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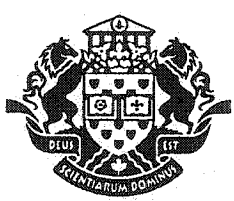

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FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

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Identification of Trail/APO2L as a Novel Therapy Targeting HIV Reservoirs and the Development of Antiapoptotic Peptides for Protection from HIV

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IDENTIFICATION OF TRAIL/APO2L AS A NOVEL THERAPY TARGETING HIV RESERVOIRS AND THE DEVELOPMENT OF ANTIAPOPTOTIC PEPTIDES FOR PROTECTION FROM HIV INDUCED CELL DEATH.

A Thesis Submitted to the
School of Graduate Studies and Research
University of Ottawa

In Partial Fulfillment for the Degree of
Doctor of Philosophy
Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine

By
Julian J. Lum

ABSTRACT

Apoptosis of CD4 T cells is the major immunological feature of HIV-1 disease. Despite the profound benefits of protease inhibitor based therapy, complete and absolute eradication of HIV is elusive. The main reason is the development of viral reservoirs including HIV infected macrophages and latently infected resting memory CD4 T cells which are refractory to all current HIV drugs. With concerns of toxicity and drug resistance, novel strategies specifically directed at reservoirs are clearly required. Dysregulation of death inducing ligands is a common characteristic of HIV infection. Here, we demonstrate altered regulation of TRAIL/Apo2L and its receptors in cells from infected patients. Moreover, *in vitro* treatment of latently infected resting memory cells with TRAIL/Apo2L significantly reduces the viral reservoir burden, in some cases to undetectable levels. This indicates that TRAIL/Apo2L possesses anti-HIV activity and implies a novel therapeutic strategy for elimination of HIV. Activation of natural killer cells with IL-15 induces a substantial increase in TRAIL/Apo2L which results in enhanced TRAIL/Apo2L specific lysis and killing of cellular reservoirs of HIV. We also suggest a possible mechanism whereby cells resistant to TRAIL/Apo2L killing acquire a sensitive phenotype, through a direct interaction with the chemokine receptor CXCR4. Finally, we show that TRAIL/Apo2L may be a regulator of inflammatory responses.

HIV-1 Vpr is an important accessory gene which contributes to the destruction of CD4 T cells. Analysis of long-term asymptomatic patients found a high frequency of a specific polymorphism within a region responsible for activating mitochondria death signals. This mutation, R77Q had a diminished ability to mediate death of T cells when compared to the wildtype virus suggesting that other mutations may possess dominant negative activity. In this case, we generated a peptide termed DN2 that blocked apoptosis irrespective of HIV both *in vitro* and *in vivo*. The generalized ability of this peptide to inhibit apoptosis suggests it may prove useful for diseases where there is evidence of excessive apoptosis.

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Finally, to my grandfather who passed away before witnessing the completion of this work – he would be proud.

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LIST OF ABBREVIATIONS

$\Delta\psi_{tm}$ – loss of mitochondrial transmembrane potential
AICD – activation induced cell death
AIDS – acquired immunodeficiency syndrome
AIF – apoptosis inducing factor
ANT – adenine nucleotide translocator
Apaf-1 – apoptotic protease activating factor 1
ART – antiretroviral therapy
CMV – cytomegalovirus
CPT - Camptothecin
CTL – Cytotoxic T lymphocytes
DN – dominant negative
EAE – experimental autoimmune encephalomyelitis
FADD – Fas Associated Death Domain
FLIP – FLICE inhibitor protein
GM-CSF – granulocyte macrophage colony stimulating factor
HIV – human immunodeficiency virus
Hsp70 – heat shock protein 70
Hu-PBL-SCID – human peripheral blood lymphocyte severe combined immunodeficient
IFNγ - interferon gamma
IL – interleukin
IUPM – infectious units per million
LPS – Lipopolysaccharide
LTNP – long term non progressor
LTR – long transcription repeats
LZhuTRAIL – Leucine zipper human TRAIL
M.O.I – multiplicity of infection
mAbs – monoclonal antibodies
MCF – mean channel fluorescence
MDM – monocyte derived macrophages
$NF_{\kappa}B$ – nuclear factor kappa B
NK – natural killer
PBL – peripheral blood lymphocytes
PBMC – peripheral blood mononuclear cells
PMA – phorbol myristyl acetate
PTPC – permeability transition pore complex
ROS – reactive oxygen species
TNF – Tumor Necrosis Factor
TRADD – TNF-R1 Associated Death Domain
TRAIL – Tumor Necrosis Factor Related Apoptosis Inducing Ligand
TSA – TRAIL specific Apoptosis
TUNEL – terminal deoxyuridine nucleotide end labelling
Vpr – viral protein R

Section 1:
Introduction

1.1 General Introduction: Apoptosis and HIV Infection

A central feature to HIV infection is an excessive degeneration of CD4 T cells that ultimately leads to the collapse of immune function, defence and susceptibility to opportunistic infections. Several lines of investigation now support the concept that central to the event of HIV infected and uninfected CD4 T cell depletion is the process of inappropriate induction of apoptosis (reviewed in Badley et al., 2000; Lum et al., 2001). However there is widespread divergence as to the precise pathway(s) leading to HIV induced