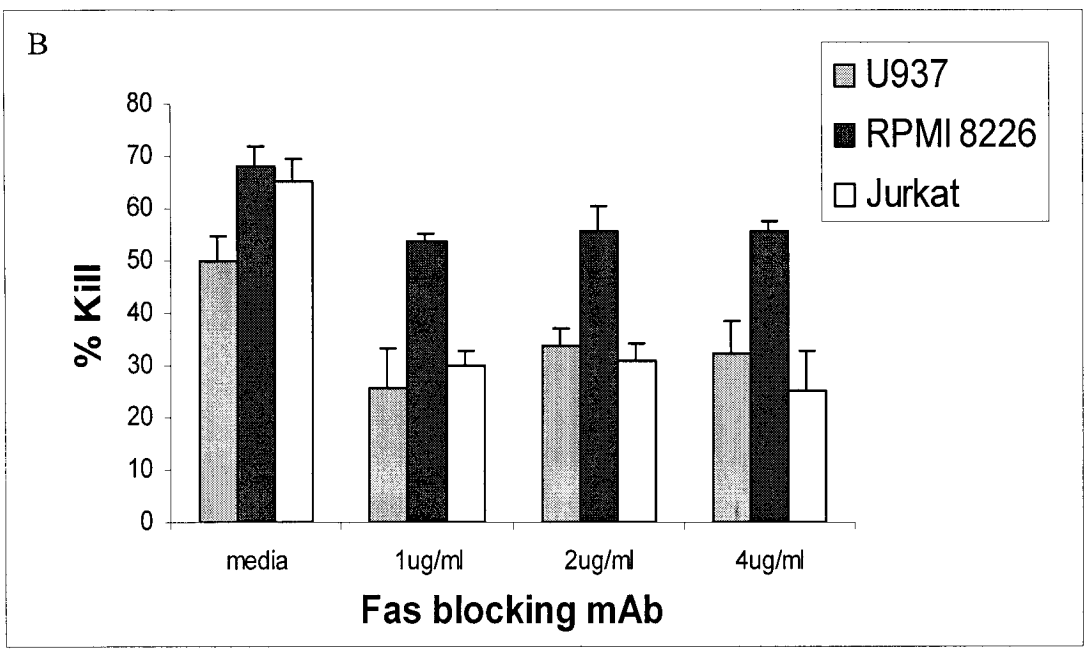
The results described in the previous sections clearly demonstrate that the Fas/FasL and the perforin/granzyme pathway are used by γδ T cells. Furthermore, it can be inferred from these results that these two pathways are simultaneously (perhaps synergistically) utilized by γδ T cells. This possibility will be discussed further in the following sections.

Figure 3-18 (A) Detection of FasL on the surface of γδ T cells. Cells were incubated with 5 mM EDTA for 2 hours, NOK-1 or NOK-2 (anti-FasL) mAb for 5 minutes, then ionomycin (1μg/ml) for 7 minutes. Bound NOK-1 or NOK-2 was detected with goat anti-mouse IgG (Fc specific; FITC). MFI=mean fluorescence intensity. (B) Titration of Fas blocking mAb. [methyl-^{3}H] thymidine labeled target cells were incubated with 1, 2, or 4 μg/ml Fas blocking mAb for 1 hour, washed, and diluted to $1x10^5$ cells/ml. Before the target cells were added to the assay wells, 5μg/ml Fas blocking mAb was added. JAM assays were carried out for 4.5 hours at an E:T ratio of 12.5:1. Results are mean % kill ±SD of triplicate wells.

A


350
Events
0
NOK-1 (MFI 1.0)
NOK-2 (MFI 0.6)
Anti-mouse IgG-FITC (MFI 0.4)
Auto (MFI 0.3)
10^0
10^1
10^2
10^3
10^4
FITC

B


U937
RPMI 8226
Jurkat
% Kill
80
70
60
50
40
30
20
10
0
media
1ug/ml
2ug/ml
4ug/ml
Fas blocking mAb

3.5.3 Cytotoxic Mechanisms: PB versus CSF

Once the optimal concentrations of anti-perforin and anti-granzyme A mAbs, Fas blocking mAb, and granzyme B inhibitor II had been determined, a large number of JAM assays were carried out to determine if the resulting inhibitions were statistically significant. The diagnoses of the patients from whom the PB and CSF γδ T cells had been derived were concealed until after the JAM assays had been performed and analyzed. The results were then divided into 5 groups (for each target): HC, MS CSF, MS PB, OND CSF, and OND PB. This allowed for statistical analyses (one tailed, paired *t* test) to determine if different cytotoxic mechanisms were responsible for the death of each of the target cells and whether the cytotoxic mechanisms used by PB versus CSF derived cells or HC versus MS versus OND derived cells were identical. The results of these assays are summarized in Tables 3-4, 3-5, and 3-6. Each table lists the mean % kill ± SEM, the number of samples, and the p values.

Table 3-4 summarizes the results of JAM assays with U937 target cells. The addition of anti-perforin or anti-granzyme A mAb did not inhibit the cytotoxicity of these target cells. However, Fas blocking mAb significantly decreased the cytotoxicity of MS PB, OND PB, MS CSF, and OND CSF samples ($p \leq 0.03$). The cytotoxicity of HC samples was also decreased, but was not statistically significant ($p=0.1$). Granzyme B inhibitor II significantly decreased the percent kill for cells derived from MS PB, OND CSF, and HC ($p \leq 0.048$). Decreased inhibition was also observed in the MS CSF and OND PB categories, but was not statistically significant ($p=0.1$ and 0.2, respectively).

Table 3-5 summarizes the results of JAM assays with Jurkat target cells. The cytotoxicity mediated by MS PB, MS CSF, OND CSF, and HC derived cell lines was

Table 3-4 JAM assays with U937 target cells and MS PB, OND PB, MS CSF, OND CSF, or HC derived γδ T cells. Cytotoxicity was inhibited with anti-perforin or anti-granzyme A mAb, Fas blocking mAb or granzyme B inhibitor II. Values shown are mean percent kill ± SEM, number of samples tested (n), and p values (one tailed, paired, t test).

U937	MS PB		OND PB		MS CSF		OND CSF		H C	
	media	mAb	media	mAb	media	mAb	media	mAb	media	mAb
Perforin										
Mean ± SEM	35.0 ± 2.1	40.0 ± 2.1	29.1 ± 2.3	31.9 ± 6.4	33.6 ± 6.2	36.6 ± 6.5	33.8 ± 5.5	44.9 ± 5.7	19.2 ± 3.3	28.9 ± 2.0
n		5		5		7		5		3
Granzyme A										
Mean ± SEM	31.4 ± 3.3	39.6 ± 2.4	25.0 ± 3.7	22.0 ± 3.8	33.6 ± 6.2	39.0 ± 4.0	34.5 ± 7.1	49.7 ± 7.5	39.0 ± 6.4	46.8 ± 7.5
n		5		5		7		4		3
Fas										
Mean ± SEM	42.9 ± 4.3	28.9 ± 4.3	34.2 ± 5.8	20.9 ± 10.4	34.7 ± 5.6	26.7 ± 5.7	37.6 ± 3.6	25.5 ± 6.9	26.8 ± 8.0	20.7 ± 6.3
n		4		4		8		7		4
p value		0.01		0.03		0.02		0.01		0.1
Granzyme B inhibitor II										
Mean ± SEM	34.4 ± 4.9	20.6 ± 1.9	27.4 ± 8.7	16.7 ± 2.7	42.2 ± 4.6	30.9 ± 11.4	39.2 ± 7.9	18.1 ± 10.0	19.2 ± 3.3	6.2 ± 3.1
n		4		5		4		4		3
p value		0.02		0.1		0.2		0.03		0.048

Table 3-5 JAM assays with Jurkat target cells and MS PB, OND PB, MS CSF, OND CSF, or HC derived γδ T cells. Cytotoxicity was inhibited with anti-perforin or anti-granzyme A mAb, Fas blocking mAb or granzyme B inhibitor II. Values shown are mean percent kill ± SEM, number of samples tested (n), and p values (one tailed, paired, t test).

Jurkat	MS PB		OND PB		MS CSF		OND CSF		H C	
	media	mAb	media	mAb	media	mAb	media	mAb	media	mAb
Perforin										
Mean ± SEM	42.0 ± 3.7	40.3 ± 4.2	47.2 ± 1.9	42.5 ± 1.6	38.4 ± 7.5	41.7 ± 7.3	50.7 ± 5.7	51.4 ± 6.9	60.5 ± 1.0	64.1 ± 4.8
n		5		5		7		5		3
p value				0.02						
Granzyme A										
Mean ± SEM	42.6 ± 6.9	39.3 ± 10.4	47.5 ± 2.8	44.4 ± 4.4	38.4 ± 7.5	36.9 ± 7.9	51.0 ± 7.3	53.8 ± 6.5	55.1 ± 2.6	44.9 ± 5.3
n		4		5		7		4		3
Fas										
Mean ± SEM	51.5 ± 6.3	30.4 ± 2.7	49.7 ± 3.1	23.9 ± 2.9	39.3 ± 6.7	27.2 ± 5.3	56.8 ± 4.3	33.8 ± 6.3	60.2 ± 0.7	40.3 ± 3.4
n		6		6		8		6		4
p value		0.008		0.001		0.01		0.0006		0.008
Granzyme B inhibitor II										
Mean ± SEM	54.5 ± 4.3	6.3 ± 3.5	44.5 ± 5.6	1.4 ± 4.8	43.4 ± 12.6	12.3 ± 6.5	58.9 ± 3.1	6.9 ± 4.8	60.5 ± 1.0	20.2 ± 6.7
n		4		5		4		4		3
p value		0.00003		0.003		0.01		0.00007		0.01

unaffected by either anti-perforin or anti-granzyme A mAb. The cytotoxicity mediated by OND PB derived cell lines was unaffected by anti-granzyme A, but significantly decreased in response to anti-perforin mAb ($p=0.02$). Both the Fas blocking mAb and granzyme B inhibitor II significantly decreased the percent kill of Jurkat target cells by all 5 categories of γδ T cell lines ($p \leq 0.01$).

Table 3-6 summarizes the results of JAM assays with RPMI 8226 target cells. The anti-perforin mAb significantly decreased cytotoxicity in each of the 5 categories ($p \leq 0.02$), as did the Fas blocking mAb ($p \leq 0.04$). Anti-granzyme A mAb significantly decreased cytotoxicity for the MS PB, OND PB, MS CSF, and OND CSF groups ($p \leq 0.04$). Decreased cytotoxicity was observed with the HC group, but was not statistically significant ($p=0.06$). Granzyme B inhibitor II significantly decreased the cytotoxicity mediated by cells derived from MS PB, OND PB, OND CSF, and HC ($p \leq 0.006$), however, the decreased cytotoxicity observed for MS CSF was not statistically significant ($p=0.2$).

The results shown in Tables 3-4 to 3-6 suggest that γδ T cells derived from different compartments (PB vs. CSF) or from healthy, MS, or OND individuals have differences in their cytotoxic mechanisms. For example, the results in Table 3-4 indicate that MS PB and OND CSF derived cells kill U937 target cells with a combination of FasL and granzyme B, OND PB and MS CSF derived cells kill U937 cells with FasL, and HC derived cells kill U937 target cells via granzyme B. Similar differences can be seen in Tables 3-5 and 3-6. These are interesting observations, with important implications in MS pathogenesis, however, a larger sample size must be analyzed before

Table 3-6 JAM assays with RPMI 8226 target cells and MS PB, OND PB, MS CSF, OND CSF, or HC derived γδ T cells. Cytotoxicity was inhibited with anti-perforin or anti-granzyme A mAb, Fas blocking mAb or granzyme B inhibitor II. Values shown are mean percent kill ± SEM, number of samples tested (n), and p values (one tailed, paired, t test).

RPMI 8226	MS PB		OND PB		MS CSF		OND CSF		H C	
	media	mAb	media	mAb	media	mAb	media	mAb	media	mAb
Perforin										
Mean ± SEM	47.5 ± 2.9	31.8 ± 5.2	55.3 ± 5.3	41.5 ± 7.2	46.2 ± 4.4	40.4 ± 4.4	56.2 ± 3.8	45.1 ± 4.6	58.7 ± 2.9	41.8 ± 6.5
n		14		8		17		13		8
p value		0.0002		0.002		0.02		0.0003		0.003
Granzyme A										
Mean ± SEM	54.9 ± 2.3	28.3 ± 8.5	57.1 ± 7.5	33.3 ± 11.5	52.4 ± 1.4	36.5 ± 8.2	48.2 ± 6.4	34.2 ± 6.7	54.0 ± 6.1	32.7 ± 2.5
n		4		4		8		6		3
p value		0.01		0.02		0.03		0.04		0.06
Fas										
Mean ± SEM	48.9 ± 5.3	41.0 ± 4.2	54.9 ± 2.1	48.5 ± 3.1	52.4 ± 1.4	44.5 ± 2.5	54.5 ± 5.3	47.6 ± 6.2	66.4 ± 1.0	55.5 ± 3.1
n		5		5		8		5		4
p value		0.003		0.04		0.008		0.04		0.02
Granzyme B inhibitor II										
Mean ± SEM	54.1 ± 2.9	15.3 ± 5.6	52.7 ± 4.4	12.0 ± 4.9	43.4 ± 12.6	28.8 ± 12.1	52.5 ± 5.9	16.4 ± 6.3	66.6 ± 1.4	30.3 ± 5.3
n		4		5		4		4		3
p value		0.003		0.001		0.2		0.002		0.006

conclusions regarding compartment or disease specific cytotoxic mechanisms can be made.

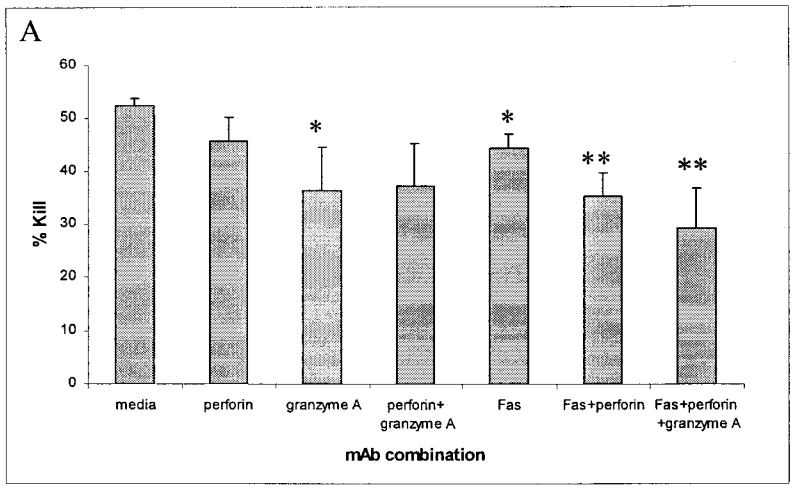
It is possible to make several general conclusions about γδ T cell mediated cytotoxicity at this time. First, granzymes A and B are important mediators of this cytotoxicity. Second, γδ T cells are capable of using both the perforin/granzyme and Fas/FasL pathways. Third, there is no "predominant" pathway, γδ T cells use the perforin/granzyme or the Fas/FasL pathway or a combination of the two. The idea of pathways acting simultaneously is explored further in the next section.

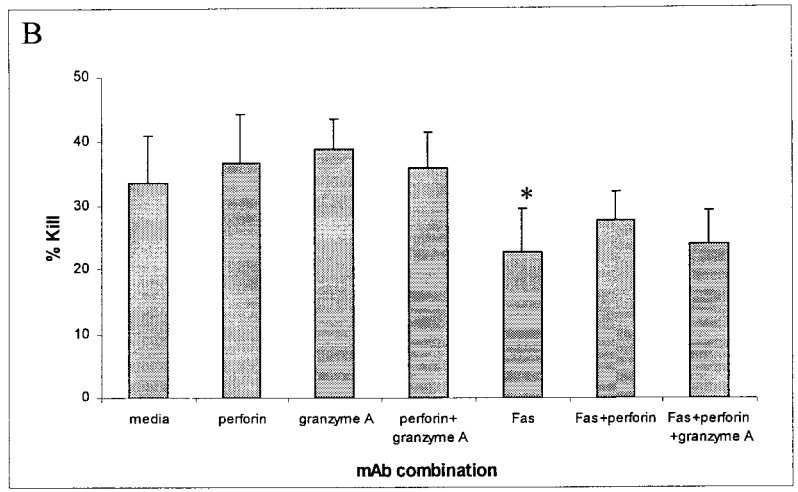
3.5.4 Are the Perforin/Granzyme and Fas/FasL Pathways Acting Together?

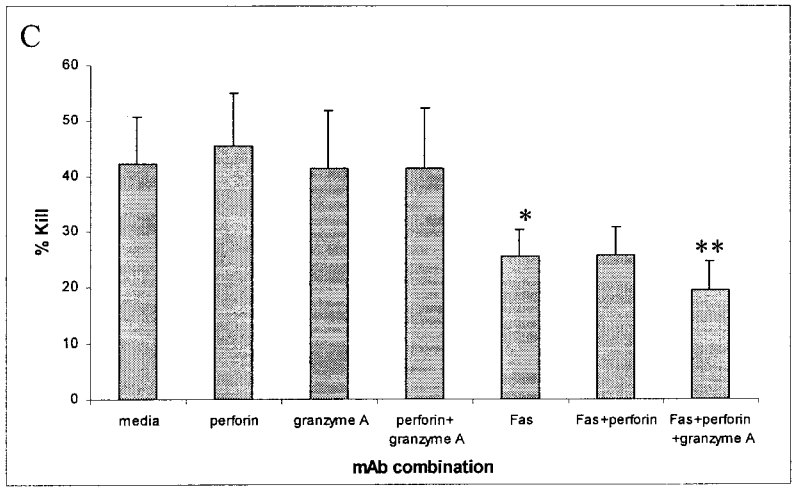
To investigate whether perforin, granzyme A, and FasL were utilized simultaneously, and perhaps synergistically, combinations of anti-perforin, anti-granzyme A, and Fas blocking mAbs were added to JAM assays with MS CSF derived γδ T cells and U937, Jurkat, or RPMI 8226 target cells. For RPMI 8226 target cells (Figure 3-19A, n=8), the combination of anti-perforin + anti-granzyme A mAbs was no more effective than anti-granzyme A alone (* p=0.03). In contrast, the combination of Fas blocking + anti-perforin mAbs or Fas blocking + anti-perforin + anti-granzyme A mAbs had a greater inhibitive effect (** p=0.02 and ** p=0.03, respectively) than Fas blocking mAb alone (* p=0.008).

The percent kill of U937 target cells (Figure 3-19B, n=6) was significantly decreased by Fas blocking mAb (* p=0.009). However, the combination of Fas blocking + anti-perforin mAbs or Fas blocking + anti-perforin + anti-granzyme A mAbs did not result in a further reduction in cytotoxicity.

Figure 3-19 JAM assays with combinations of anti-perforin, anti-granzyme A, and Fas blocking mAbs. MS CSF derived γδ T cell lines were incubated with [methyl-^{3}H] thymidine labeled RPMI 8226 (A), U937 (B), or Jurkat (C) target cells at an E:T ratio of 12.5:1. The concentrations of the mAbs used were as follows: anti-perforin 16.3μg/ml, anti-granzyme A 65.2μg/ml, and Fas blocking 2μg/ml (+5μg/ml). The results shown here represent the mean % kill ± SEM. * represents a significant decrease in % kill as compared with the media value. ** represents a significant inhibition in % kill as compared with the addition of the Fas blocking mAb alone. Statistical analyses used the one-tailed, paired t test.


A
% Kill
60
50
40
30
20
10
0
*
*
**
**
media
perforin
granzyme A
perforin+ granzyme A
Fas
Fas+perforin
Fas+perforin +granzyme A
mAb combination


B
% Kill
50
40
30
20
10
0
*
media
perforin
granzyme A
perforin+ granzyme A
Fas
Fas+perforin
Fas+perforin +granzyme A
mAb combination


C
% Kill
60
50
40
30
20
10
0
*
**
media
perforin
granzyme A
perforin+ granzyme A
Fas
Fas+perforin
Fas+perforin +granzyme A
mAb combination

The addition of anti-perforin or anti-granzyme A mAb or a combination of the two had no effect on the cytotoxicity of Jurkat target cells (Figure 3-19C, n=5). The addition of Fas blocking mAb significantly decreased cytotoxicity (* p=0.02). A further decrease in cytotoxicity was observed with the Fas blocking + anti-perforin + anti-granzyme A mAbs combination (** p=0.02), but not with the Fas blocking + anti-perforin mAbs combination.

The results summarized in Tables 3-4 to 3-6 and Figure 3-19 demonstrate that γδ T cell lines use the perforin/granzyme and the Fas/FasL pathways simultaneously and in some instances have a synergistic effect. In addition, these results show that the targets are killed by a different mixture of cytotoxic components: RPMI 8226 target cells by a mix of perforin, granzymes A and B, and Fas L, Jurkat and U937 cells by FasL and granzyme B. This leads to the question of whether γδ T cells are capable of "recognizing" that target cell lines have different cytotoxic susceptibilities and adjusting their cytotoxic mechanisms accordingly.

3.5.5 Are γδ T cells Capable of "Cytotoxic Decision Making"? Granule Enzyme Exocytosis

In order to address the question of γδ T cell "recognition" of target cell susceptibility, I decided to examine the granule enzyme exocytosis by γδ T cells in response to RPMI 8226, U937, and Jurkat target cells.

3.5.5.1 Technique and Background

The granule enzyme exocytosis assay measures the amount of serine esterase released from the cytoplasmic granules of cytotoxic cells in response to a stimulus, such as an antibody or another cell. The supernatant is collected and the substrate (BLT) and

colouring agent (DTNB) are added. The enzyme activity is measured spectrophotometrically and the percentage of mAb- or cell-induced secretion is calculated. A negative value indicates that the stimulus did not induce a level of secretion higher than the spontaneous secretion of the cell.

In this study, the granule enzyme exocytosis assays were carried out in response to target cells, therefore, JAM assays were set-up with extra wells for enzyme analysis. Before the secretion induced by the target cells could be measured, it was necessary to determine whether the target cells themselves released serine esterases. To investigate this possibility, $2x10^6$ cells/ml of each target cell (and one PB derived γδ T cell line, positive control) were incubated with media alone or with Triton X-100 for 4 hours at 37°C, in triplicate (Triton X-100 induces maximum granule release). Table 3-7 lists the mean absorbances ± SD for each target and the PB derived γδ T cell line. The absorbances for the target cells were the same in the presence or absence of Triton X-100 indicating that the target cells do not secrete serine esterases. The absorbance for the PB derived γδ T cell line increased with Triton X-100 (from 0.06 to 0.14) indicating that the γδ T cells are secreting serine esterases.

Table 3-7 Results of granule enzyme exocytosis assay to test serine esterase secretion by target cells.

	Cell Line				
Treatment	Hut 78	U937	RPMI 8226	Jurkat	MS PB1
media	0.01 ± 0.009	0 ± 0.006	0.01 ± 0.004	0.02 ± 0.005	0.06 ± 0.003
Triton X-100	0.01 ± 0.008	0.01 ± 0.01	0.01 ± 0.008	0.02 ± 0.002	0.14 ± 0.06

3.5.5.2 Granule Enzyme Exocytosis: Results

Granule enzyme exocytosis assays were carried out simultaneously with JAM assays using 3 PB and 2 CSF derived γδ T cell lines (2 PB/CSF pairs) with Jurkat, U937, and RPMI 8226 target cells. The calculated percent secretion (and the mean absorbance

± SD) for each γδ T cell line in response to each of the target cells is listed in Table 3-8. (The number of MS CSF1 cells remaining after the JAM assay was set-up did not allow the granule enzyme exocytosis assay to be carried out in triplicate for this particular line). The amount of serine esterase released from a particular γδ T cell line varied considerably depending on the target cell which induced the secretion. For example, the percent secretion by the OND CSF1 line was -7.4% for Jurkat, 38.2% for RPMI 8226, and 5.9% for U937. The percent secretion also differed between the PB/CSF cell line pairs in response to the same target cell, for example, in response to U937 the percent secretion was 1.1% for the MS PB1 line and 67.9% for the MS CSF1 line.

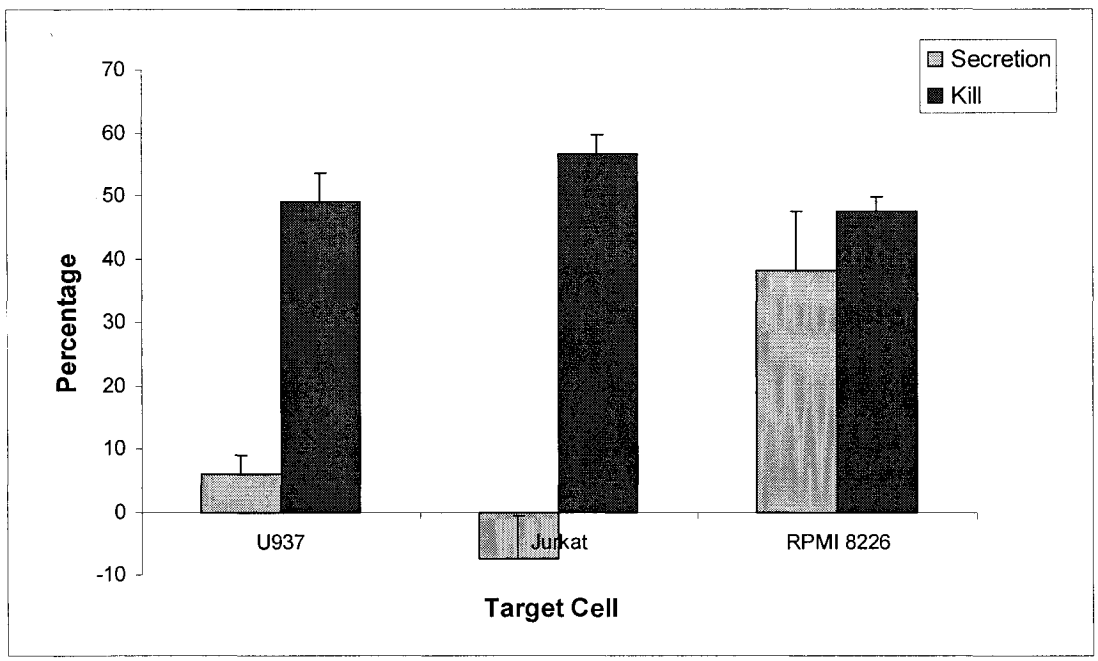
Table 3-8 Results of granule enzyme exocytosis assay for γδ T cell lines stimulated with U937, RPMI 8226, and Jurkat target cells.

	Cell Line				
Target Cell	MS PB1	MS CSF1	OND PB1	OND CSF1	OND PB2
U937	1.1 (0.02±0.005)	67.9	16.8 (0.02±0.008)	5.9 (0.03±0.002)	-1.7 (0.04±0.001)
Jurkat	4.1 (0.02±0.004)	10.7	12.9 (0.02±0.01)	-7.4 (0.03±0.007)	-4.4 (0.03±0.03)
RPMI 8226	14.9 (0.03±0.009)	42.9	24.5 (0.03±0.004)	38.2 (0.04±0.005)	2.6 (0.04±0.003)

Parentheses contain mean absorbances ± SD for triplicate results
MS CSF1 did not have sufficient cells for assay to be carried out in triplicate

Statistical comparisons of the percent secretion and percent kill for each of the target cell lines did not identify a correlation between the two. As Figure 3-20 (representative, n=5) shows the percent secretion for cell line OND CSF1 was 38.2% with 47.4% lysis for RPMI 8226 target cells, however, Jurkat cells did not induce any further secretion by these cells (-7.4%) but 56.7% of the targets were killed. It is possible that there is no correlation between the percent secretion and the percent kill because the percent secretion is a measure of the total serine esterase secretion by the cells. Perhaps a correlation exists between the individual cytotoxic mediators and the percent kill.

Figure 3-20 Percent secretion of granule esterases versus percent kill for γδ T cell line incubated with RPMI 8226, Jurkat, or U937 target cells. JAM assays were set-up with [methyl-^{3}H] thymidine labelled target cells at an E:T ratio of 12.5:1 and incubated at 37°C for 4.5 hours. The percent secretion was calculated from the results of granule enzyme exocytosis experiments. In each JAM assay plate were wells for "blank", "total", and target cell induced enzyme exocytosis. Following the incubation, 50μl of supernatant was removed from the exocytosis wells before the contents of the plate were harvested onto glass fibre filters. The supernatant was treated with BLT substrate solution followed by PMSF and absorbances were measured at 412nm. Results represent mean % kill or mean % secretion ± SD of triplicate wells.


Secretion
Kill
70
60
50
40
30
20
10
0
-10
Percentage
U937
Jurkat
RPMI 8226
Target Cell

3.5.6 Relative Concentration of Cytotoxic Molecules versus Percent Kill

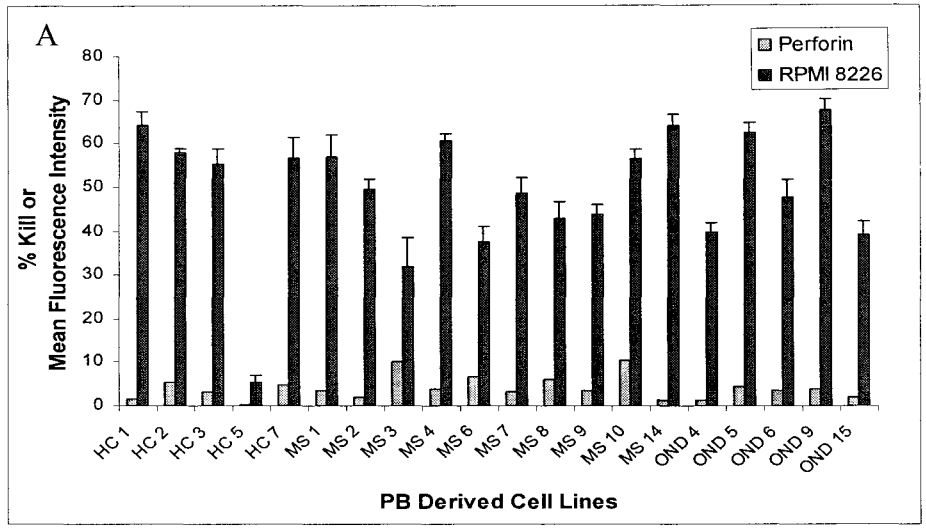
To investigate the possibility that the relative level of perforin, granzyme A, B, or M, or granulysin stored within the cells (represented by the mean fluorescence intensity) could be directly related to the percent kill, JAM assays and intracellular flow cytometry were performed simultaneously. The results are compiled in Figures 3-21, 3-22, and 3-23. The Pearson Product-Moment Correlation Coefficient® and a two tailed *t* test were used to determine if a correlation existed between the two variables.

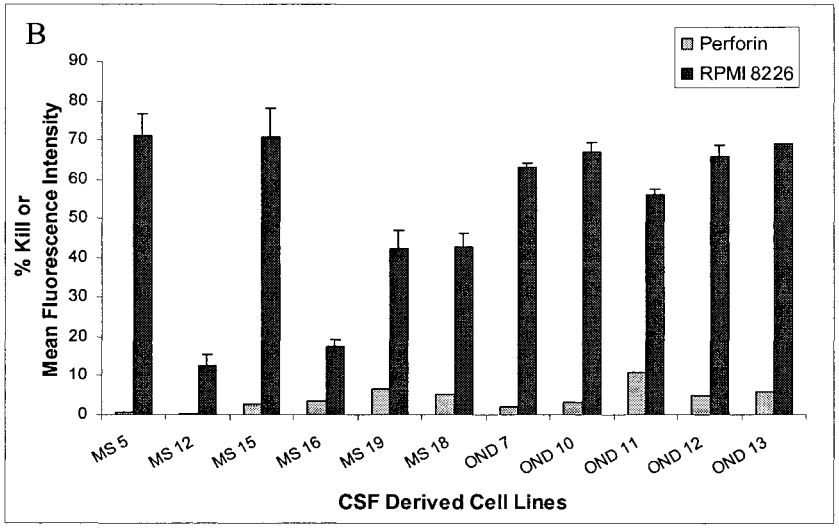
Figure 3-21 depicts the MFI of perforin in PB (A) and CSF (B) derived γδ T cell lines versus the percent kill of the RPMI 8226 target cell line. The MFI of perforin for PB and CSF derived pairs versus the percent kill of RPMI 8226 target cells is shown in Figure 3-21C. Statistical analyses of these results did not identify a correlation between the relative concentration of perforin stored in the cells and the percent kill of RPMI 8226 cells. Similar comparisons of the relative levels of granzyme A (Figure 3-21D and E), granzyme M (H and I), and granulysin (J and K) contained within the PB and CSF derived cells, respectively, versus the cytotoxicity of RPMI 8226 also showed no correlations. However, a correlation was identified between the MFI of granzyme B (for PB derived lines) and the percent kill of RPMI 8226 (Figure 3-21F, $r=0.61$, $p<0.05$).

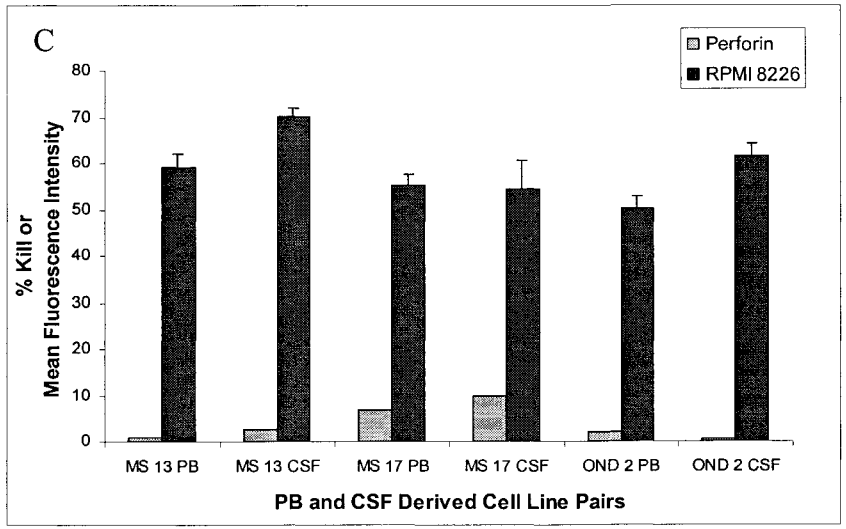
The cytotoxicity of Jurkat target cells was not directly related to the level of perforin (Figure 3-22A and B), granzyme A (C and D), granzyme B (E and F), granzyme M (G and H) or granulysin (I and J) detected in the PB or CSF γδ T cell lines.

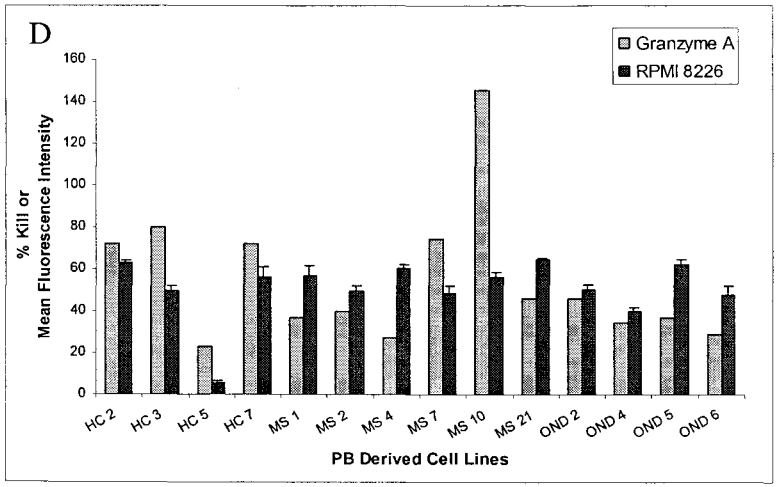
Another correlation was identified in this study between the relative concentration of granzyme M stored in the PB derived γδ T cells and the cytotoxicity of U937 target cells (Figure 3-23G, $r=0.57$, $p<0.05$), no such correlation was found to exist with the CSF

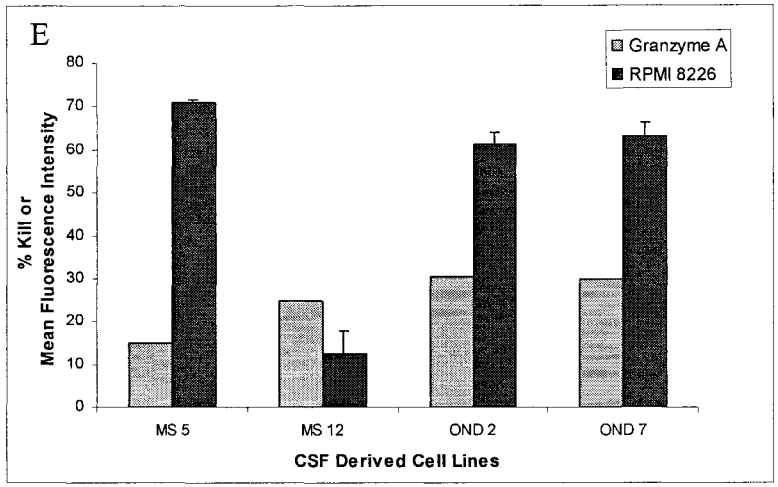
Figure 3-21 Comparison of relative concentration of perforin (A, B, C), granzyme A (D and E), granzyme B (F and G), granzyme M (H and I), and granulysin (J and K) versus the percent kill of RPMI 8226 target cells. JAM assays were carried out with γδ T cell lines derived from PB and CSF of MS and OND patients and PB of HC. Intracellular flow cytometry analyses were performed on the day of the JAM assay. The results depicted are the mean % kill ± SD of triplicate wells and the mean fluorescence intensity representing the relative concentration of each molecule inside the cells. See text for explanation of statistical analyses.

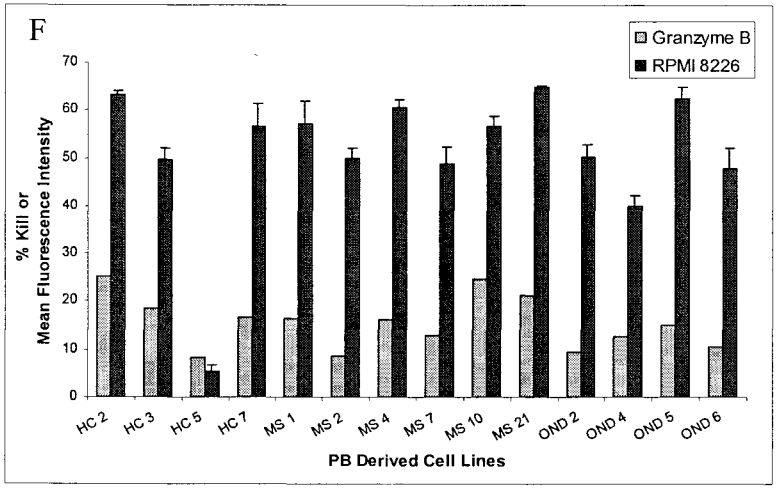

A
Perforin
RPMI 8226
% Kill or
Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
HC 1
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 3
MS 4
MS 6
MS 7
MS 8
MS 9
MS 10
MS 14
OND 4
OND 5
OND 6
OND 9
OND 15
PB Derived Cell Lines

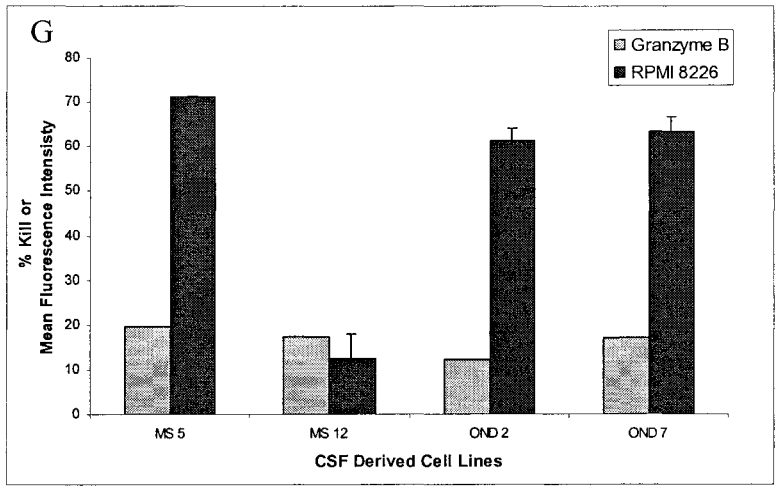

B
Perforin
RPMI 8226
% Kill or
Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
90
MS 5
MS 12
MS 15
MS 16
MS 19
MS 18
OND 7
OND 10
OND 11
OND 12
OND 13
CSF Derived Cell Lines

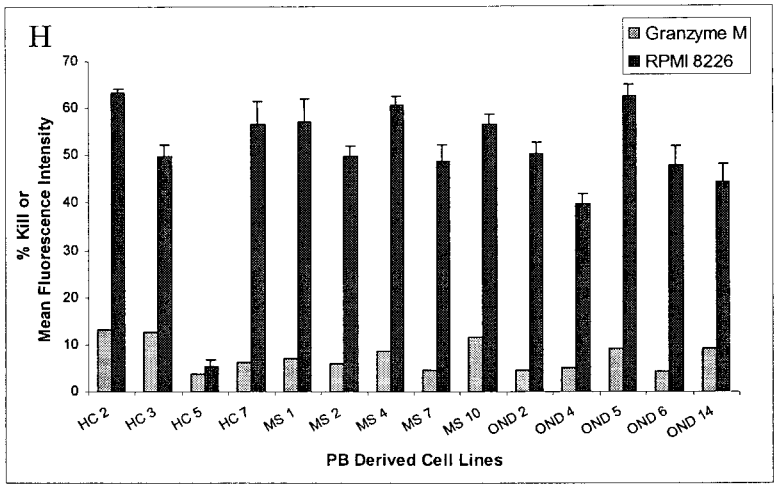

C
Perforin
RPMI 8226
% Kill or
Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
MS 13 PB
MS 13 CSF
MS 17 PB
MS 17 CSF
OND 2 PB
OND 2 CSF
PB and CSF Derived Cell Line Pairs

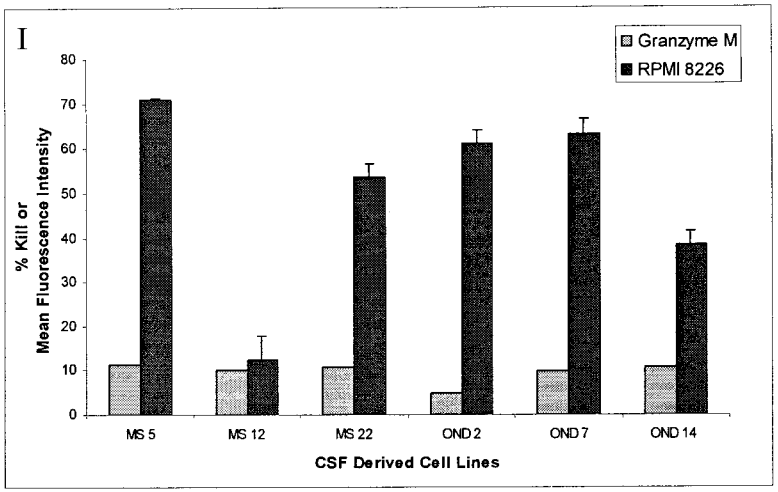

D
Granzyme A
RPMI 8226
% Kill or
Mean Fluorescence Intensity
0
20
40
60
80
100
120
140
160
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
MS 21
OND 2
OND 4
OND 5
OND 6
PB Derived Cell Lines

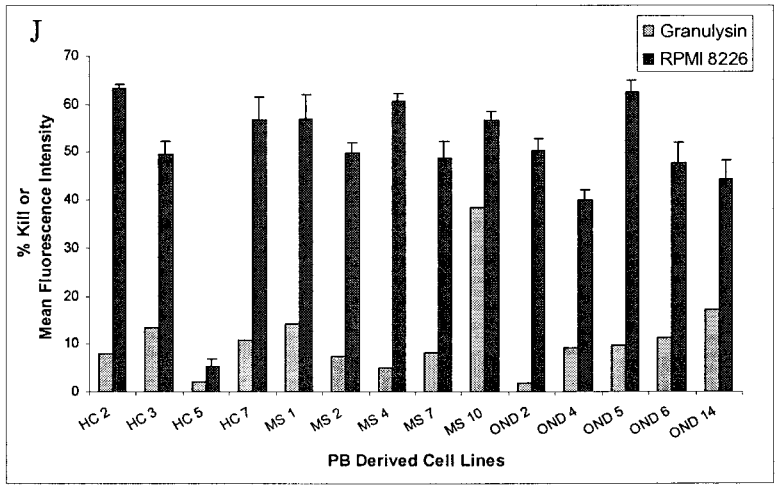

E
Granzyme A
RPMI 8226
% Kill or
Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
MS 5
MS 12
OND 2
OND 7
CSF Derived Cell Lines


F
Granzyme B
RPMI 8226
% Kill or
Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
MS 21
OND 2
OND 4
OND 5
OND 6
PB Derived Cell Lines


G
Granzyme B
RPMI 8226
% Kill or
Mean Fluorescence Intensisty
0
10
20
30
40
50
60
70
80
MS 5
MS 12
OND 2
OND 7
CSF Derived Cell Lines


H
Granzyme M
RPMI 8226
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
OND 2
OND 4
OND 5
OND 6
OND 14
PB Derived Cell Lines


I
Granzyme M
RPMI 8226
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
MS 5
MS 12
MS 22
OND 2
OND 7
OND 14
CSF Derived Cell Lines


J
Granulysin
RPMI 8226
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
OND 2
OND 4
OND 5
OND 6
OND 14
PB Derived Cell Lines

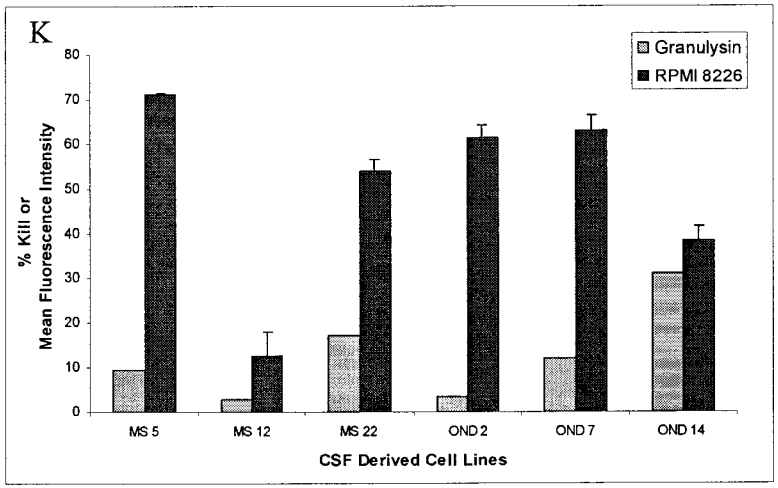
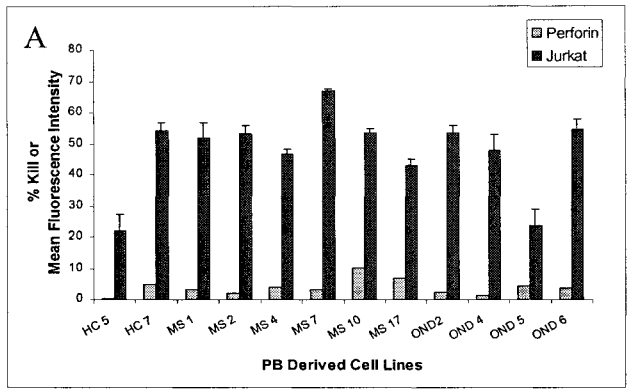
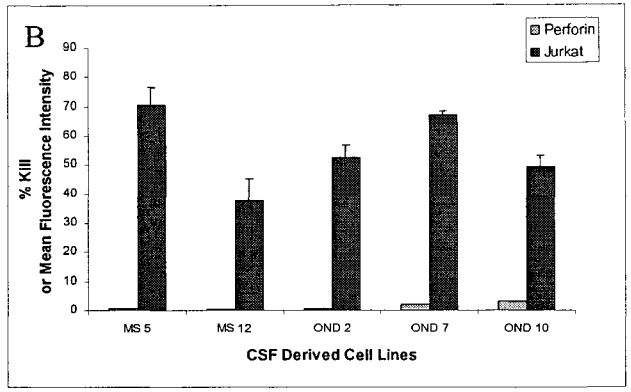
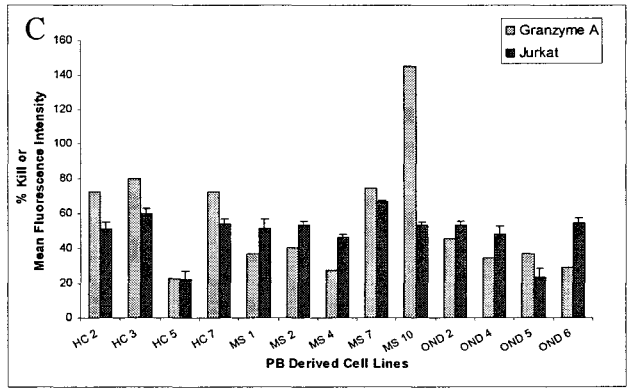
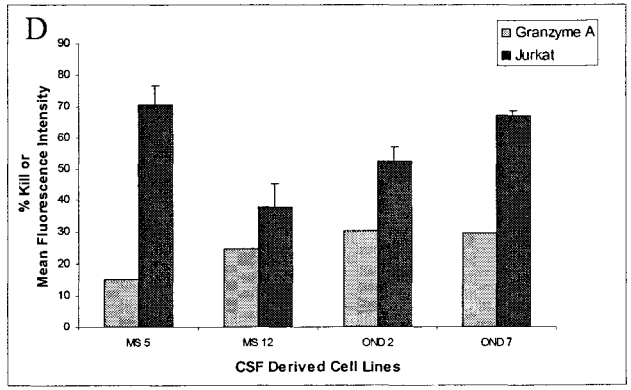

K
Granulysin
RPMI 8226
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
MS 5
MS 12
MS 22
OND 2
OND 7
OND 14
CSF Derived Cell Lines

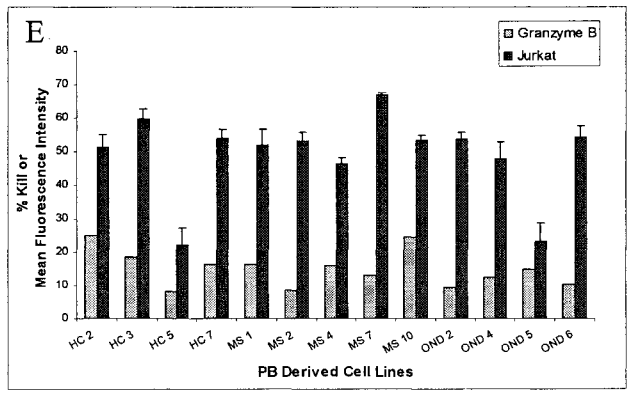
Figure 3-22 Comparison of relative concentration of perforin (A and B), granzyme A (C and D), granzyme B (E and F), granzyme M (G and H), and granulysin (I and J) versus the percent kill of Jurkat target cells. JAM assays were carried out with γδ T cell lines derived from PB and CSF of MS and OND patients and PB of HC. Intracellular flow cytometry analyses were performed on the day of the JAM assay. The results depicted are the mean % kill ± SD of triplicate wells and the mean fluorescence intensity representing the relative concentration of each molecule inside the cells. See text for explanation of statistical analyses.

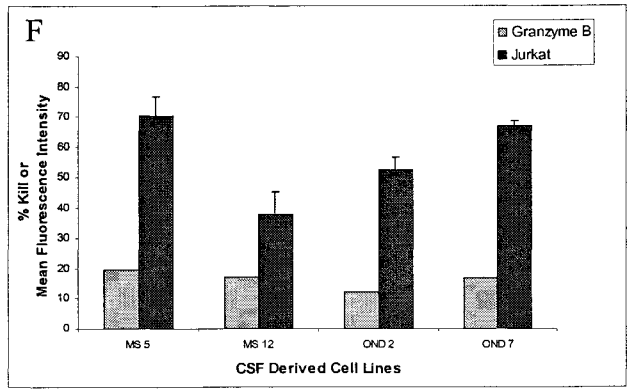

A
Perforin
Jurkat
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
MS 17
OND2
OND 4
OND 5
OND 6
PB Derived Cell Lines

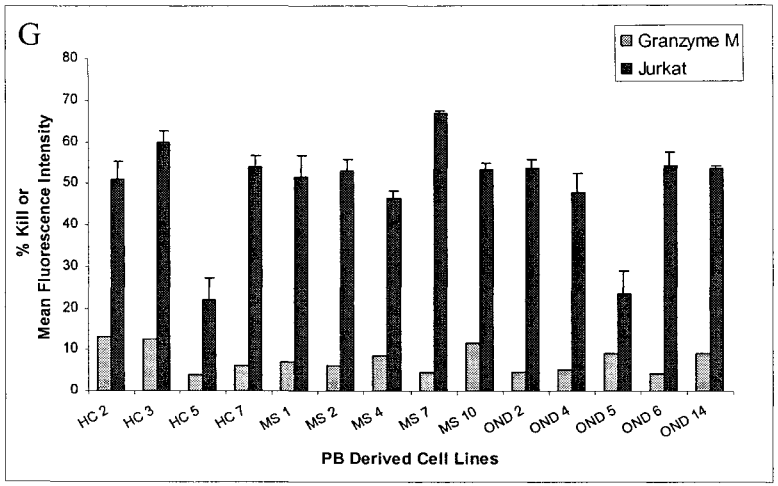

B
Perforin
Jurkat
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
90
MS 5
MS 12
OND 2
OND 7
OND 10
CSF Derived Cell Lines

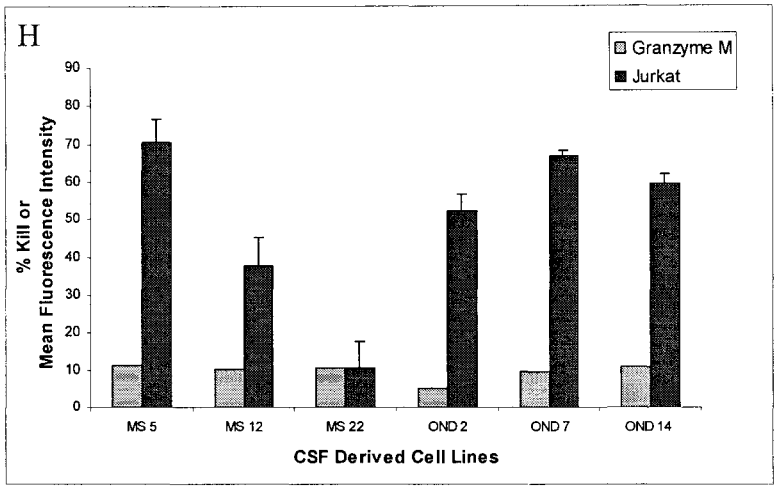

C
Granzyme A
Jurkat
% Kill or Mean Fluorescence Intensity
0
20
40
60
80
100
120
140
160
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
OND 2
OND 4
OND 5
OND 6
PB Derived Cell Lines

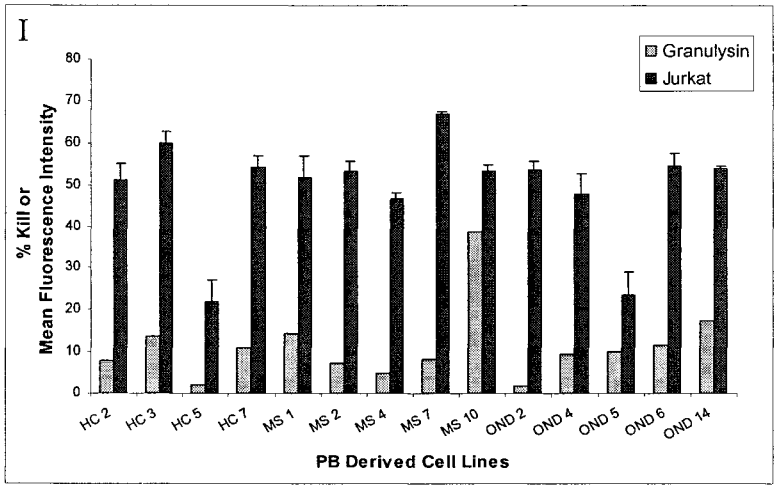

D
Granzyme A
Jurkat
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
90
MS 5
MS 12
OND 2
OND 7
CSF Derived Cell Lines


E
Granzyme B
Jurkat
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
OND 2
OND 4
OND 5
OND 6
PB Derived Cell Lines


F
Granzyme B
Jurkat
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
90
MS 5
MS 12
OND 2
OND 7
CSF Derived Cell Lines


G
Granzyme M
Jurkat
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
OND 2
OND 4
OND 5
OND 6
OND 14
PB Derived Cell Lines


H
Granzyme M
Jurkat
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
90
MS 5
MS 12
MS 22
OND 2
OND 7
OND 14
CSF Derived Cell Lines


I
Granulysin
Jurkat
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
OND 2
OND 4
OND 5
OND 6
OND 14
PB Derived Cell Lines

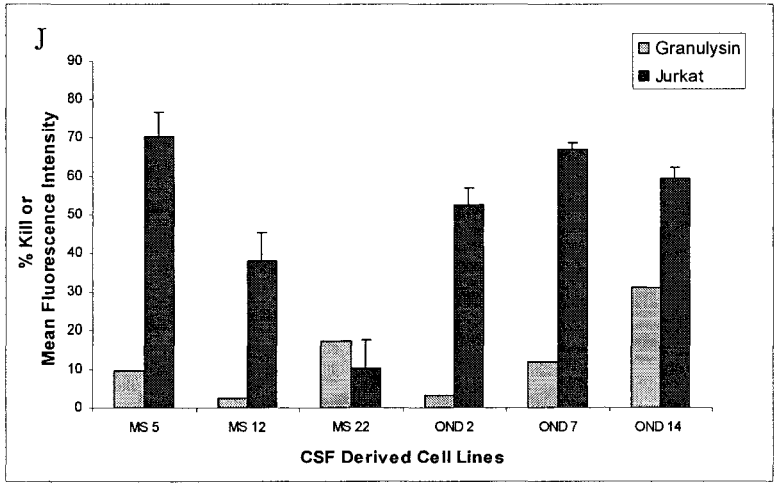
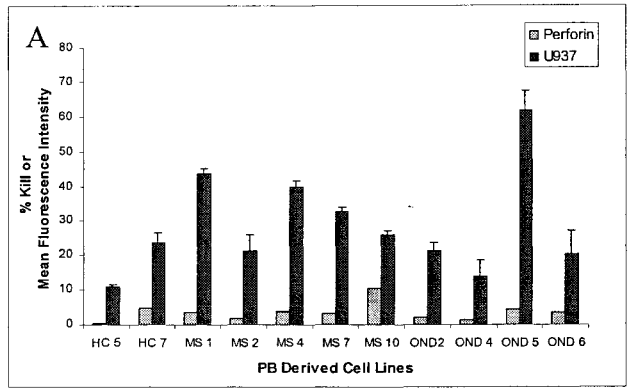
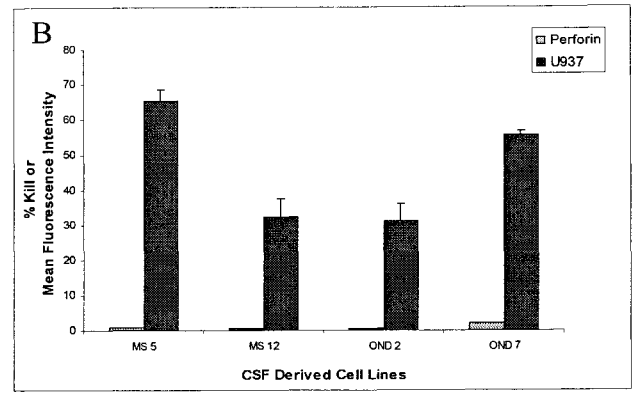
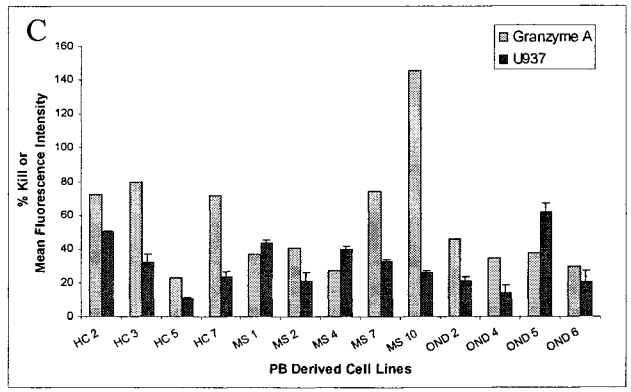
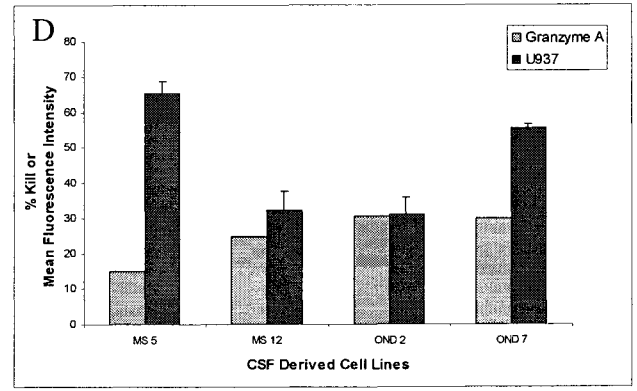

J
Granulysin
Jurkat
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
90
MS 5
MS 12
MS 22
OND 2
OND 7
OND 14
CSF Derived Cell Lines

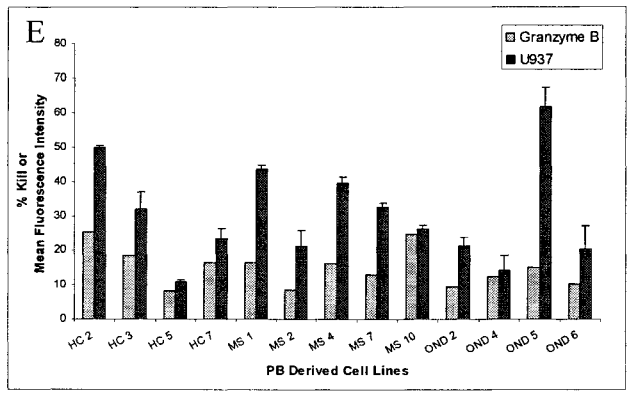
Figure 3-23 Comparison of relative concentration of perforin (A and B), granzyme A (C and D), granzyme B (E and F), granzyme M (G and H), and granulysin (I and J) versus the percent kill of U937 target cells. JAM assays were carried out with γδ T cell lines derived from PB and CSF of MS and OND patients and PB of HC. Intracellular flow cytometry analyses were performed on the day of the JAM assay. The results depicted are the mean % kill ± SD of triplicate wells and the mean fluorescence intensity representing the relative concentration of each molecule inside the cells. See text for explanation of statistical analyses.

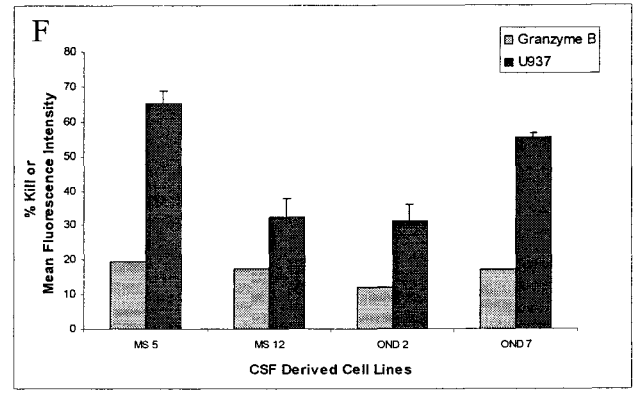

A
Perforin
U937
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
OND2
OND 4
OND 5
OND 6
PB Derived Cell Lines

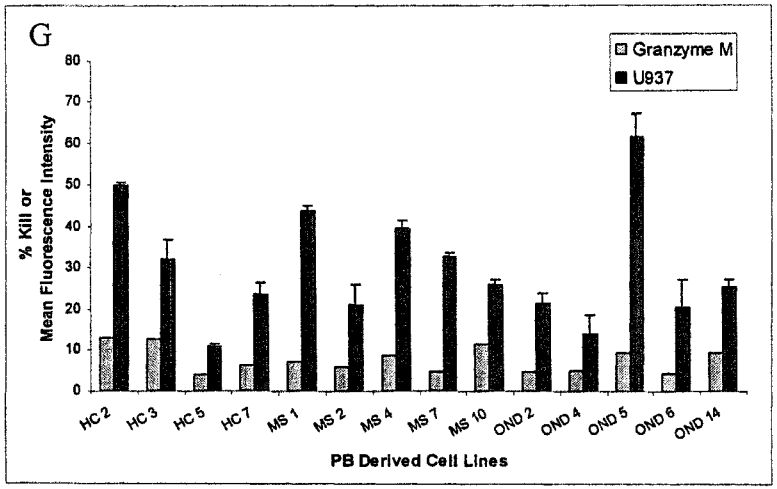

B
Perforin
U937
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
MS 5
MS 12
OND 2
OND 7
CSF Derived Cell Lines

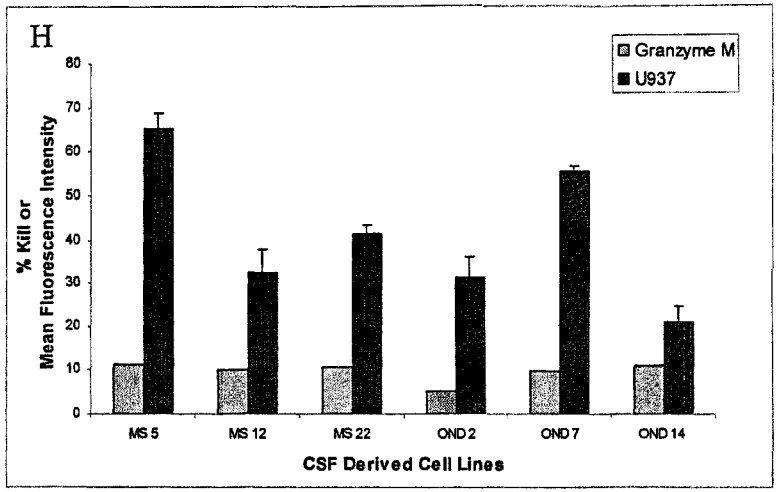

C
Granzyme A
U937
% Kill or Mean Fluorescence Intensity
0
20
40
60
80
100
120
140
160
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
OND 2
OND 4
OND 5
OND 6
PB Derived Cell Lines

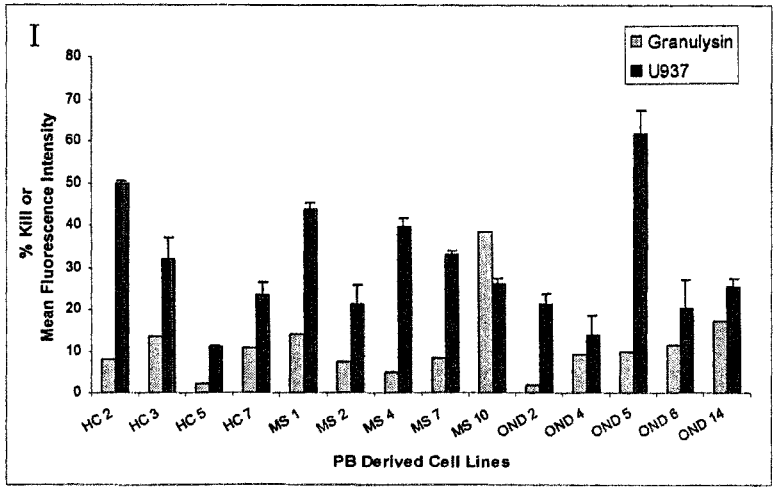

D
Granzyme A
U937
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
MS 5
MS 12
OND 2
OND 7
CSF Derived Cell Lines

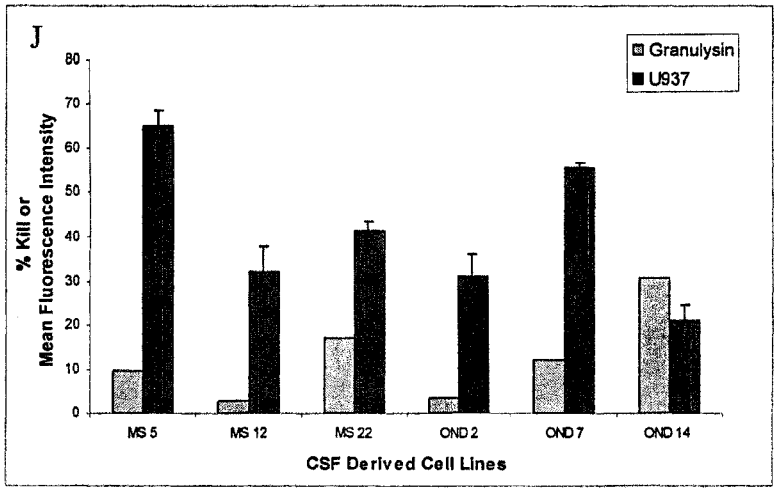

E
Granzyme B
U937
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
OND 2
OND 4
OND 5
OND 6
PB Derived Cell Lines


F
Granzyme B
U937
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
MS 5
MS 12
OND 2
OND 7
CSF Derived Cell Lines


G
Granzyme M
U937
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
OND 2
OND 4
OND 5
OND 6
OND 14
PB Derived Cell Lines


H
Granzyme M
U937
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
MS 5
MS 12
MS 22
OND 2
OND 7
OND 14
CSF Derived Cell Lines


I
Granulysin
U937
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
HC 2
HC 3
HC 5
HC 7
MS 1
MS 2
MS 4
MS 7
MS 10
OND 2
OND 4
OND 5
OND 6
OND 14
PB Derived Cell Lines


J
Granulysin
U937
% Kill or Mean Fluorescence Intensity
0
10
20
30
40
50
60
70
80
MS 5
MS 12
MS 22
OND 2
OND 7
OND 14
CSF Derived Cell Lines

derived cells (Figure 3-23H, r=0.3). The level of perforin (Figure 3-23A and B), granzyme A (C and D), granzyme B (E and F), or granulysin (I and J) could not be directly related to the cytotoxicity of U937 cells.

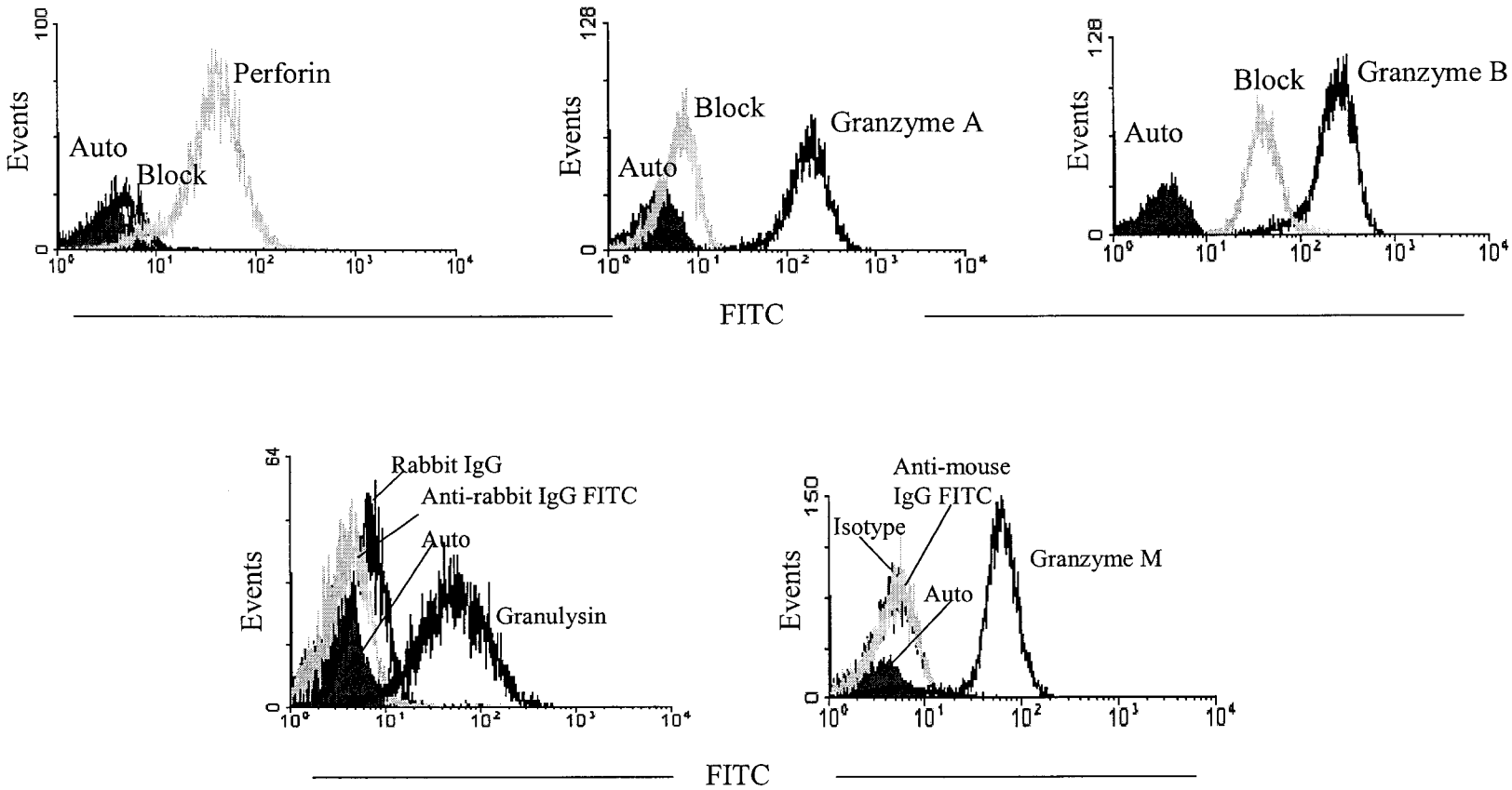
In summary, the cytotoxicity mediated by γδ T cell lines (1) uses both the Fas/FasL and the perforin/granzyme pathways, (2) is largely mediated by granzyme B, (3) is influenced (particularly the serine esterase secretion) by the target cell, (4) cannot be correlated with either the percent secretion of serine esterases nor with the relative concentration of perforin, granzymes A, B, or M, or granulysin, and (5) may be compartment or disease specific.

3.6 Cytotoxic Mechanisms Utilized by Transformed γδ T cell clones

3.6.1 Detection of Perforin, Granzymes, and Granulysin

The first step in identifying the cytotoxic mechanisms utilized by the transformed clones was to determine which cytotoxic mediators these cells expressed. Intracellular flow cytometry analyses (Figure 3-24, representative) showed that the transformed γδ T cell clones expressed perforin (n=12), granzymes A, B, and M, and granulysin (n=4). This figure also demonstrates the specificity of the antibodies as previously shown with the non-transformed lines. (The incomplete blocking of the anti-granzyme B-FITC mAb was due to the small volume of unlabelled mAb used, also the anti-granzyme M antibody had not yet been labelled with FITC when this experiment was performed and so goat anti-mouse IgG (Fc specific, FITC) was used in the detection of granzyme M).

Figure 3-24 Detection of cytotoxic molecules stored in transformed γδ T cell clones. Cells were surface stained with anti-CD3-QR and anti-γδ TCR-PE mAbs, permeabilized with a 0.1% saponin solution, and intracellularly labelled with anti-perforin or anti-granzymes A, B, or M FITC conjugated mAbs. Granulysin was detected with a combination of polyclonal rabbit anti-granulysin antibody and goat anti-rabbit IgG FITC. The specificity of the antibodies was confirmed with isotype controls, non-specific rabbit IgG, or blocking with unlabelled mAbs. Flow cytometry analysis was gated on CD3 and γδ TCR positive cells. Representative results are shown (n=4).


Events
Auto
Block
Perforin
Granzyme A
Granzyme B
Rabbit IgG
Anti-rabbit IgG FITC
Granulysin
Anti-mouse
IgG FITC
Isotype
Granzyme M
FITC

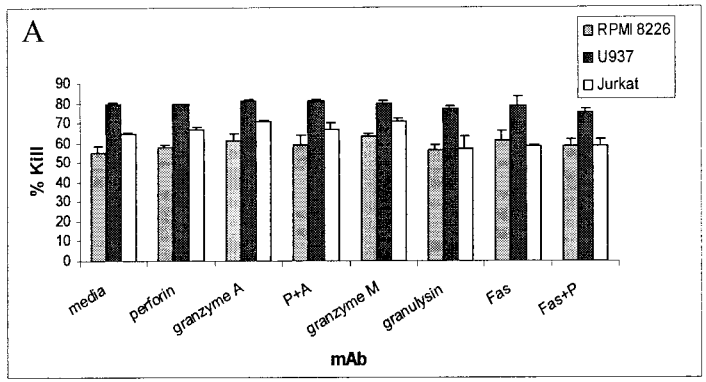
3.6.2 Inhibition of Cytotoxicity Mediated by Transformed γδ T cell clones

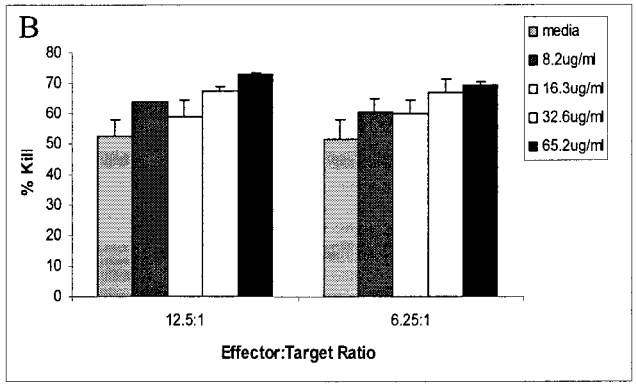
The goal of the inhibition assays was not only to elucidate the cytotoxic mechanism(s) used by the transformed γδ T cell clones, but also to determine if these mechanisms differed between clones derived from PB and CSF or HC, MS, and OND samples. Therefore, these assays were carried out with a minimum of 4 clones: MS PB clone (47-7), HC PB clone (70-10-5), OND PB clone (80-10-5), and MS CSF clone (60-10-10). (RPMI 8226, Jurkat, and U937 target cells were used in each assay).

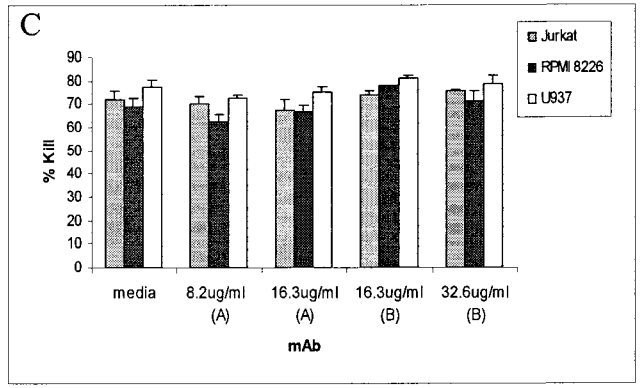
Initial attempts to inhibit the cytotoxicity mediated by transformed γδ T cell clones used the concentrations that had been effective for the non-transformed cell lines: 16.3μg/ml anti-perforin mAb, 65.2μg/ml anti-granzyme A mAb, 2μg/ml (+5μg/ml) Fas blocking mAb. Combinations of anti-perforin + anti-granzyme A mAbs or Fas blocking + anti-perforin mAbs and 1:100 dilutions of anti-granzyme M ascites and anti-granulysin polyclonal rabbit antibody were also used. Figure 3-25A (representative, n=5) demonstrates that the percent kill of U937, RPMI 8226, and Jurkat target cells was not significantly affected by any of these treatments.

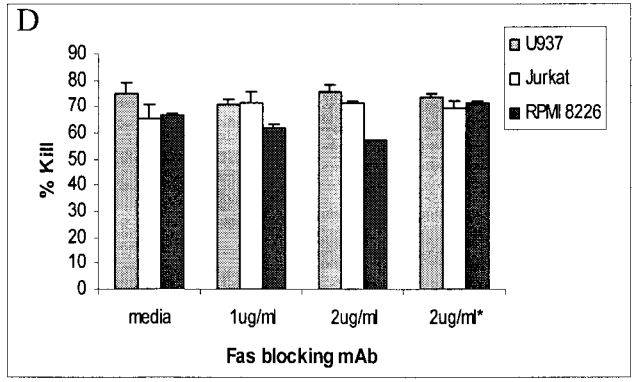
Further experiments were carried out to determine whether different concentrations of mAb were required to achieve inhibition. Titration of anti-perforin mAb (Figure 3-25B, representative, n=3) with RPMI 8226 target cells showed increased kill with increasing concentrations. The cytotoxicity of U937, Jurkat, and RPMI 8226 target cells was not inhibited by 8.2 or 16.3μg/ml of anti-granzyme A mAb or by 16.3 or 32.6μg/ml of anti-granzyme B mAb (Figure 3-25C, representative, n=2). Fas blocking mAb did not have a significant effect on the percent kill of U937, RPMI 8226, or Jurkat

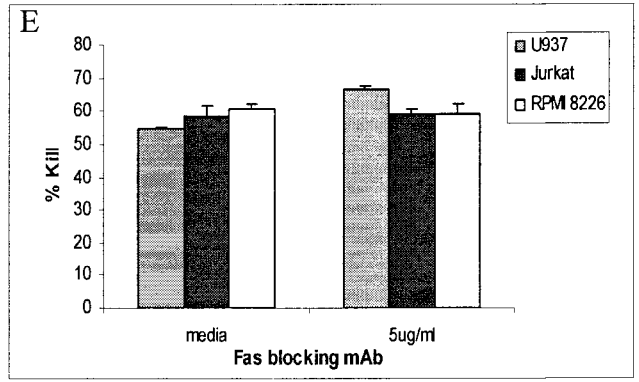
Figure 3-25 Cytotoxicity assays with antibodies specific to cytotoxic molecules expressed by transformed γδ T cell clones. JAM assays were carried out for 4.5 hours with [methyl-^{3}H] thymidine labelled targets at an E:T ratio of 12.5:1, with 10 000 target cells per well. (A) JAM assay with RPMI 8226, Jurkat, and U937 target cells, concentrations of antibodies were as follows: anti-perforin (P) 16.3μg/ml, anti-granzyme A (A) 65.2μg/ml, Fas blocking 2μg/ml (+5μg/ml), anti-granzyme M and anti-granulysin 10μ of 1:100 dilution. (B) Titration of anti-perforin mAb with RPMI 8226 cells. (C) Titration of anti-granzyme A and anti-granzyme B (2C4/F5) mAb with U937, Jurkat, and RPMI 8226 target cells. (D and E) Titration of Fas blocking mAb. Target cells were incubated with 1, 2, or 5μg/ml Fas blocking mAb for 1 hour, washed, diluted to 1x10^5 cells/ml, and 5μg/ml Fas blocking mAb was added. * denotes cells which received 500ng/ml Fas blocking mAb rather than the typical 5μg/ml. Results shown are mean % kill ± SD of triplicate wells.


A
RPMI 8226
U937
Jurkat
% Kill
media
perforin
granzyme A
P+A
granzyme M
granulysin
Fas
Fas+P
mAb


B
media
8.2ug/ml
16.3ug/ml
32.6ug/ml
65.2ug/ml
% Kill
12.5:1
6.25:1
Effector:Target Ratio


C
Jurkat
RPMI 8226
U937
% Kill
media
8.2ug/ml (A)
16.3ug/ml (A)
16.3ug/ml (B)
32.6ug/ml (B)
mAb


D
U937
Jurkat
RPMI 8226
% Kill
media
1ug/ml
2ug/ml
2ug/ml*
Fas blocking mAb


E
U937
Jurkat
RPMI 8226
% Kill
media
5ug/ml
Fas blocking mAb

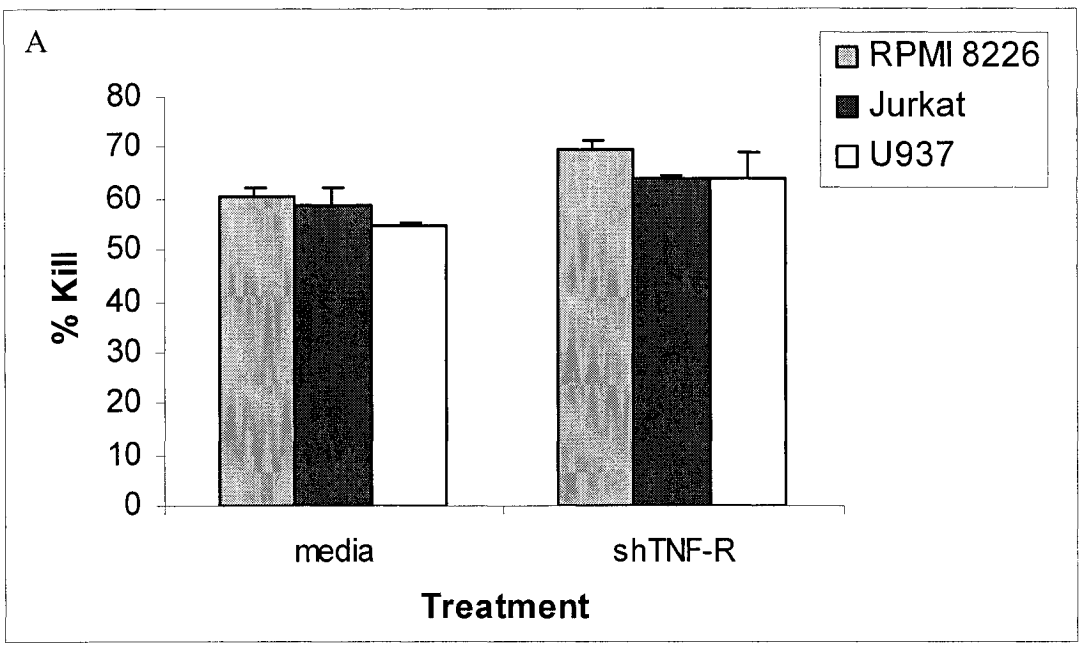
cells at 1, 2, or 5μg/ml (Figure 3-25D and E; * indicates that the second addition of Fas blocking mAb was 500ng/ml rather than 5μg/ml).

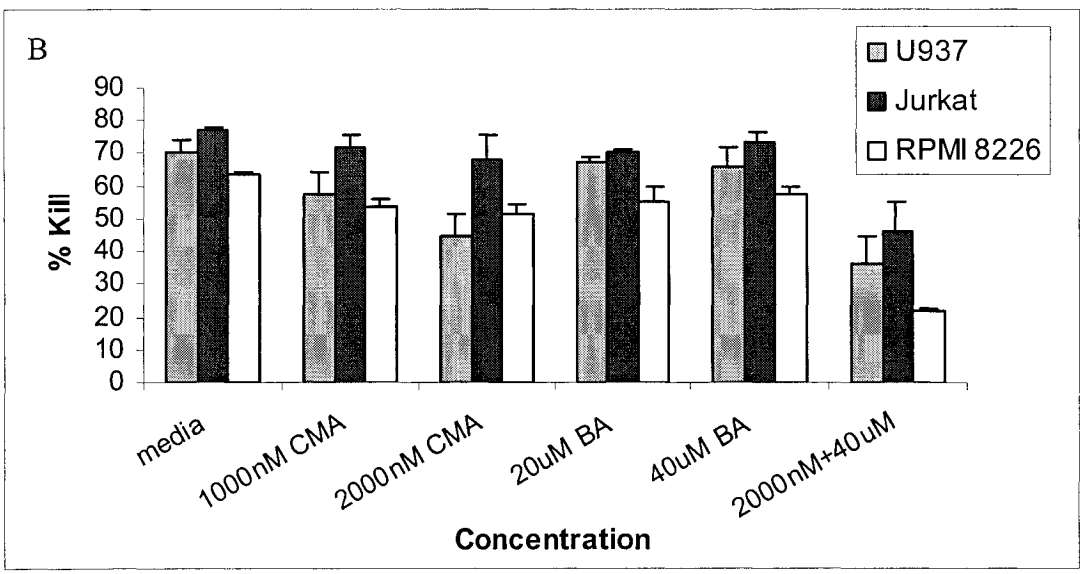
This inability to significantly inhibit the cytotoxicity mediated by the transformed clones with anti-perforin, anti-granzyme A or Fas blocking mAbs led to questions of whether the clones were actually using the perforin/granzyme or Fas/FasL pathways or whether another cytotoxic pathway was being employed.

The TNF/TNF-R pathway had not been considered as a possible cytotoxic mechanism for the γδ T cell lines or the transformed clones because it has a much longer time frame than the perforin/granzyme or Fas/FasL pathways. Cytotoxicity assays investigating the TNF/TNF-R pathway typically last 48 to 72 hours, the JAM assays discussed in this thesis were carried out in 4.5 to 5 hours. It was not believed that the TNF/TNF-R pathway could be significant in such a short assay. However, this possibility needed to be eliminated. As shown in Figure 3-26A (representative, n=2), the addition of soluble human TNF-R (shTNF-R; 1:1000 dilution) to JAM assays with U937, Jurkat, or RPMI 8226 target cells did not decrease the cytotoxicity mediated by the clones. Experiments with 1:100, 1:1000, and 1:10 000 dilutions of shTNF-R were also carried out, but inhibition of cytotoxicity was not observed (data not shown).

Prior to the availability of mAbs specific for cytotoxic molecules, numerous investigations had used chemical means to decipher the cytotoxic pathways used by γδ T cell lines. At this point in my work, I decided to use these less specific methods to obtain a broader view of the cytotoxicity mediated by the clones. The clones were pre-incubated with concanamycin A or brefeldin A or a combination of the two using the protocols cited by Zeine et al. (339), the results are shown in Figure 3-26B (it had been

Figure 3-26 Cytotoxicity assays with transformed γδ T cell clones and U937, Jurkat, or RPMI 8226 target cells labelled with [methyl-^{3}H] thymidine. JAM assays were run for 5 hours at an E:T ratio of 12.5:1. Clones were incubated with a 1:1000 dilution of shTNF-R for 30 minutes prior to their addition to the assay (A). In (B) the clones were incubated with concanamycin A (CMA) or brefeldin A (BA) or a combination of the two for 2 hours prior to their addition to the assay. Results shown represent mean % kill ± SD of triplicate wells.


A
RPMI 8226
Jurkat
U937
80
70
60
50
40
30
20
10
0
% Kill
media
shTNF-R
Treatment


B
U937
Jurkat
RPMI 8226
90
80
70
60
50
40
30
20
10
0
% Kill
media
1000nM CMA
2000nM CMA
20uM BA
40uM BA
2000nM+40uM
Concentration

previously determined that this combination of concanamycin A and brefeldin A was not toxic to γδ T cells, data not shown). Pre- incubation with concanamycin A (especially 2000nM) decreased the cytotoxicity of U937 (70% to 44.4%), was less effective for RPMI 8226, and had no effect on the percent kill of Jurkat target cells. The use of brefeldin A had no significant effect on the cytotoxicity of any of the target cells. However, pre-incubation with a combination of brefeldin A and concanamycin A (40μM and 2000nM, respectively) gave the largest decrease in cytotoxicity (U937: 70.0% to 36%; Jurkat: 77.2% to 46.4%; RPMI 8226: 63.4% to 21.6%). These results suggested that both the perforin/granzyme and Fas/FasL pathways were being used by the clones. Since the cytotoxicity mediated by the clones had not been inhibited by anti-perforin, anti-granzyme, or Fas blocking mAb, I decided to examine the granule enzyme exocytosis of the clones in more detail.

3.6.3 Transformed γδ T cell clones: Granule Secretion

Granule enzyme exocytosis assays were carried out with 4 clones to determine if the contents of their cytoplasmic granules were being released in response to stimulation with RPMI 8226, Jurkat, or U937 cells. The results of these assays showed that the clones were capable of secreting serine esterases and that the percent secretion varied with the "stimulating" cell (Table 3-9). For example, the percent secretion for clone 60-10-10 was -9.5% in response to U937, 3.4% in response to Jurkat, and 55.9% in response to RPMI 8226. This suggested that the clones were able to adjust the secretion of their cytoplasmic granules depending on the target cell encountered.

Table 3-9 Results of granule enzyme exocytosis assay for γδ T cell clones stimulated with U937, RPMI 8226, and Jurkat target cells.

	Clone			
Target Cell	47-7	60-10-10	70-10-5	80-10-5
U937	10.4 (0.02±0.01)	-9.5 (0.01±0.0)	1.6 (0.06±0.006)	-15.2 (0.03±0.003)
Jurkat	11.4 (0.02±0.01)	3.4 (0.02±0.03)	9.2 (0.07±0.004)	-9.1 (0.04±0.003)
RPMI 8226	16.1 (0.03±0.01)	55.9 (0.05±0.02)	17.1 (0.07±0.002)	-18.2 (0.03±0.002)

Parentheses contain mean absorbances ± SD for triplicate results

As for the non-transformed γδ T cells, granule enzyme exocytosis and JAM assays were performed simultaneously. However, a correlation between the percent secretion and the percent kill was not found. As shown in Figure 3-27 (representative, n=4), 17.1% secretion gave 76.1% kill for RPMI 8226 cells, while 1.6% secretion gave 79.4% kill for U937 cells.

3.6.4 Specific Inhibition of γδ T cell clone Mediated Cytotoxicity

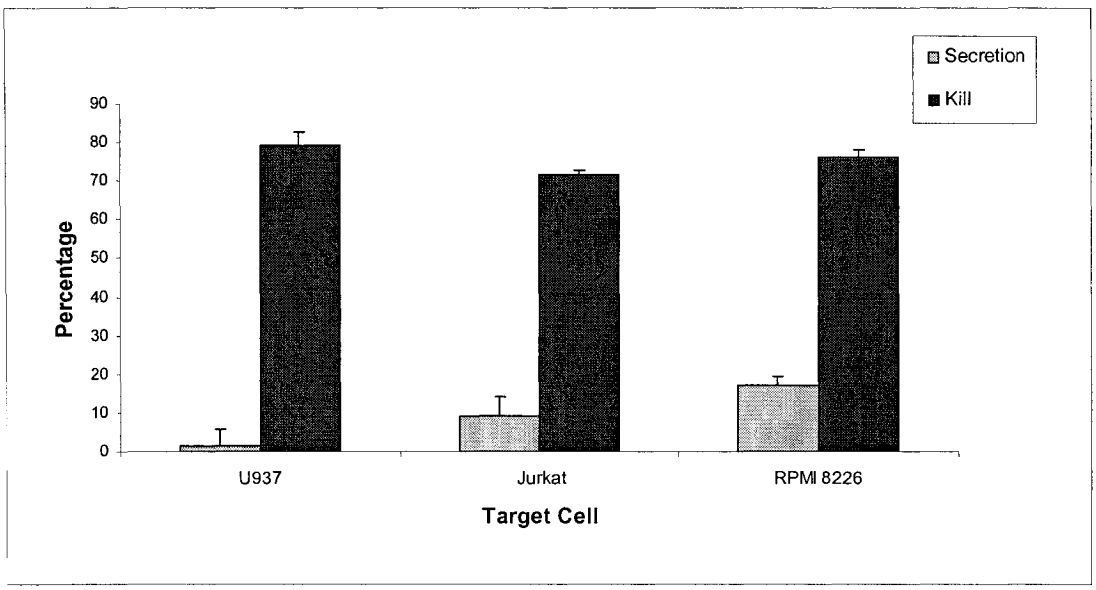
At this time point, I had just tested granzyme B inhibitor II with the non-transformed γδ T cell lines, and had been very successful in inhibiting the cytotoxicity of Jurkat, U937, and RPMI 8226 cells. Based on these results, JAM assays were performed with the transformed clones and granzyme B inhibitor II (Figure 3-28, representative, n=4). Significant inhibition was achieved with 100μM of granzyme B inhibitor II for each target cell line, U937 (82.7% to 25.7%), Jurkat (72.9% to 43.7%), and RPMI 8226 (71.5% to 17.0%), indicating that the majority of cytotoxicity mediated by the transformed γδ T cell clones was due to granzyme B.

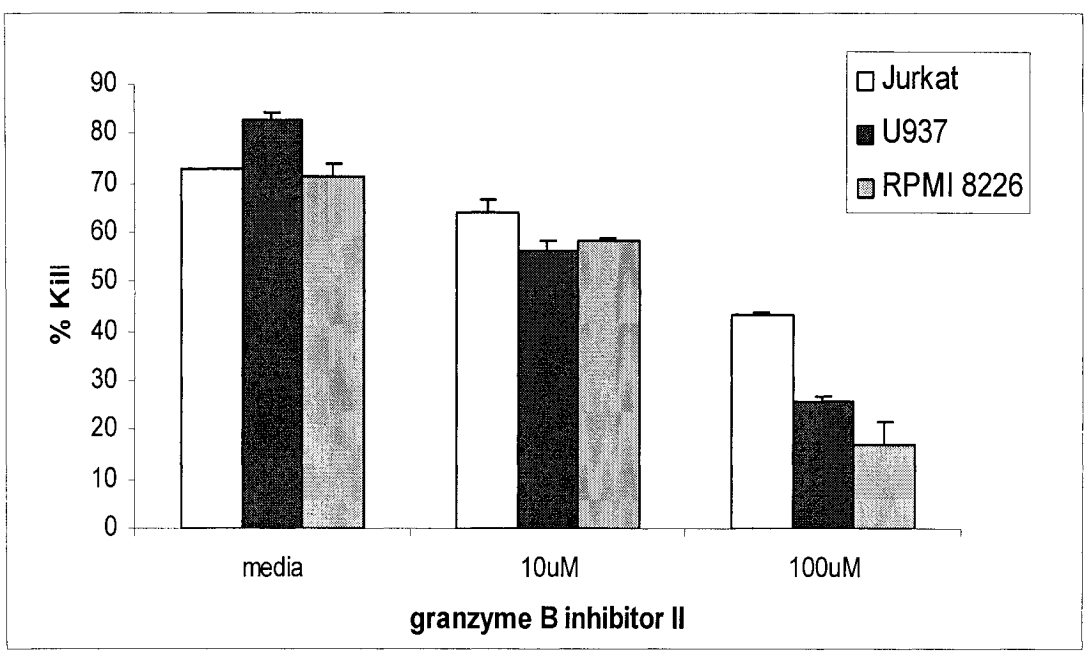
3.6.5 Further Investigation of Transformed γδ T cell clone Mediated Cytotoxicity

The results obtained to date indicated that the cytotoxicity mediated by the transformed clones was significantly different from that of the non-transformed lines. Further experiments were carried out to characterize this cytotoxicity in more detail.

Figure 3-27 Percent secretion of granule esterases versus percent kill for transformed γδ T cell clone incubated with RPMI 8226, Jurkat, or U937 target cells. JAM assays were carried out with [methyl-^{3}H] thymidine labelled target cells at an E:T ratio of 12.5:1 and allowed to proceed for 4.5 hours. The percent secretion was calculated from the results of granule enzyme exocytosis experiments. In each JAM assay plate were wells for "blank", "total", and target cell induced enzyme exocytosis. Following the 5 hour incubation, 50μl of supernatant was removed from each of these wells and the contents of the plate were harvested onto glass fibre filters. The supernatant was treated with BLT substrate solution followed by PMSF. Absorbances were measured at 412nm. Results represent mean % kill or mean % secretion ± SD of triplicate.

Figure 3-28 Effect of granzyme B inhibitor II on 4.5 hour cytotoxicity assay with transformed γδ T cell clones. JAM assays were carried out with [methyl-^{3}H] thymidine labeled Jurkat, U937, or RPMI 8226 target cells. Granzyme B inhibitor II was added to the assay wells at a concentration of 10 or 100μM. Results shown are mean % kill ± SD of triplicate wells.


Secretion
Kill
Percentage
90
80
70
60
50
40
30
20
10
0
U937
Jurkat
RPMI 8226
Target Cell

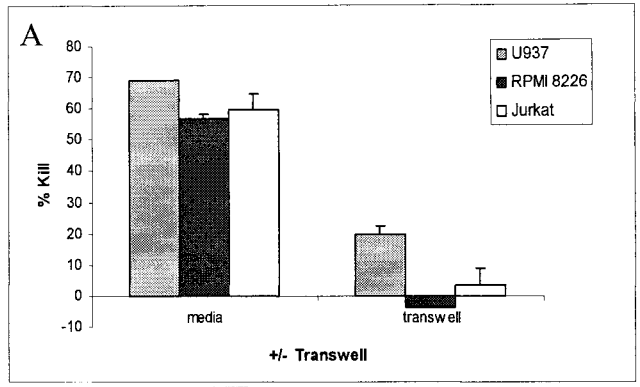

Jurkat
U937
RPMI 8226
% Kill
90
80
70
60
50
40
30
20
10
0
media
10uM
100uM
granzyme B inhibitor II

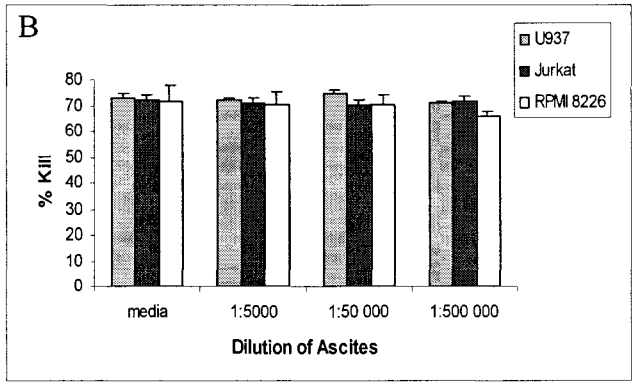
To investigate whether cell to cell contact was necessary for γδ T cell clone mediated cytotoxicity, JAM assays were performed with transwells. U937, Jurkat, or RPMI 8226 target cells were added to wells of the assay plate, the effectors were added to the transwells, and inserted into the wells of the plate. The presence of the transwells caused a significant decrease in the cytotoxicity of U937 (69.2% to 19.0%), RPMI 8226 (57.1% to -3.7%), and Jurkat (59.5% to 3.5%) cells (Figure 3-29A, representative, n=4).

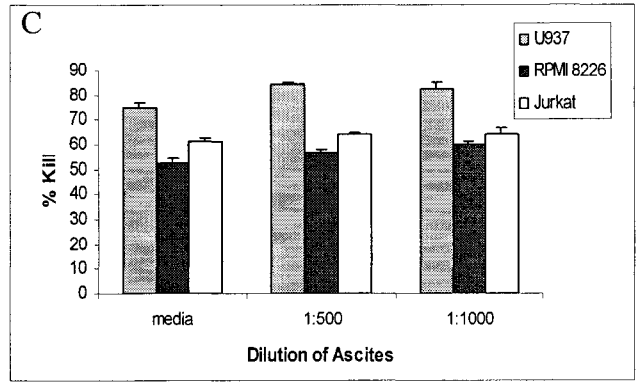
It appeared that cell to cell contact was important, but which surface molecules were involved? Previous experiments in the lab had attempted to regulate the cytotoxicity mediated by γδ T cell clones with anti-γδ TCR, anti-CD2, and anti-CD58 mAbs, but had been unsuccessful. Many labs have reported cytotoxic inhibition with anti-γδ TCR ascites, perhaps the purified mAb was not as effective as the ascites fluid. To investigate this possibility clones 47-7 and 70-10-5 were pre-incubated with 1:500, 1:1000, 1:5000, 1:50 000, or 1:500 000 dilutions of anti-γδ TCR ascites and used in JAM assays. This pre-treatment had no effect on the cytotoxicity of Jurkat, U937 or RPMI 8226 target cells (Figure 3-29C and D, representative).

Another surface molecule of interest was CD150 (signalling lymphocyte activation marker or SLAM). SLAM is a CD2-related surface marker expressed by T cells, B cells, and NK cells. It has been shown that T cells infected with *Herpesvirus saimiri* are triggered to cytotoxicity by SLAM-engagement alone (357). Flow cytometry analysis with anti-CD150-PE mAb demonstrated that U937, Jurkat, RPMI 8226, and Hut 78 did not express CD150 (Figure 3-30A). Although the clones did express CD150 (Figure 3-30B, representative, n=4), anti-CD150 mAb had no effect on the cytotoxicity of RPMI 8226, Jurkat, or U937 target cells (Figure 3-29D, representative, n=4).

Figure 3-29 Examination of cell-to-cell contact in cytotoxicity mediated by transformed γδ T cell clones. JAM assays were carried out at E:T ratios of 12.5:1, with [methyl-^{3}H] thymidine labeled U937, Jurkat, or RPMI 8226 target cells. (A) Target cells were aliquoted into the wells of the assay plate. Transformed γδ T cell clones were added to tissue culture inserts (transwells; 0.2μm anopore membranes) and the transwells were placed into the assay wells. (B and C) γδ T cell clones were incubated with a 1:500, 1:1000, 1:5000, 1:50 000, or 1:500 000 dilution of anti-γδ TCR ascites for 30 minutes prior to their use in JAM assays. (D) Anti-CD150 mAb (500ng/ml or 1μg/ml) or an isotype control was added to γδ T cell clones in the assay plate immediately before the addition of target cells. Results shown are representative (n=4) mean % kill ± SD of triplicate wells.


A
U937
RPMI 8226
Jurkat
% Kill
80
70
60
50
40
30
20
10
0
-10
media
transwell
+/- Transwell


B
U937
Jurkat
RPMI 8226
% Kill
80
70
60
50
40
30
20
10
0
media
1:5000
1:50 000
1:500 000
Dilution of Ascites


C
U937
RPMI 8226
Jurkat
% Kill
90
80
70
60
50
40
30
20
10
0
media
1:500
1:1000
Dilution of Ascites

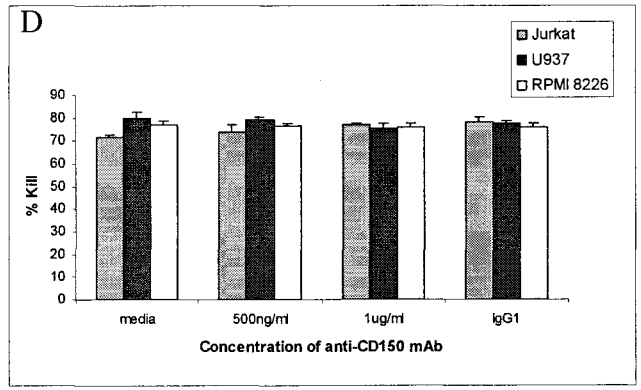
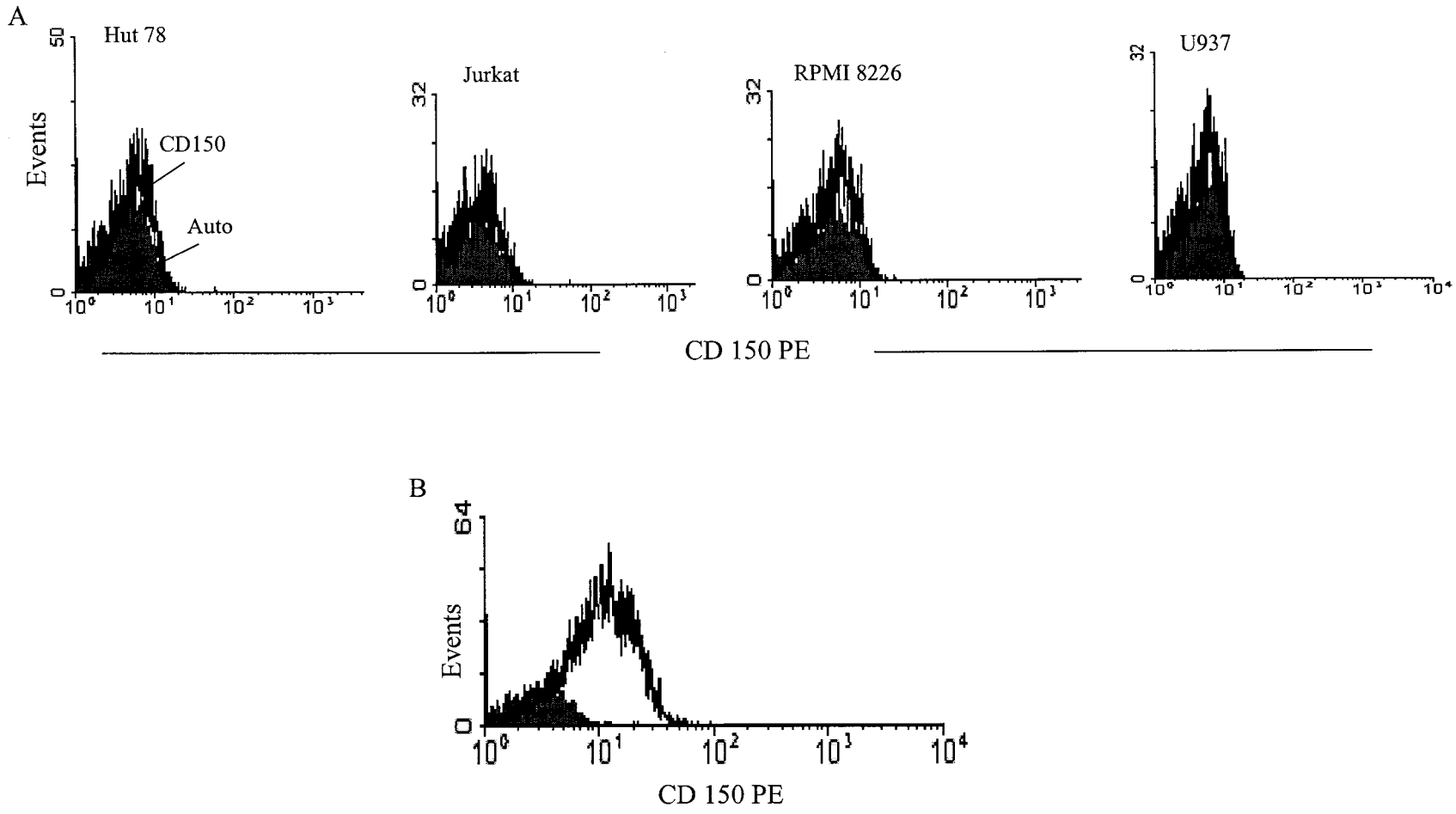
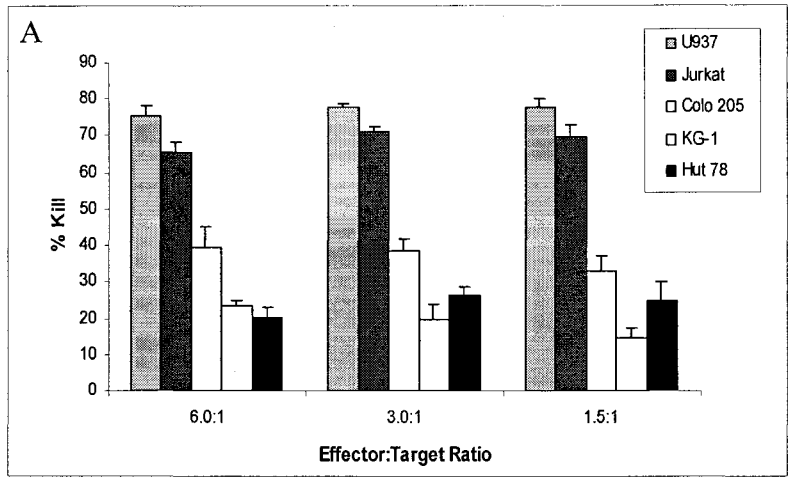

D
Jurkat
U937
RPMI 8226
% Kill
90
80
70
60
50
40
30
20
10
0
media
500ng/ml
1ug/ml
IgG1
Concentration of anti-CD150 mAb

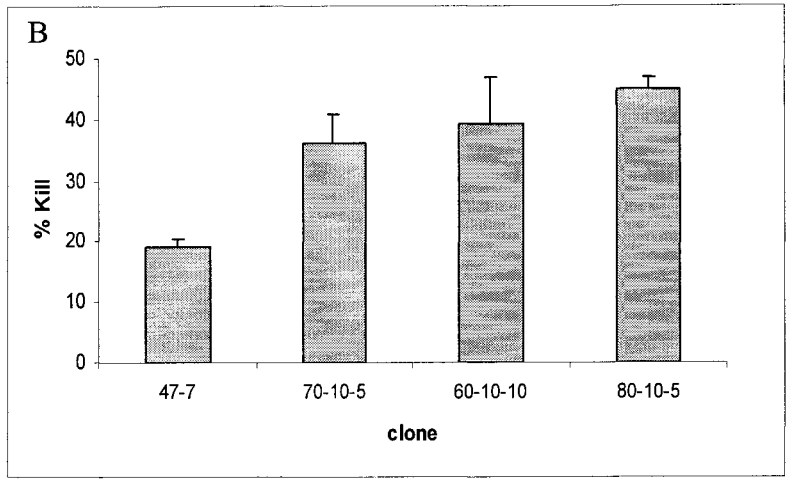
Figure 3-30 Examination of CD150 expression on the surface of Hut 78, Jurkat, RPMI 8226, and U937 target cells (A) or transformed γδ T cell clones (B, n=4). Cells were blocked with non-specific mouse IgG and labeled with anti-CD150 PE.

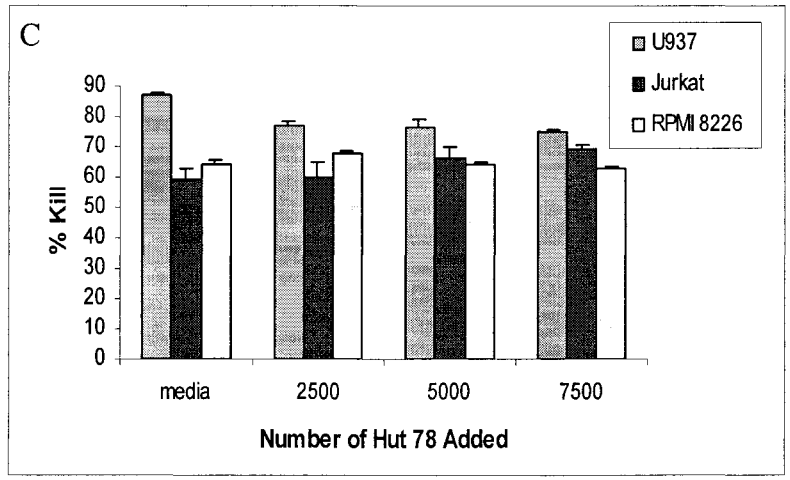

A
Hut 78
Jurkat
RPMI 8226
U937
Events
CD150
Auto
CD 150 PE
B
Events
CD 150 PE

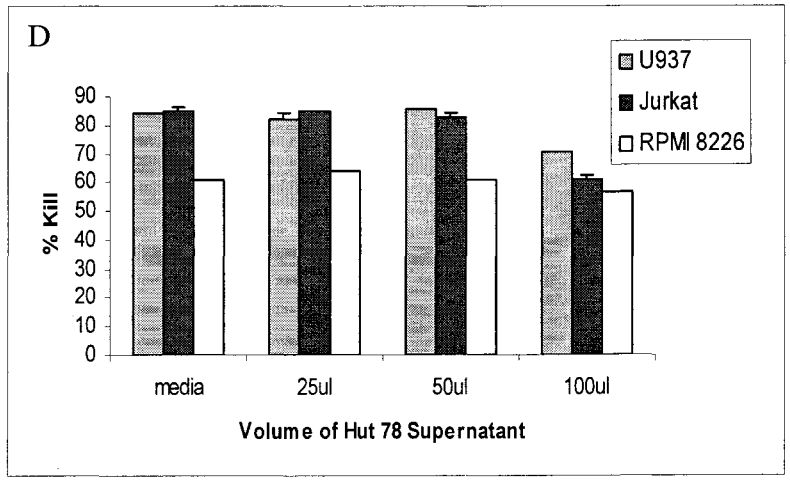
Early studies to identify target cells easily cytolysed by clone 47-7 identified Hut 78 as the least susceptible to cytotoxicity mediated by this clone when compared with Jurkat, U937, KG-1, and Colo 205 (Figure 3-31A). Clones 70-10-5, 80-10-5, and 60-10-10 were unable to kill more than 45% of Hut 78 cells (Figure 3-31B). To investigate the possibility that Hut 78 cells expressed a protective surface molecule(s), or secreted such a molecule into the supernatant, JAM assays were set-up with Hut 78 cells or supernatant. Neither the Hut 78 cells nor supernatant was able to significantly inhibit lysis of Jurkat, U937, or RPMI 8226 target cells (Figure 3-31C and D, representative, $n=4$, $p \geq 0.2$).

Figure 3-31 Investigation of the cytotoxicity mediated by transformed γδ T cell clones against Hut 78 target cells. All JAM assays were carried out for 4.5 hours with [methyl-^{3}H] thymidine labeled target cells at an E:T ratio of 6.25:1. (A) Cytotoxicity mediated by clone 47-7 in response to Hut 78, U937, Jurkat, Colo 205, and KG-1 target cells. (B) Comparison of cytotoxicity mediated by clones 47-7, 70-10-5, 60-10-10, and 80-10-5 in response to Hut 78. (C) Effect of Hut 78 cells on cytotoxicity of U937, Jurkat, and RPMI 8226 target cells. Increasing numbers of cold Hut 78 cells were added to 10 000 [methyl-^{3}H] thymidine labeled target cells in the assay wells. (D) Effect of Hut 78 supernatant on cytotoxicity of U937, Jurkat, and RPMI 8226. Increasing volumes of supernatant isolated from cold Hut 78 cultures were added to assay wells with target and clone cells. Results shown are representative (n=4) mean % kill ± SD of triplicate wells.


A
U937
Jurkat
Colo 205
KG-1
Hut 78
% Kill
90
80
70
60
50
40
30
20
10
0
6.0:1
3.0:1
1.5:1
Effector:Target Ratio


B
% Kill
50
40
30
20
10
0
47-7
70-10-5
60-10-10
80-10-5
clone


C
U937
Jurkat
RPMI 8226
% Kill
90
80
70
60
50
40
30
20
10
0
media
2500
5000
7500
Number of Hut 78 Added

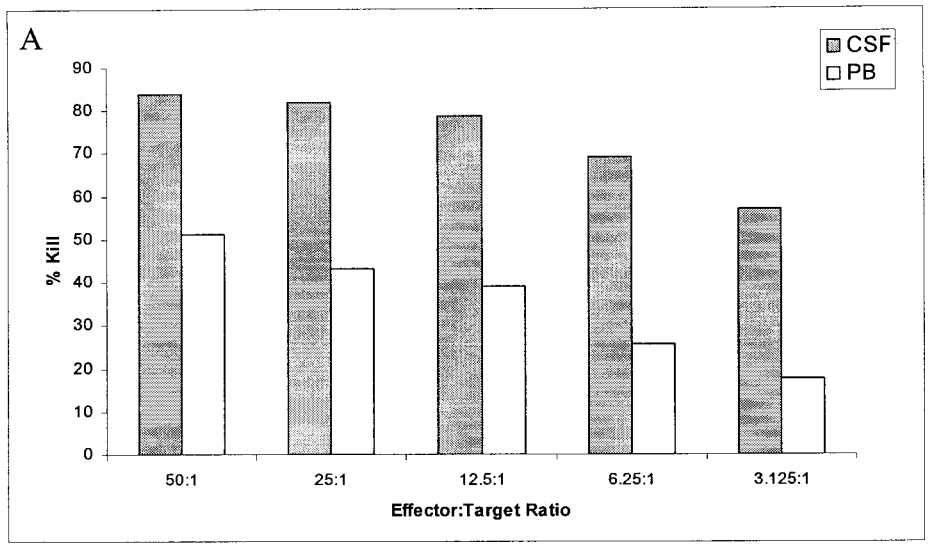

D
U937
Jurkat
RPMI 8226
% Kill
90
80
70
60
50
40
30
20
10
0
media
25ul
50ul
100ul
Volume of Hut 78 Supernatant

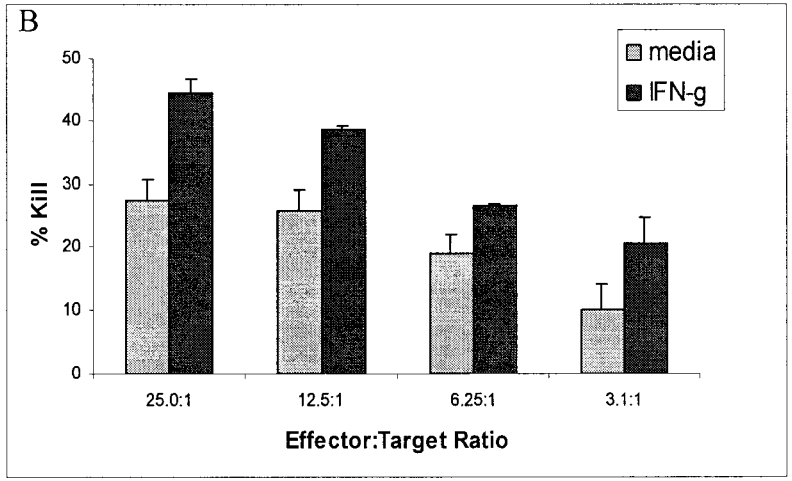
3.7 2-Dimensional Electrophoretic Analysis of γδ T cell Lines

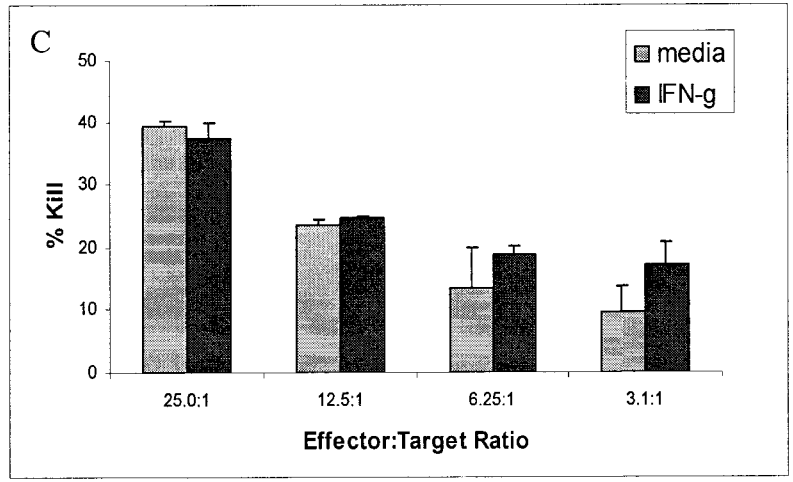
During my research I observed several functional and phenotypic differences between γδ T cell lines derived from PB and CSF of the same individual:

1. The iNKR phenotype (Figures 3-2 and 3-3). Typically, the percentage of p70, p58.1, p58.2, and p140 positive cells was lower in the CSF derived γδ T cells, while the percentage of CD94 and NKG2A expressing cells was lower in the PB derived lines. In a number of samples the CSF cells did not express particular iNKR that were expressed by the PB counterparts.
2. The cytotoxicity (Figure 3-32A). In the majority of cases, the cytotoxicity of the CSF derived γδ T cell lines was greater than that of the matching PB lines.
3. The cytotoxicity of the cell lines in response to target cells pre-treated with IFN-γ. The example shown in Figure 3-32B and C demonstrates that the cytotoxicity mediated by the CSF (B) derived line increases in response to U937 target cells pre-treated with 60U/ml IFN-γ. In contrast, the cytotoxicity of the PB (C) derived line is unchanged.
4. The relative concentration of perforin, granzyme A, or granulysin stored within the cells, as indicated by the mean fluorescence intensity, often differed between PB and CSF derived cell line pairs (Figure 3-21). For example, the MFI of granulysin was 30.9 for the OND CSF14 line and 17.2 for its PB match.
5. The percent secretion of serine esterases. Table 3-8 demonstrates that the percent secretion, in response to the same target cell, differs between PB and CSF derived cell lines. Stimulation with U937 target cells induced 1.1% secretion by the MS PB1 cell line and 67.9% for the MS CSF1 line.

Figure 3-32 Cytotoxicity assays with γδ T cell lines derived from PB and CSF of patient MS 13 (A) or MS 5 (B and C). JAM assays were carried out for 5 hours with [methyl-^{3}H] thymidine labeled U937 target cells. (A) Comparison of percent kill due to PB or CSF derived cell line. Comparison of response of CSF (B) and PB (C) derived γδ T cell lines to target cells treated overnight with 60U/ml IFN-γ.


A
CSF
PB
% Kill
90
80
70
60
50
40
30
20
10
0
50:1
25:1
12.5:1
6.25:1
3.125:1
Effector:Target Ratio


B
media
IFN-g
% Kill
50
40
30
20
10
0
25.0:1
12.5:1
6.25:1
3.1:1
Effector:Target Ratio

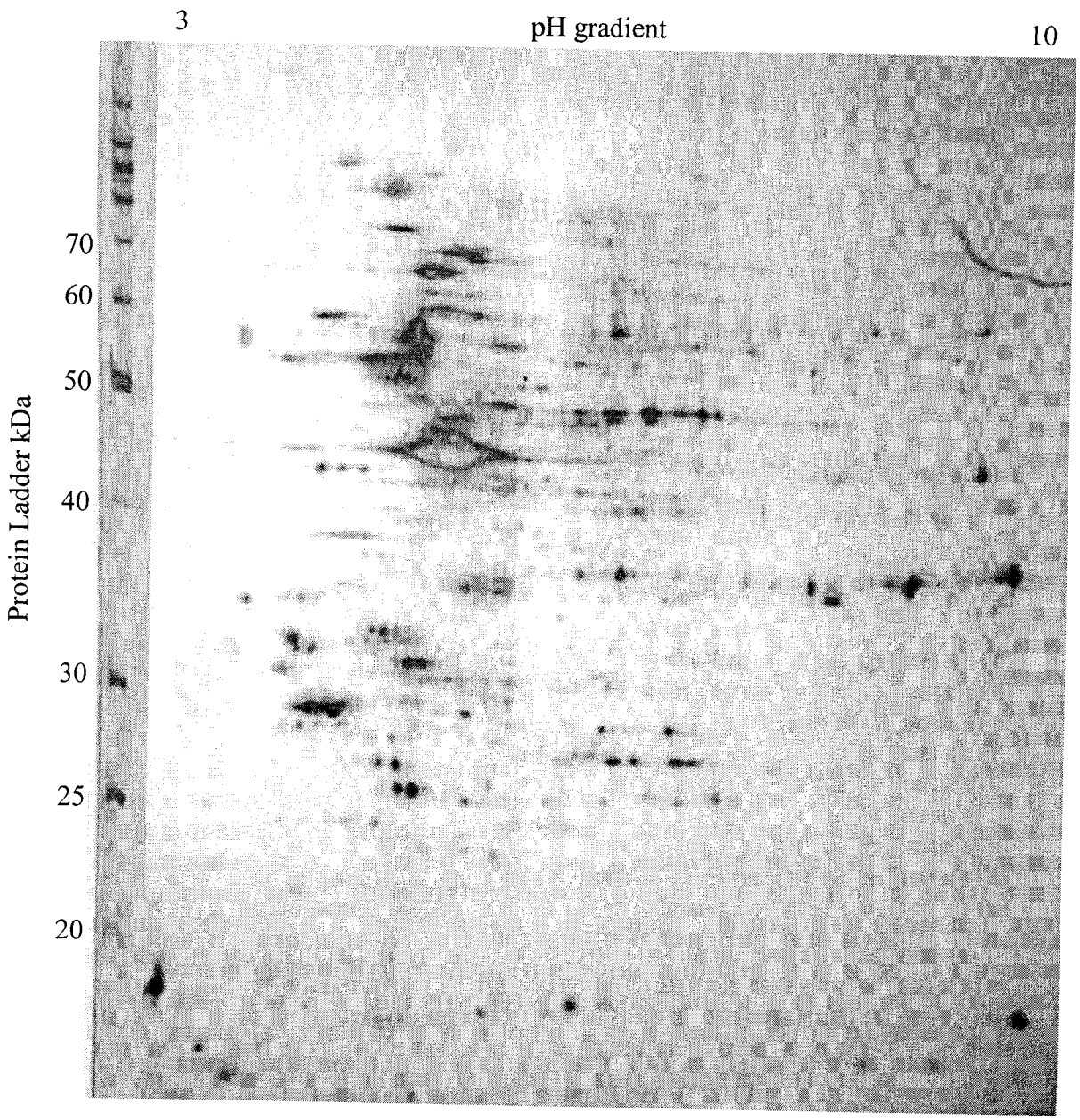

C
media
IFN-g
% Kill
50
40
30
20
10
0
25.0:1
12.5:1
6.25:1
3.1:1
Effector:Target Ratio

6. The cytotoxic mechanisms used by the γδ T cell lines. It was speculated in section 3.5.3, that the cytotoxic mechanisms differed slightly between HC, OND, and MS derived lines and between PB and CSF samples.

These phenotypic and functional differences, while not statistically significant, led to the question of whether there were actual differences in the protein expression of the cell lines that could distinguish PB and CSF (HC, OND, and MS) derived γδ T cells. To answer this question, 2-dimensional electrophoresis was performed with protein isolated from γδ T cell lines derived from HC PB, OND PB, OND CSF, MS PB, and MS CSF. Simply stated, this technique involves isolating proteins and electrophoresing them in two dimensions: first the proteins are aligned according to their pI (isoelectric focusing) and then the proteins are aligned according to their size (SDS-PAGE). The resulting gels are fixed and stained with silver nitrate. Digital images of the gels are made and compared using software such as PDQuest (a typical gel image is depicted in Figure 3-33).

Preliminary work showed that the optimal amount of protein to load per gel was 50μg and that the resolution of the gels was not improved with the PlusOne™ 2-D Clean-Up kit. Analysis of the gel images involves designating one gel image as the template to which all other gels are matched. Any spots which are not present on the template image, but are present on other images, can be added manually. The PDQuest program numbered each spot and generated a frequency report for each one. This report shows the presence or absence of each spot and its relative intensity for all 24 gels (in total 554 spots were analyzed). These reports were translated into the number of cell lines in each category which expressed that particular spot. Spots with frequencies of 0, 1, 4, or 5 cell

Figure 3-33 Representative image of 2 dimensional gel (n=24). Protein was isolated from PB and CSF derived γδ T cell lines, 50μg was loaded onto IPG strips, and isoelectric focusing was carried out for 24 hours. The strips were loaded onto acrylamide gels and SDS-PAGE was carried out. The resulting gels were fixed and silver stained and digital images were made for comparison.


3
pH gradient
10
Protein Ladder kDa
70
60
50
40
30
25
20

lines per category and that differed between the comparison groups were selected for mass spectrometry analysis, for example a spot which was detected in 4 MS PB samples but in only 1 MS CSF sample was selected. Using these criteria, 40 spots were sent for mass spectrometry analysis and the resulting peptides were compared with sequences in the NCBI database. The protein identification, the mass/pI, the peptide sequences, and the MOWSE scores for each of these spots are given in Tables 3-10 to 3-15. Each table lists the spots selected from one of 6 comparisons: MS PB to MS CSF, MS CSF to OND CSF, MS PB to OND PB, OND CSF to OND PB, HC to MS PB, or HC to OND PB. Although the number of cell lines in which that spot was detected is given for each category, the categories compared in each table have been highlighted.

Comparison of MS PB and OND PB (Table 3-10) gel images showed that the "protein profiles" of these two groups were very similar. Only 3 spots were found to have different frequencies. Spot 5602 has a low MOWSE score, typically MOWSE scores of 40 and above are considered good and reliable matches, however, a manual match of spot 5602 determined it to be a good match.

The profiles of the HC and OND PB gels were also very similar, as only 6 spots were found to have different frequencies (Table 3-11). However, the comparison of HC and MS PB showed 13 spots with different frequencies (Table 3-12). With one exception (9701) these spots were detected in 4 of the 5 MS PB samples but in only 1 (or none) of the HC samples. It is interesting to note that 4 spots were common to Tables 3-11 and 3-12, perhaps suggesting that certain proteins may be specific to the "disease state".

Only 7 proteins with differing frequencies were identified in the OND CSF to OND PB image comparison (Table 3-13). Six of these proteins were detected in 4 of the

Table 3-10 Analysis of Spots Identified from Comparison of OND PB and MS PB 2D Electrophoresis gels.

Spot Number	HC	OND CSF	OND PB	MS CSF	MS PB	Protein Candidate	Mass (kDa) / pI	Peptide Sequence	Mowse Score
1504	3	4	0	4	4	40S ribisomal protein P40 (C10 protein) (NCBI- 125968)	32.88 / 4.8	-LLVVTDPR -FAAATGATPIAGR -DPEEIEKEEQAAAEK -AIVAIENPADVSVISSR	224
5602	2	4	4	1	1	chaperonin subunit 8 (theta) (NCBI- 12846632)	49.86 / 5.55	-FAEAFEAIPR	30
8202	1	3	1	1	4	alpha tubulin subunit (NCBI- 13124850)	50.09 / 4.94	-DVNAAIATIK -AVFVDLEPTVIDEVR	67

Table 3-11 Analysis of Spots Identified from Comparison of HC and OND PB 2D Electrophoresis gels.

Spot Number	HC	OND CSF	OND PB	MS CSF	MS PB	Protein Candidate	Mass (kDa) / pI	Peptide Sequence	Mowse Score
2402	1	5	4	4	4	heterogeneous nuclear ribonucleoperotein C (C1/C2) (NCBI- 14249959)	32.37 / 5.00	-VPPPPPIAR -VDSLLENLEK	83
3201	4	2	0	0	2	1 - RAN binding protein 1 (NCBI- 20346197)	23.58 / 5.15	-TLEEDEEELFK	63
						2 - actin 1 (NCBI- 3907620)	41.72 / 5.23	-GYSFTTTAER	46
4201	1	5	4	4	4	1- heterogeneous nuclear ribonucleoprotein C2 (NCBI- 8393544)	34.36 / 4.92	-VFIGNLNTLVVK -GFAFVQYVNER -MIAGQVLDINLAAEPK	238
						2 - actin (NCBI- 3182892)	41.79 / 5.23	-AGFAGDDAPR -DLTDYLMK -VAPEEHPVLLTEAPLNPK	150
						3 - NADH dehydrogenase (ubiquinone) Fe-S protein 3 (30kD) (NADH-coenzyme Q reductase) (NCBI- 4758788)	30.22 / 6.99	-SLVDLTAVDVPTR -VVAEPVELAQEFR	143
7606	0	5	4	3	4	1 - annexin XI (56kD autoantigen) (NCBI- 4557317)	54.36 / 7.53	-LLISLSQGNR -DAQELYAAGENR	69
						2 - coronin (NCBI- 4585215)	20.10 / 8.33	-DAGPLLISLK	51
8608	1	5	5	5	4	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1, cardiac muscle (NCBI- 4757810)	59.71 / 9.16	-VLSIGDGIAR -AVDSLVPIGR -TSIAIDTIINQK -TGTAEMSSILEER -ILGADTSVDLEETGR -TGAIVDVPVGEELLR	205
8108	1	4	4	5	3	casein alpha S1 (NCBI- 225632)	24.42 / 4.85	-YLGYLEQLLR	41

Table 3-12 Analysis of Spots Identified from Comparison of HC and MS PB 2D Electrophoresis gels.

Spot Number	HC	OND CSF	OND PB	MS CSF	MS PB	Protein Candidate	Mass (kDa) / pI	Peptide Sequence	Mowse Score
0002	0	3	3	2	4	smooth muscle and non-muscle myosin alkali light chain, isoform 2 (NCBI- 17986260)	12.96 / 4.65	-ALGQNPTNAEVLK	45
1405	1	5	3	1	4	HSPC263 (NCBI- 6841176)	31.67 / 4.90	-LLTSGYLQR -EYAEDDNIYQQK -IQQEIAVQNPLVSER	168
2402	1	5	4	4	4	heterogeneous nuclear ribonucleoprotein C (C1/C2) (NCBI- 14249959)	32.37 / 5.00	-VPPPPPIAR -VDSLLENLEK	83
4201	1	5	4	4	4	1- heterogeneous nuclear ribonucleoprotein C2 (NCBI- 8393544)	34.36 / 4.92	-VFIGNLNTLVVK -GFAFVQYVNER -MIAGQVLDINLAAEPK	238
						2 - actin (NCBI- 3182892)	41.79 / 5.23	-AGFAGDDAPR -DLTDYLMK -VAPEEHPVLLTEAPLNPK	150
						3 - NADH dehydrogenase (ubiquinone) Fe-S protein 3 (30kD) (NADH-coenzyme Q reductase) (NCBI- 4758788)	30.22 / 6.99	-SLVDLTAVDVPTR -VVAEPVELAQEFR	143
5710	0	3	2	4	4	1 - matricin (NCBI- 631730)	60.45 / 6.23	-AVAQALEVIPR	61
						2 -dihydropyrimidinase-like 2; collapsin response mediator protein hCRMP-2 (NCBI- 4503377)	62.26 / 5.95	-SSAEVIAQAR -GSPLVVISQGK	47
7303	1	5	3	3	4	alternative splicing factor ASF-2 (NCBI- 105294)	31.98 / 5.61	-DAEDAVYGR -IYVGNLPPDIR	54
7606	0	5	4	3	4	1 - annexin XI (56kD autoantigen) (NCBI- 4557317)	54.36 / 7.53	-LLISLSQGNR -DAQELYAAGENR	69
						2 - coronin (NCBI- 4585215)	20.10 / 8.33	-DAGPLLISLK	51
7712	1	3	3	3	4	1 - heterogeneous nuclear ribonucleoprotein C (NCBI- 133263)	30.93 / 4.91	-MIAGQVLDINLAAEPK	73
						2 - alpha 1 type I collagen preprotein (NCBI- 4502945)	138.83 / 5.66	-GQAGVMGFPGPK + Ox. Of M -SGDRGETGPAGPAGPVGPVGAR	52
						3 - ezrin (NCBI- 4514720)	69.32 / 5.90	-APDFVFYAPR	43

Table 3-12 cont'd

8202	1	3	1	1	4	alpha tubulin subunit (NCBI- 13124850)	50.09 / 4.94	-DVNAAIATIK -AVFVDLEPTVIDEVR	67
8301	1	1	2	0	4	1 - proline synthetase co-transcribed homolog (bacterial) (NCBI- 6005842)	30.33 / 7.09	-APLEVAQEH	53
						2 - guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 (NCBI- 18543331)	35.07 / 7.60	-DETNYGIPQR -DVLSVAFSSDNR	51
8608	1	5	5	5	4	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1, cardiac muscle (NCBI- 4757810)	59.71 / 9.16	-VLSIGDGIAR -AVDSLVPIGR -TSIAIDTIINQK -TGTAEMSSILEER -ILGADTSVDLEETGR -TGAIVDVPVGEELLGR	205
9701	4	2	2	5	1	pyruvate kinase, muscle (NCBI- 4505839)	57.88 / 7.95	-GSGTAEVELK -GDYPLEAVR -GDLGIEIPAEK -SVETLKEMIK -LDIDSPPITAR -NTGIICTIGPASR + Carb. Of C	224
9702	1	4	2	0	4	pyruvate kinase, M1 ioszyme (pyrvate kinase muscle isozyme) (cystolic thyroid hormone-binding protein) (CTHBP) (NCBI- 20178296)	57.90 / 7.96	-LDIDSPPITAR -NTGIICTIGPASR + Carb. Of C -GADFLVTEVENGGSLGSK	52

Table 3-13 Analysis of Spots Identified from Comparison of OND CSF and OND PB 2D Electrophoresis gels.

Spot Number	HC	OND CSF	OND PB	MS CSF	MS PB	Protein Candidate	Mass (kDa) / pI	Peptide Sequence	Mowse Score
1504	3	4	0	4	4	40S ribisomal protein P40 (C10 protein) (NCBI- 125968)	32.88 / 4.8	-LLVVTDPR -FAAATGATPIAGR -DPEEIEKEEQAAAEK -AIVAIENPADVSVISSR	224
2107	2	4	1	1	2	sorcin (NCBI- 4507207)	21.66 / 5.32	-SGTVDPQELQK	76
2304	1	4	1	1	1	1 - alpha-S1 casein precursor (NCBI- 115646)	24.51 / 4.98	-YLGYLEQLLR -FFVAPFPEVFGK -VPQLEIVPNSAEER -HQGLPQEVLNENLLR -DIGSESTEDQAMEDIK -YKVPQLEIVPNSAEER -HPIKHQGLPQEVLNENLLR	432
						2 - beta-lactoglobulin (NCBI- 530873)	6.50 / 4.60	-TPEVDDEALEK -TPEVDDEALEKFDK	141
						3 - alpha-S2 casein precursor (NCBI-115654)	26.00 / 8.54	-FALPQYLK -NAVPITPTLNR -ALNEINQFYQK	127
						4 - beta-casein (NCBI- 4495057)	24.85 / 5.26	-FQSEEQQQTEDELQDK -DMPIQAFLLYQEPVLGPVR + Ox. Of M	124
						5 - kappa-casein C (NCBI- 300459)	18.91 / 6.09	-YIPIQYVLSR -SPAQILQWQVLSNTVPAK	88
6509	3	4	1	2	2	1 - heterogeneous nuclear ribonucleoprotein: type A/B hnRNP p38 (NCBI- 6562847)	30.83 / 7.68	-FGEVVDCTIK + Carb. Of C -EVYQQQQYGSGGR	116
						2 - poly(rC)-binding protein 2, isoform a (NCBI- 14141168)	38.63 / 6.33	-IANPVEGSTDR -INISEGNCPER + Carb. Of C	81
						3 - testis mitotic checkpoint BUB3 (NCBI- 3378104)	36.93 / 6.36	-VYTLSVSGDR -LNQPPEDGISSVK	49
6708	3	4	1	3	2	lamin A/C (NCBI- 5031875)	65.10 / 6.40	-LAVYIDR -DLEDSLAR -LSPSPTSQR -LADALQELR -SLETENAGLR -ITESEEVVSR -SGAQASSTPLSPTR -LQEKEDLQELNDR -NSNLVGAAHEELQQSR	476
8306	2	4	1	2	3	1 - porin isoform 1 (NCBI- 6063691)	30.70 / 8.63	-VTQSNFAVGYK	82
						2 - voltage-dependent anion-selective channel protein 2 (VDAC-2) (Outer mitochondrial membrane protein porin 2) (B36-VDAC) (NCBI- 6093769)	31.70 / 7.44	-LTLSALVDGK -YQLDPTASISAK	51
8607	4	1	4	4	3	2-phosphopyruvate-hydratase alpha-enolase; carbonate dehydratase (NCBI- 693933)	47.08 / 7.01	-IEEELGSK -GNPTVEVDLFTSK	111

5 OND CSF samples but in only 1 (0 for 1504) OND PB sample. The 7th protein was detected in 4 OND PB samples and only 1 OND CSF sample.

As hypothesized, the comparison of MS CSF and MS PB gels showed the "protein profiles" of these two groups to be quite different, 14 spots were found to have different frequencies (Table 3-14). The majority of these spots were present in 1 (or none) of the CSF samples but in 4 or 5 of the PB samples. Three spots were present in 4 or 5 of the CSF samples but only 1 PB sample. The last comparison, that of OND CSF to MS CSF (Table 3-15) also showed a difference in "protein profiles", the frequencies of 15 spots were found to be different. Thirteen of these spots were detected in 4 or 5 of the OND CSF gels, but in only 1 (or none) MS CSF gel. Closer examination of Tables 3-14 and 3-15 revealed that 7 spots were common to both. The frequencies of these 7 spots are listed in Table 3-16. The MS CSF category immediately "stands out", as only 1 cell line expresses any of these spots (0 for 9702). [Note: the single MS CSF derived line differs for each spot]. These 7 proteins may represent a protein profile specific to γδ T cells derived from the cerebrospinal fluid of multiple sclerosis patients.

Statistical analyses of these results (Fisher's exact test and two tailed *t* test) confirmed that the absence of spots 1405, 1802, 6502, and 9702 from MS CSF samples was MS specific ($p=0.047$) and that spots 1209 and 9702 were MS CSF specific ($p=0.047$). However, it would be prudent to analyse a larger number of samples before excluding spots 1209, 4603, and 8504.

Table 3- 14 Analysis of Spots Identified from Comparison of MS CSF and MS PB 2D Electrophoresis gels.

Spot Number	HC	OND CSF	OND PB	MS CSF	MS PB	Protein Candidate	Mass (kDa) / pI	Peptide Sequence	Mowse Score
1209	3	4	2	1	5	Chain A, crystal structure of truncated human Rhogdi quadruple mutant (NCBI- 14278159)	15.81 / 5.45	-AEEYEFLTPVEEAPK	45
3105	2	2	3	4	1	1 - heterogeneous nuclear ribonucleoprotein C (C1/C2) (NCBI- 14250048)	33.58 / 4.99	-MIAGQVLDINLAAEPK -MIAGQVLDINLAAEPK + Ox. Of M	66
						2 - adenine phosphoribosyltransferase (APRT) (NCBI- 1351958)	19.70 / 6.31	-IDYIAGLDSR	57
4603	2	4	4	1	4	1 - ubiquinol-cytochrome c reductase core protein I (NCBI- 4507841)	52.59 / 5.94	-SLLTYGR -IAEVDASVVR -ADLTEYLSTHYK -NALVSHLDGTTPVCEDIGR + Carb. Of C	162
						2 - translation initiation factor eIF4A II (NCBI- 2370593)	46.90 / 5.12	-VLITTDLLAR -GYDVIAQAQSGTGK	115
						3 - heat shock protein 90 (NCBI- 72221)	84.07 / 5.01	-GVVDSEDLPLNISR	68
						4 - nuclear RNA helicase (DEAD family) homolog (NCBI- 539961)	45.59 / 6.65	-ILVATNLFGR	67
4704	2	2	3	1	4	1 - dJ14N1.2 (novel S-100/ICaBP type calcium binding domain protein, similar to trichohyalin) (NCBI- 12314268)	24.33 / 7.27	-SVVTVIDVFYK	82
						2 - NADH dehydrogenase (ubiquinone) Fe-S protein 3 (30kD) (NADH-coenzyme Q reductase) (NCBI- 4758788)	30.22 / 6.99	-SLVDLTAVDVPTR	70
						3 - t-complex protein 1 (NCBI- 7305565)	60.30 / 5.76	-EQLAIAEFAR -ICDDELILIK + Carb of C.	61
						4 - albumin (NCBI- 229552)	66.09 / 5.76	-KVPQVSTPTLVEVSR	57
6804	2	3	2	1	4	moesin (NCBI- 4505257)	67.78 / 6.08	-SGYLAGDK -QLFDQVVK -ALELEQER -EDAVLEYLK -AQMVQEDLEK -AQMVQEDLEK + Ox. of M -QEAEEAKEALLQASR	237

Table 3-14 cont'd

8301	1	1	2	0	4	1 - proline synthetase co-transcribed homolog (bacterial) (NCBI- 6005842)	30.33 / 7.09	-APLEVAQEH	53
						2 - guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 (NCBI- 18543331)	35.07 / 7.60	-DETNYGIPQR -DVLSVAFSSDNR	51
						3 - serum albumin precursor (NCBI-2492797)	67.84 / 5.85	-LVNEVTEFAK	48
1802	3	5	2	1	4	similar to 40S ribosomal protein SA (P40) (34/67 KD laminin receptor) (NCBI- 20821866)	27.11 / 6.24	-FTPGTFTNQIQAAFR	82
8504	2	4	2	1	4	1 - similar to proteasome (prosome, macropain) 26S subunit, ATPase, 6 (NCBI- 19263883)	33.03 / 5.73	-LSDGFNGADLR -GCLLYGPPGTGK + Carb. of C -HGEIDYEAIVK -AVASQLDCNFLK + Carb. Of C	133
						2 - ovarian/breast septin delta (NCBI- 14530107)	38.46 / 7.13	-VVNIVPVIAK -SITHDIEEK -YLQEEVNINR	93
9701	4	2	2	5	1	pyruvate kinase, muscle (NCBI- 4505839)	57.88 / 7.95	-GSGTAEVELK -GDYPLEAVR -GDLGIEIPAEK -SVETLKEMIK -LDIDSPPITAR -NTGIICTIGPASR + Carb. Of C	224
9702	1	4	2	0	4	pyruvate kinase, M1 ioszyme (pyrvate kinase muscle isozyme) (cystolic thyroid hormone-binding protein) (CTHBP) (NCBI-20178296)	57.90 / 7.96	-LDIDSPPITAR -NTGIICTIGPASR + Carb. Of C -GADFLVTEVENGGSLGSK	52
6502	3	5	4	1	4	beta-actin (NCBI- 6716561)	41.65 / 5.38	-AGFAGDDAPR -DLTDYLMK -DLTDYLMK + Ox. Of M -GYSFTTTAER -DSYVGDEAQSK	109
7803	1	2	2	4	1	1 - actin (NCBI- 5114428)	41.72 / 5.46	-SYELPDGQVITIGNER	58
						2 - alpha-S1-casein (NCBI- 2119398)	24.48 / 4.85	-YLGYLEQLLR	42
8202	1	3	1	1	4	alpha tubulin subunit (NCBI- 13124850)	50.09 / 4.94	-DVNAAIATIK -AVFVDLEPTVIDEVR	67
1405	1	5	3	1	4	HSPC263 (NCBI- 6841176)	31.67 / 4.90	-LLTSGYLQR -EYAEDDNIYQQK -IQQEIAVQNPLVSER	168

Table 3- 15 Analysis of Spots Identified from Comparison of MS CSF and OND CSF 2D Electrophoresis gels.

Spot Number	HC	OND CSF	OND PB	MS CSF	MS PB	Protein Candidate	Mass (kDa) / pI	Peptide Sequence	Mowse Score
1204	1	5	3	1	2	heterogeneous nuclear ribonucleoprotein C (C1/C2) (NCBI- 13097279)	32.32 / 4.94	-VPPPPPIAR	42
1209	3	4	2	1	5	Chain A, crystal structure of truncated human Rhogdi quadruple mutant (NCBI- 14278159)	15.81 / 5.45	-AEEYEFLTPVEEAPK	45
2107	2	4	1	1	2	sorcin (NCBI- 4507207)	21.66 / 5.32	-SGTVDPQELQK	76
4603	2	4	4	1	4	1 - ubiquinol-cytochrome c reductase core protein I (NCBI- 4507841)	52.59 / 5.94	-SLLTYGR -IAEVDASVVR -ADLTEYLSTHYK -NALVSHLDGTTPVCEDIGR + Carb. Of C	162
						2 - translation initiation factor eIF4A II (NCBI- 2370593)	46.90 / 5.12	-VLITTDLLAR -GYDVIAQAQSGTGK	115
						3 - heat shock protein 90 (NCBI- 72221)	84.07 / 5.01	-GVVDSEDLPLNISR	68
						4 - nuclear RNA helicase (DEAD family) homolog (NCBI- 539961)	45.59 / 6.65	-ILVATNLFGR	67
6201	2	1	3	5	4	1-dJ14N1.2 (novel S-100/ICaBP type calcium binding domain protein, similar to trichohyalin) (NCBI- 12314268)	24.33 / 7.27	-SVVTVIDVFYK	54
8204	1	4	2	1	1	adenylate kinase 2 isoform b (NCBI- 7524346)	25.60 / 7.71	-AVLLGPPGAGK -APSVPAAEPEYPK	62
8607	4	1	4	4	3	2-phosphopyruvate-hydratase alpha-enolase; carbonate dehydratase (NCBI- 693933)	47.08 / 7.01	-IEEELGSK -GNPTVEVDLFTSK	111
1802	3	5	2	1	4	similar to 40S ribosomal protein SA (P40) (34/67 KD laminin receptor) (NCBI- 20821866)	27.11 / 6.24	-FTPGTFTNQIQAAFR	82
8504	2	4	2	1	4	1 - similar to proteasome (prosome, macropain) 26S subunit, ATPase, 6 (NCBI- 19263883)	33.03 / 5.73	-LSDGFNGADLR -GCLLYGPPGTGK + Carb. of C -HGEIDYEAIVK -AVASQLDCNFLK + Carb. Of C	133
						2 - ovarian/breast septin delta (NCBI- 14530107)	38.46 / 7.13	-VVNIVPVIAK -SITHDIEEK -YLQEEVNINR	93

Table 3-15 cont'd

6502	3	5	4	1	4	beta-actin (NCBI- 6716561)	41.65 / 5.38	-AGFAGDDAPR -DLTDYLMK -DLTDYLMK + Ox. Of M -GYSFTTTAER -DSYVGDEAQSK	109
9702	1	4	2	0	4	pyruvate kinase, M1 ioszyme (pyruvate kinase muscle isozyme) (cystolic thyroid hormone-binding protein) (CTHBP) (NCBI-20178296)	57.90 / 7.96	-LDIDSPPITAR -NTGIICTIGPASR + Carb. Of C -GADFLVTEVENGGSLGSK	52
1405	1	5	3	1	4	HSPC263 (NCBI- 6841176)	31.67 / 4.90	-LLTSGYLQR -EYAEDDNIYQQK -IQQEIAVQNPLVSER	168
2304	1	4	1	1	1	1 - alpha-S1 casein precursor (NCBI- 115646)	24.51 / 4.98	-YLGYLEQLLR -FFVAPFPEVFGK -VPQLEIVPNSAEER -HQGLPQEVLNENLLR -DIGSESTEDQAMEDIK -YKVPQLEIVPNSAEER -HPIKHQGLPQEVLNENLLR	432
						2 - beta-lactoglobulin (NCBI- 530873)	6.50 / 4.60	-TPEVDDEALEK -TPEVDDEALEKFDK	141
						3 - alpha-S2 casein precursor (NCBI- 115654)	26.00 / 8.54	-FALPQYLK -NAVPITPTLNR -ALNEINQFYQK	127
						4 - beta-casein (NCBI- 4495057)	24.85 / 5.26	-FQSEEQQQTEDELQDK -DMPIQAFLLYQEPVLGPVR + Ox. Of M	124
						5 - kappa-casein C (NCBI- 300459)	18.91 / 6.09	-YIPIQYVLSR -SPAQILQWQVLSNTVPAK	88
8501	3	4	2	0	3	isovaleryl Coenzyme A dehydrogenase (NCBI- 16877964)	46.21 / 8.42	-TDLAAVPASR	37
5602	2	4	4	1	1	chaperonin subunit 8 (theta) (NCBI- 12846632)	49.86 / 5.55	-FAEAFEAIPR	30

Table 3-16 Frequencies of Spots Common to MS CSF/MS PB and OND CSF/MS CSF Comparisons.

Spot	HC	OND CSF	OND PB	MS CSF	MS PB
1209	3	**4**	2	**1**	**5**
1405	1	**5**	3	**1**	**4**
1802	3	**5**	2	**1**	**4**
4603	2	**4**	4	**1**	**4**
6502	3	**5**	4	**1**	**4**
8504	2	**4**	2	**1**	**4**
9702	1	**4**	2	**0**	**4**

The observation that 7 spots (or rather the lack of expression of these 7 proteins) were specific to γδ T cells derived from the CSF of MS patients is extremely intriguing, especially since proteins involved in signal transduction, the mitochondrial respiratory pathway, protein turnover, and glycolysis have been identified. These proteins and their possible relevance to MS are described in the next chapter.

Chapter Four- Discussion

4.1 Overview

The working hypothesis of this study was that γδ T cells contribute to MS pathogenesis through the initiation and/or perpetuation of an immune-mediated attack against the oligodendrocyte/myelin unit and perhaps the axon itself. A complete understanding of the regulation of γδ T cell mediated cytotoxicity, via inhibitory natural killer cell receptors, and the cytotoxic mechanisms utilized by these cells is required before therapies to either slow the damage to the oligodendrocyte-myelin unit or prevent it altogether can be developed. It is also important to determine whether there are fundamental differences in the cytotoxicity mediated by γδ T cells derived from PB and CSF of MS patients versus those derived from OND patients and healthy controls.

My results have clearly demonstrated that even though γδ T cells express a number of iNKR (p70, p58.1, p58.2, p140, CD94, NKG2A), control of the cytotoxic response of these cells is more complex than the interaction of iNKR on the γδ T cells with the appropriate HLA class I molecules on the target cells. I have also shown that γδ T cell mediated cytotoxicity makes use of both the perforin/granzyme and the Fas/FasL pathways, rather than a single pathway acting as the "predominant" pathway, the cytotoxic mechanism utilized by the γδ T cells is influenced by the target cell. Numerous phenotypic and functional differences were noted between PB and CSF derived γδ T cells, including differences in iNKR repertoires, cytotoxic potential and mechanisms, percent secretion of serine esterases, and the relative concentration of perforin, granzyme A, and granulysin. Two dimensional electrophoretic analysis of protein samples isolated from γδ T cells (derived from MS PB and CSF, OND PB and CSF, and HC PB)

identified differences in protein expression for cells in each of these categories. Most important was the discovery of a multiple sclerosis cerebrospinal fluid γδ T cell specific protein profile. This not only supports my hypothesis that γδ T cells derived from MS patients are MS disease state specific, but also expands it to include the possibility that γδ T cells are MS disease state specific and CSF compartment specific.

4.2 γδ T cells and Natural Killer cell Receptors

Inhibitory natural killer cell receptors scan the surface of potential target cells for normal expression of HLA class I molecules. If the "appropriate" expression is identified, an inhibitory signal is generated, and the target cell is spared, if not the cell is lysed. The observations that γδ T cells are cytotoxic to target cells with low levels (or no expression) of HLA class I molecules (161,174,241) and that the cytotoxicity mediated by a γδ T cell clone correlated with the level of expression of HLA class I molecules (243) implied that this cytotoxicity is regulated via iNKR. Indeed, I showed that γδ T cells express iNKR, including p70, p140, p58.1, p58.2, CD94, and NKG2A (see section 3.3; 122,138,161,193,244). Numerous studies have shown that the cytotoxicity mediated by γδ T cells can be regulated via *in vitro* manipulation of the iNKR/HLA class I molecule interaction (161,174,186,210,219,227,228,231,237-239; summarized in Figure 1-1). For example, a target cell with the appropriate expression of HLA class I molecules becomes susceptible to lysis if the interaction between the iNKR and HLA molecules is prevented with an anti-HLA or anti-iNKR mAb and an inhibitory signal cannot be generated. In contrast, the cross-linking of an anti-iNKR mAb leads to the generation of

an inhibitory signal, even if the target cell has no (or very little) HLA class I expression, and the target cell is spared.

The results of my cytotoxicity experiments with polyclonal γδ T cell lines and transformed γδ T cell clones demonstrated that the specific lysis of U937, RPMI 8226, and K562 target cell lines is not regulated exclusively by iNKR (Figures 3-6 and 3-7). Neither pre-incubation of γδ T cells with anti-iNKR mAbs nor the immobilization of these mAbs onto the assay wells led to significant and repeatable modulation of cytotoxicity. In addition, pre-incubation of U937 target cells with IFN-γ (or -β) increased the expression of HLA class I molecules, but did not significantly and repeatedly inhibit the specific lysis (Figures 3-12 and 3-13).

There are several technical aspects of these experiments that must be considered as possible reasons for this inability to control γδ T cell mediated cytotoxicity via iNKR. First, the anti-iNKR mAbs used in these assays may not have been effective. This seems an unlikely possibility, as many other investigators have used the same mAb clones and have not reported any problems (138,161,174,237,244). Second, the cross-linking of the mAbs was not sufficient. Moretta et al. (186) found that the percent lysis of U937 target cells increased, rather than decreased, in the presence of anti-p58 mAbs (GL183 and EB6). They suggested this was the result of "inefficient cross-linking due to the low affinity of the human FcγR for the murine IgG1 subclass", and that the mAbs had actually masked the iNKR and thereby led to an increase in kill. To eliminate this possibility I immobilized the anti-iNKR mAbs on the assay plate (Figure 3-6D and E), however, significant mAb specific inhibition was not observed. Third, the iNKR present on the γδ T cells are not functional, unfortunately I had no way of verifying or disproving

this. Fourth, another iNKR not examined in this study (or presently unknown) is responsible for the regulation of γδ T cell mediated cytotoxicity. The iNKR studied here do not represent a complete list of inhibitory receptors.

With respect to the IFN-γ (or -β) experiments, pre-incubating the target cells with interferons did increase the expression of HLA class I molecules, however, it is possible that a "critical" HLA class I molecule(s) was either not up-regulated or not sufficiently up-regulated (243). For example, if the effector cell expresses the p58.1 receptor and the interferon does not increase the expression of HLA-Cw2, 4, 5, or 6, or does not increase the expression to the required level, then the p58.1 receptor is not activated. (The flow cytometry analyses of the U937 target cells were carried out with anti-HLA-ABC mAb, and therefore, show an increase in "total" HLA class I expression rather than specific HLA class I molecules; Figure 3-12). It should also be considered that treatment of the U937 cells with interferon may have affected the expression of other molecules important in γδ T cell mediated cytotoxicity but not analyzed in my experiments.

Perhaps iNKR control of γδ T cell mediated cytotoxicity is not a universal phenomenon, but instead is effector or target cell specific. The majority of reports which have demonstrated iNKR regulation of γδ T cell cytotoxicity employ HLA class I negative target cells, such as 721.221, Daudi, and C1R, and transfect these cells with HLA class I alleles or β_2 microglobulin to induce class I expression (161,174,193,242-244). The literature concerning γδ T cell mediated cytotoxicity of U937, RPMI 8226, and K562 target cells and the role of iNKR is sparse and contradictory. Poccia et al. (142) report that anti-CD94 mAb had no effect on the lysis of K562 cells, while Mingari et al. (229) show that both anti-p58 and anti-CD94 mAbs inhibited the cytotoxicity of

K562 cells. My results clearly show that RPMI 8226 cells are lysed by γδ T cells (Figure 3-6A). In contrast, Halary et al. (138) found that γδ T cell mediated lysis of RPMI 8226 target cells required anti-CD94 or anti-HLA class I mAbs. Each of these labs isolated γδ T cells from different sources, including patients with HIV-1, pure red cell aplasia, or leukemia, or healthy controls, and used different methodologies to generate and expand the γδ T cells. These variables could bias the iNKR repertoire and/or function of the resulting lines and clones.

The most logical interpretation of my results is that γδ T cell mediated cytotoxicity is not exclusively regulated by the interaction of iNKR and HLA class I molecules. Many authors had noted that NK cell mediated cytolysis could not be explained solely by the presence or absence of HLA class I molecules and suggested that it is the balance of the inhibitory and activating signals that determines the response of the cell (358-361). This theory has also been used to explain the regulation of γδ T cell mediated cytotoxicity (243,362,363). γδ T cells also express activating receptors, such as NKRP1A (Figure 3-8; 122,154) and NKG2D (214), both capable of overriding the inhibitory signals of iNKR. In addition, U937 cells express MICA, the ligand for NKG2D (364). The balance of the activating and inhibitory signals is also influenced by the sheer number and strength of the signals. Inhibitory signals typically overcome activation signals by recruiting tyrosine phosphatase (SHP-1 and/or SHP-2) which de-phosphorylates a number of local substrates and interrupts activation signalling. However, if the number of phosphorylated substrates or the rate at which they are phosphorylated is too great, the phosphatases cannot de-phosphorylate all of the substrates and an activation signal prevails (365). Unfortunately, the study of activating

receptors has lagged behind that of iNKR, as it is only recently that reagents to study activating receptors at the single cell level have become available (365). Further work with these receptors will shed more light on the regulation of NK and γδ T cell mediated cytotoxicity.

The preliminary work to study the role of iNKR in γδ T cell mediated cytotoxicity included flow cytometry analyses of 13 γδ T cell clones transformed with *Herpesvirus saimiri*. These studies revealed that none of the clones expressed p70, p58.1, p58.2, or p140. Further investigation showed that the iNKR phenotype of γδ T cell lines transformed with *Herpesvirus saimiri* differed from that of their non-infected parent lines (Table 3-2). To my knowledge this is the first report of changes to cell surface molecules in general, and the iNKR repertoire in particular, due to infection with *Herpesvirus saimiri*. There is much evidence that immortalisation of T cells (αβ and γδ) with *Herpesvirus saimiri* does not affect the "original characteristics" of the cell (including cell surface molecule expression) and is therefore a useful tool for functional analyses of these cells (366-368, Dr. R. Pon personal communication). Comparison of the iNKR phenotypes of a larger number of infected and non-infected γδ T cell line pairs is needed to determine whether *Herpesvirus saimiri* infection directly affects the iNKR repertoire.

4.3 iNKR Phenotype and IFN-β Treatment

IFN-β is the first immunomodulatory treatment proven to be effective for MS (353,354). The exact mechanisms underlying its therapeutic effects have not been fully elucidated, however, this cytokine modulates T cell activities and regulates the secretion of other cytokines and chemokines or their receptors (355,356). I wanted to determine

whether the beneficial effects of IFN-β treatment were due to an ability to manipulate iNKR expression by γδ T cells (*in vivo* and *in vitro*) and thereby affect the cytotoxicity mediated by γδ T cells. The iNKR phenotypes of γδ T cell lines derived from PB of MS patients undergoing treatment with IFN-β, were unchanged throughout the 6 month follow-up period, as compared with baseline levels (Figure 3-9). It is possible that IFN-β treatment does alter the iNKR phenotype of γδ T cells, but that these changes were not observed in my studies for one of the following reasons: (1) the changes are short lived and therefore, were not observed at the time points investigated in this study, (2) the protocol used to generate and expand the γδ T cells may have biased the resulting lines, for example, IL-2 is required to sustain the γδ T cell cultures, but it has been shown to up-regulate the expression of p58.1 and p58.2 (369), (3) IFN-β affects iNKR not studied in this investigation or activating NKR, or (4) the γδ T cells with altered iNKR expression may concentrate in the CNS.

Incubation of γδ T cell lines with IFN-β did not alter the iNKR phenotype at the doses and times analyzed (Figure 3-10). It is possible that: (1) a different dose or time frame is required for an increase or decrease in expression to occur, (2) IFN-β may induce the secretion of another cytokine which directly affects iNKR expression, such as IL-15 or TGF-β (228,370) which could not be induced *in vitro*, or (3) IFN-β may affect the expression of another iNKR not included in this study.

These results illustrate that the therapeutic effect of IFN-β treatment for patients with MS is not directly related to the expression of p70, p58.1, p58.2, p140, CD94, or NKG2A. Since weekly injection of Avonex® is considered a "lower dosed" IFN-β,

compared with other forms of IFN-β therapy, it is possible that an effect on iNKR expression (both *in vivo* and *in vitro*) would be observed with one of the other "higher dosed" IFN-β regimens.

4.4 Mechanisms of γδ T cell Mediated Cytotoxicity

Lymphocyte mediated cytotoxicity typically proceeds via the perforin/granzyme or the Fas/FasL pathway (246). γδ T cells display a profound cytotoxic activity against tumour cell lines (102,117,130,131) and virus or bacteria infected cells (97,100,132-136) and express FasL, perforin, granulysin, and granzymes A, B, and M mRNA and protein (124,141,153,330,331-334; Figure 3-14). However, the cytotoxic mechanisms utilized by γδ T cells are not completely understood. This is due, in large part, to the fact that the majority of reports describing γδ T cell mediated cytotoxicity rely on results obtained from a single target cell line and/or chemical compounds, such as Mg^{2+}EGTA, isocoumarin compounds, brefeldin A, strontium ions, and concanamycin A (136,141,332, 338,339). The use of such chemical compounds is an effective method of inhibiting cytotoxicity, and at one time was the only method available, however, it is not a useful means of identifying specific molecules involved in cytolysis. For example, the addition of Mg^{2+}EGTA blocks granule exocytosis, if cytotoxicity is inhibited by the addition of this compound it would be concluded that the perforin/granzyme pathway is being used. Unfortunately, this does not give the investigator any information as to which cytotoxic molecule (perforin, granzymes, granulysin, FasL, or another molecule) is actually responsible for the death of the target cell.

The inherent problem with using a single target cell line is that the experiment is restricted by the cytotoxic susceptibility of that target cell. For example, Jurkat target

cells are susceptible to Fas mediated cytotoxicity. The addition of Fas blocking mAb will decrease the percent lysis observed in the cytotoxicity assay, while the addition of anti-perforin mAb will have no effect on lysis. In this case, the investigator is likely to conclude that the cytotoxicity mediated by γδ T cells uses the Fas/FasL pathway and rule out the use of the perforin/granzyme pathway. However, he/she is only investigating half of the story as they cannot sufficiently investigate the perforin/granzyme pathway in this experimental set-up.

My working hypothesis was that γδ T cell mediated cytotoxicity utilizes both the perforin/granzyme and the Fas/FasL pathways, and that neither of these pathways predominates, but rather the cells tailor their cytotoxic response to the target cells. My examination of γδ T cell mediated cytotoxicity, was designed to simultaneously compare the cytotoxicity of 3 targets with different cytotoxic susceptibilities and to use specific cytotoxic inhibitors, monoclonal antibodies, to gain a more in depth understanding of the γδ T cell cytotoxic pathways.

This work has led to several interesting and novel conclusions. First, that γδ T cell mediated cytotoxicity makes use of both the perforin/granzyme and the Fas/FasL pathways (Tables 3-4 to 3-6 and Figure 3-19). Perhaps more important is the finding that these pathways are not mutually exclusive and often operate simultaneously. This is particularly evident for RPMI 8226 target cells, as the greatest inhibition in percent kill is obtained with a combination of Fas blocking, anti-perforin, and anti-granzyme A mAb (Figure 3-19A). Second, that the granule enzyme exocytosis of an individual γδ T cell line is influenced by the target cell (Table 3-8). This suggests that γδ T cells are capable of recognizing that target cells have different cytotoxic susceptibilities and adjusting their

cytotoxic attack accordingly. Unfortunately, the ligand(s) and receptors which relay this information to the γδ T cells remain unidentified. The final observation is that granzyme B is an important cytotoxic mediator of γδ T cell mediated cytotoxicity (Figure 3-17C and D and Tables 3-4 to 3-6).

These conclusions are in direct contrast with previous work, which had concluded that the role of the Fas/FasL pathway in γδ T cell mediated cytotoxicity was "minor" and was "seen when the (predominant) perforin based component was eliminated", and that granzymes did not play an important role in γδ T cell cytotoxicity (339). These discrepancies are most likely due to the use of non-specific methods in the experiments, including Mg^{2+}EGTA, isocoumarin compounds, brefeldin A, and concanamycin A, as specific mAbs were not yet readily available. Such compounds are often not absolutely specific for one protein and therefore may lead to inaccurate conclusions.

I was unable to find an anti-granzyme B mAb that would inhibit the activity of granzyme B. Therefore, I decided to experiment with a granzyme B inhibitor. Since the granzyme B inhibitors are also caspase inhibitors I wanted to choose an enzyme which was not cell permeable so that inhibition of the caspase would not be an issue. The inhibitor which matched my criteria was granzyme B inhibitor II (caspase-8 inhibitor I). Although all 3 target cell lines were susceptible to granzyme B mediated cytolysis, only 1 of the lines was susceptible to perforin. This is particularly interesting as granzyme B cannot induce apoptosis in the absence of perforin or another endosomolytic agent (261,263,265-267). Perhaps the anti-perforin mAb did not inactivate all of the perforin present and enough remained for granzyme B assistance, or the anti-perforin mAb does not affect the granzyme B related function of this enzyme, or the γδ T cells contain

another enzyme capable of aiding granzyme B. Experiments in which both anti-perforin and granzyme B inhibitor II are added to the assay wells are needed to study this problem further.

With few exceptions, 100% inhibition of cytotoxicity was not achieved through the addition of anti-perforin, anti-granzyme A, or Fas blocking mAb, or granzyme B inhibitor II. Even combinations of anti-perforin, anti-granzyme A, and Fas blocking mAb were not able to give 100% inhibition. Unfortunately, time and resources did not permit experiments with combinations of granzyme B inhibitor II and mAbs. Perhaps such combinations would fully inhibit γδ T cell mediated cytotoxicity. It is also possible that granulysin or granzyme M (or another unidentified cytotoxic molecule) is partially responsible for the "remaining" cytotoxicity as I was not able to determine whether these enzymes are important mediators of γδ T cell mediated cytotoxicity against Jurkat, U937, or RPMI 8226 target cells. The correlation observed between the relative level of granzyme M stored in the PB derived γδ T cell lines and the percent lysis of the U937 target cells certainly implies that granzyme M has a role in the specific lysis of these cells (Figure 3-23G). There is evidence to support a role for granulysin in γδ T cell mediated cytotoxicity, Dieli et al. (333) used an immunoaffinity absorption approach to demonstrate that granulysin is responsible for γδ T cell mediated killing of *M. tuberculosis* infected macrophages and intracellular *M. tuberculosis*, [as previously described, killing of intracellular *M. tuberculosis* also requires perforin (298)]. Until a suitable anti-granulysin or anti-granzyme M mAb becomes available, it may be useful to apply this immunoaffinity absorption technique (de-granulate the cells with Sr^{2+}, absorb

the supernatants on plates coated with anti-granulysin or anti-granzyme M mAb, collect supernatants and use in cytotoxicity assays) to studies in our laboratory.

Reports of the cytotoxicity mediated by *Herpesvirus saimiri* transformed γδ T cell clones are sparse and non-specific methods of cytotoxic inhibition were employed. For example, Narazaki et al. (371) concluded that the cytotoxicity mediated by a transformed Vγ1Vδ1 clone was perforin-dependent since it was inhibited by pre-treatment with Sr^{2+}. This experiment implies that the perforin/granzyme pathway is being used by the clone, however, it does not identify the molecule(s) in the perforin/granzyme pathway which is the cytotoxic mediator.

I hypothesized that the cytotoxic mechanisms utilized by γδ T cell clones would be identical to those used by the non-transformed parental lines. This was not the case. Cytotoxicity experiments carried out with 4 γδ T cell clones transformed with *Herpesvirus saimiri* demonstrated that the clones expressed perforin, granzymes, and granulysin (Figure 3-24), and were capable of secreting the contents of their cytotoxic granules (Table 3-9). However, the cytotoxicity mediated by these clones against Jurkat, RPMI 8226, and U937 was not abrogated by antibodies to any of these molecules (Figure 3-25). This cytotoxicity was also resistant to Fas blocking mAb and shTNF-R (Figures 3-25E and 3-26A).

Further experiments with granzyme B inhibitor II demonstrated that all 4 clones used granzyme B as the major cytotoxic mediator (Figure 3-28). Although each of the clones was derived from a different sample (HC PB, MS CSF, OND PB, or OND CSF) the cytotoxic mechanism was the same. As observed with the non-transformed lines, the requirement for perforin was not obvious. Whether this use of granzyme B is the result of

viral transformation, a bias in the clonal selection protocol, or a coincidence remains to be determined. However, it should not be concluded that the clones "exclusively" use granzyme B until a closer investigation of granzyme M and granulysin has been carried out. It is also possible that some as yet unidentified cytotoxic molecule is contributing to the cytotoxicity mediated by the transformed clones.

4.5 Two Dimensional Electrophoresis

During my investigations with γδ T cells, I noticed several phenotypic and functional differences between lines derived from PB and CSF of the same individual. The iNKR repertoires were not identical (Figures 3-2 and 3-3), the cytotoxic ability of the CSF line was often greater than that of the PB line (Figure 3-32A), the relative concentration of perforin, granzyme A, or granulysin stored within the cells was not equal (Figure 3-21), and the cells responded differently to target cells that had been pre-incubated with interferon (Figure 3-32B and C). In addition, there were differences in the cytotoxic mechanisms utilized by γδ T cells derived from PB and CSF samples or from MS, HC, and OND samples (Tables 3-4 to 3-6).

There are reports of differences in the γδ V gene repertoire of γδ T cells derived from CSF versus the paired PB samples (99,347) and of clonal expansion of γδ T cells isolated from the CSF of MS patients, while γδ present in the PB of MS patients and the PB and CSF of control patients are not oligoclonal (348).

I theorized that the protein expression of CSF derived γδ T cells was different than that of PB derived cells. In order to test this hypothesis, two dimensional

electrophoretic analyses were carried out with protein samples isolated from γδ T cell lines derived from MS PB, MS CSF, OND PB, OND CSF, and HC PB specimens.

Two way comparisons of the 2D gels revealed that 7 spots were common to the MS PB to MS CSF and OND CSF to MS CSF comparisons. These 7 spots (or proteins) were detected in 4 or 5 out of 5 MS PB or OND CSF samples, but in only 1 of the 5 MS CSF samples (spot 9702 does not appear in any of the MS CSF profiles). Therefore, the absence of these 7 proteins represents a "protein profile" specific to γδ T cells derived from the CSF of MS patients. This disease and compartment specific profile supports the theory that γδ T cells isolated from the CSF of MS patients are not simply the result of an influx of activated PB cells, but instead represent a group of cells specifically activated in (and perhaps specifically recruited to) the CNS (347).

Spot 1209 was identified by the NCBI database (372) as "chain A, crystal structure of truncated human RhoGDI quadruple mutant". In their attempts to crystallize human RhoGDI, Longenecker et al. (373) discovered that nice crystals were obtained by removing the first 23 or 66 residues and introducing lysine to alanine mutations into these truncated proteins. A query of the NCBI database using the BLAST searching algorithms (and a manual match) identified spot 1209 as Rho GDP dissociation inhibitor alpha (GDI). The Rho proteins are a family of GTP-binding proteins (GTPases) which regulate the signal transduction pathways involved in the re-organization of the actin cytoskeleton, gene transcription, regulation of cell polarity, G1 cell cycle progression, vesicular transport pathways, microtubule dynamics, and a variety of enzymatic activities (374,375). These proteins are regulated by guanine nucleotide dissociation inhibitors (α, β, and γ) which inhibit the dissociation of the nucleotide bound to Rho and thereby

regulate the activation of Rho proteins (between active GTP-bound and inactive GDP-bound states) and their translocation between the cytosol and the cell membrane (374,376). Given that Rho GTPases are important regulators of numerous signal transduction pathways, it is almost impossible to anticipate how the absence of RhoGDI will affect the γδ T cell. There are no reports of such a deficiency in any human system, however, mice RhoGDIα mutants displayed progressive impairment of the kidneys and reproductive organs (377). Interestingly, spot 6502 was identified by the NCBI database as beta-actin, a non-muscle cytoskeletal actin. The absence of beta-actin and a regulator of the actin cytoskeleton suggests that MS CSF γδ T cells may have impaired motility and shape control, granule exocytosis, and membrane receptor rearrangement.

Spot 9702 was identified by the NCBI database as pyruvate kinase, M1 isoenzyme (pyruvate kinase muscle isoenzyme). Pyruvate kinase catalyzes the reaction of phosphoenolpyruvate and ADP to pyruvate and ATP (378). Since pyruvate feeds into a number of metabolic pathways, pyruvate kinase represents a primary metabolic intersection. There are 4 pyruvate kinase isoenzymes in mammals, the M1 isozyme is localized in muscle, heart, brain, and lymphocytes, M2 is present in the kidneys and lungs, R in red blood cells, and L in the liver (379,380). The absence of pyruvate kinase impairs glycolysis leading to abnormal levels of ATP and NAD, a build up of glycolytic intermediates, and possibly improper functioning of the cell (378,379). While there are no reports of pyruvate kinase deficiency in white cells, pyruvate kinase R deficiency is a hereditary condition that leads to non-spherocytic anemia (381).

Spot 1405 was identified by the NCBI database as HSPC263, also known as otubain 1 (ovarian tumor domain ubiquitin aldehyde binding protein 1; 382). [The

ovarian tumor superfamily is composed of a group of putative cysteine proteases homologous to the ovarian tumor gene product of *Drosophila* (383)]. Otubain 1 is a deubiquitinating enzyme with cysteine protease activity. In contrast to other deubiquitinating enzymes, which cleave the ubiquitin-peptide bond, otubain 1 is a highly specific ubiquitin isopeptidase and is thought to be important in specific ubiquitin dependent pathways, particularly signalling pathways, rather than the general protein degradation machinery (382). This enzyme is also important in the regulation of $CD4^+$ T cell anergy induction (384,385).

Spot 8504 appears to be a mixture of 2 proteins. The NCBI database identified this spot as both "similar to proteasome 26S subunit, ATPase,6" and ovarian/breast septin delta. A query of the NCBI database using the BLAST searching algorithms (and a manual match) confirmed that one of the proteins was indeed human proteasome 26S subunit ATPase 6. Before any conclusions can be made regarding this "spot", 2D electrophoresis must be repeated with IPG strips with a smaller pH gradient so that these proteins (pIs of 5.73 and 7.13) can be separated and it can be conclusively determined which of the proteins is indeed absent from the MS CSF derived γδ T cells. That said, both of these proteins are potentially interesting.

The 26S subunit ATPase 6 is part of the 26S proteasome which degrades ubiquitinated proteins, typically those with a K48 polyubiquitin chain (reviewed by 386,387). [Activated ubiquitin is covalently conjugated to the substrate via an isopeptide bond between a lysine residue in the substrate and the C terminus of ubiquitin. Polyubiquitination is achieved by the formation of an isopeptide bond between glycine 76 of activated ubiquitin and the ε group of one of three lysines (K29, K48, or K63) of the

ubiquitin protein already attached to the substrate]. Otubain 1 has deubiquitinating activity towards K48 linked polyubiquitin chains (385). The absence of both ATPase 6 and otubain 1 could have far reaching effects on protein turnover and various signal transduction pathways. The ovarian/breast septin delta is part of the septin family, a group of molecules (putatively GTPases) that function in cytokinesis and lymphocyte adhesion and transmigration across endothelial membranes (reviewed by 388,389). The lack of a protein involved in adhesion and migration would certainly affect the activation and functioning of γδ T cells.

Spot 1802 was identified by the NCBI database as "similar to 40S ribosomal protein SA (34/67 KD laminin receptor), a query of the NCBI database using the BLAST searching algorithms (and a manual match) identified the protein as human 67 kDa laminin receptor. The laminins are a family of extracellular matrix proteins and structural elements of the basal laminae (390,391). The laminin receptors are non-integrin matrix protein receptors that mediate T cell adherence to laminin and basement membrane invasion (392-394). The absence of this laminin receptor would be expected to impair the migration and mobility of the cell.

Unfortunately, spot 4603 was identified by the NCBI database as a mixture of 4 proteins: ubiquinol-cytochrome c reductase core protein 1, translation initiation factor eIF4A II, heat shock protein 90, and nuclear RNA helicase (DEAD family) homolog. This spot must be re-investigated by 2D electrophoresis (using IPG strips with a smaller pH gradient) before the protein which is absent from the MS CSF γδ T cell protein profile can be determined.

The results of the 2 dimensional electrophoresis work have demonstrated that there are differences in the protein expression of γδ T cells isolated from PB and those derived from CSF, particularly with respect to MS. This suggests that the γδ T cells present in the CSF of MS patients are not PB cells that have simply "wandered" through the blood-brain-barrier and been removed with the CSF, but rather that the CSF derived γδ T cells are actually a subset of cells functioning within the BBB. The differences in protein expression may be due to: (1) activation of the CSF derived cells by an antigen(s) present within the CNS, (2) regulation of the γδ T cells by other cells or their secreted mediators (such as cytokines or chemokines) or neurological molecules present in the CNS, (3) a fundamental difference (genetic mutation) between the cells which leads to their separation and/or dissimilar protein expression patterns, (4) an unknown mechanism.

4.6 Implications for Multiple Sclerosis Pathogenesis

In Chapter One I hypothesized that γδ T cells were instrumental in the initiation and/or perpetuation of injury to myelin, oligodendrocytes, and axons observed in MS brain. This hypothesis was based, in part, on the observations that γδ T cells accumulate in MS lesions (47,344-346) and inflict damage upon oligodendrocytes *in vitro* (349,350). My work has primarily focussed on gathering information regarding the regulation and mechanisms of γδ T cell mediated cytotoxicity, such information is crucial to the development of therapies aimed at treating or preventing the damage observed in MS.

It had been reported that the actions of γδ T cells, including cytotoxicity, were controlled via inhibitory natural killer cell receptors and could be modulated *in vitro* by

manipulating the iNKR/HLA class I molecule interaction (138,142,161,174,244). Since oligodendrocytes express HLA class I molecules *in vitro* (58,59), it was plausible that the cytolysis of oligodendrocytes mediated by γδ T cells was controlled via iNKR, and could be abrogated by treatments designed to modulate iNKR stimulation/signalling. My experiments, aimed at manipulating γδ T cell mediated cytotoxicity through the use of anti-iNKR mAbs and IFN-induced up-regulation of HLA class I expression, have demonstrated that the control of γδ T cell mediated cytotoxicity is much more complex than originally thought. The interaction between iNKR and HLA class I molecules is only part of the story, it is the balance of the activating and inhibitory signals the cell receives that determines its response (243,358-363). Of course, more work is required before therapies to modulate γδ T cell cytotoxicity via iNKR, or other natural killer cell receptors, can be successfully developed.

The neutralization of cytotoxic mediators released by γδ T cells may also prove to be a useful treatment for MS. Of course, this requires in-depth knowledge of the cytotoxic mechanisms utilized by γδ T cells and the cytotoxic susceptibilities of oligodendrocytes. A number of studies of γδ T cell mediated cytotoxicity have been published, however, none of these studies was able to discuss the cytotoxic mechanisms in any great detail (97,136,141,332,333,336-340). I have demonstrated that γδ T cells express perforin, granzymes A, B, and M, granulysin, and FasL and are capable of using combinations of perforin, granzymes A and B, and FasL to kill target cells. [I was not able to demonstrate a cytotoxic role for either granzyme M or granulysin. These molecules require further study as discussed in a preceding section]. Oligodendrocytes are susceptible to Fas and perforin mediated injury, indicating that perhaps both pathways

are involved in the cytotoxicity of these cells (57,74,339,395-397). Although this is a good start in the identification of cytotoxic molecules which may make appropriate targets for MS therapies, further work with oligodendrocyte target cells is required to determine exactly how γδ T cells are responsible for their demise.

Another aim of my project was to investigate whether certain molecular interactions and cytotoxic mechanisms were specific to MS or to the PB or CSF compartment. I have already noted that differences were observed between PB and CSF derived cells with respect to iNKR phenotype, cytotoxic potential, and concentration of perforin, granzyme A, and granulysin. The results compiled in Tables 3-4 to 3-6 also suggest that the cytotoxic mechanisms utilized by γδ T cell lines are specific to the compartment from which the lines are derived or the disease state of the donor. However, it is the results of the protein analyses that demonstrate the most significant difference in γδ T cells derived from PB or CSF (and also between MS, OND, and HC). Table 3-16 lists the 7 spots present in the protein profiles of MS PB and OND CSF samples and absent from MS CSF samples. These 7 spots represent a disease and compartment specific profile. Two of these spots were determined to be a mixture of proteins and require further processing and analysis (spots 4603 and 8504). Of the remaining spots, 3 were identified as proteins involved in migration and mobility of the cells (1209, 1802, and 6502). The absence of one or all of these proteins would be expected to significantly impair the migration of the cells. A better understanding of the exact roles played by these proteins would aid in the quest to understand how and why MS CSF γδ T cells enter and remain within the CNS. The last two proteins were identified as pyruvate kinase and otubain 1. Pyruvate kinase deficiency leads to

abnormal levels of ATP and a build-up of glycolytic intermediates. The absence of otubain 1 disrupts protein turnover and various signal transduction pathways. Each of these proteins is important in a number of processes and it is difficult to speculate as to how their absence could and would affect the γδ T cell and even more difficult to predict the role these absences may play in the development of MS. Certainly, more work is required before the importance of each of these proteins to MS pathogenesis can be fully understood.

4.7 Summary

The work described in this thesis was undertaken to gain a more detailed understanding of the regulation and mechanisms of γδ T cell mediated cytotoxicity, and to search for disease and compartment specific functional and phenotypic properties of γδ T cells. Results have been presented which dispute the theory that the cytotoxicity attributed to γδ T cells is controlled solely via the interaction of inhibitory natural killer cell receptors and HLA class I molecules. Instead the cells are influenced by the signals transduced by a number of inhibitory and activating receptors. It is the balance of these signals that ultimately determines the response of the γδ T cell to the stimulus.

Cytotoxic γδ T cells possess all of the cytotoxic molecules necessary for a perforin/granzyme or a Fas/FasL mediated attack, and indeed use both of these pathways, often simultaneously. The choice of pathway is influenced by the target cell, as the percent secretion of serine esterases varies with the target cell encountered.

γδ T cell lines derived from PB and CSF samples display differences in iNKR phenotypes, cytotoxic potential, concentrations of stored cytotoxic molecules, and

cytotoxic mechanisms. In addition, protein expression differs not only between PB and CSF derived cells, but also between HC, MS, and OND specimens. This is the first report of 2 dimensional analyses of γδ T cells and of an MS CSF specific protein expression profile. The absence of 7 proteins from the MS CSF samples, represents a disease and compartment specific protein profile. Although the significance of these proteins to γδ T cells, and indeed to MS, requires further analysis, I would suggest that their absence impairs the mobility and migration of these cells and disrupts a number of signalling pathways.

This work represents a significant contribution to the understanding of γδ T cell mediated cytotoxicity (regulation and mechanisms), and has illustrated that the γδ T cells present in the CSF of patients with MS are specific to the MS disease state and to the CSF compartment.

4.8 Future Work

The results of the JAM assays shown in Tables 3-4 to 3-6 suggested that the cytotoxic mechanisms differed between γδ T cells derived from PB versus CSF. It would be interesting to continue these assays with a larger sample size to see if this observation is statistically significant.

This first attempt to analyse the protein expression by γδ T cells derived from PB and CSF (and MS, HC, and OND) has generated some intriguing results, however, a greater number of samples needs to be analyzed to ensure their validity. It would also be advantageous to repeat the 2 dimensional analyses with protein samples isolated via another extraction methodology that allows the isolation of transmembrane proteins.

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Contribution of Collaborators

This thesis represents original work performed solely by Melanie L.D. Green, with the exception of the mass spectrometry analysis which was carried out by Dr. John Kelly at the National Research Council of Canada, Ottawa, Ontario.

Green M. and Freedman MS. (2001) γδ T cell mediated cytotoxicity in multiple sclerosis (MS): comparison of cytotoxic mechanisms. At the International Society of Neuroimmunology, VIth International Congress, Edinburgh, Scotland.

Matusevicius D. Green MLD. and Freedman MS. (2001) IFN-β (Avonex) treatment modulates cytokine profile produced by γδ T cells. At the 17th congress of the European Committee for Treatment and Research in Multiple Sclerosis, Dublin, Ireland.

Green MLD. Matusevicius D. and Freedman MS. (2001) IFN-β (Avonex) treatment alters the expression of killer inhibitory receptors (KIR) on MS γδ T cells. At the 17th congress of the European Committee for Treatment and Research in Multiple Sclerosis, Dublin, Ireland

Awards and Distinctions

1991	Governor General Award for Scholastic Excellence
1991-1992 and 1992-1993	Dr. Leslie Harris Memorial University of NF Alumni Scholarship
1991-1992	Canada Scholarships Program
1994-1995	Faculty of Science Dean's List, Memorial University of Newfoundland
1996-1997 1997-1998 and 1998-1999	School of Graduate Studies and Office of Research and Graduate Studies (Faculty of Medicine) Graduate Student Fellowship
1999-2000 to 2003-2004	MRC level Research Studentship from the Multiple Sclerosis Society of Canada. Annual competition.
1999-2000	University of Ottawa Admission Scholarship
1999-2000 to 2003-2004	University of Ottawa Excellence Scholarship. Renewed annually.
2001	University of Ottawa, Faculty of Graduate and Postdoctoral Studies, 3rd and 4th Year Doctoral Research Scholarship.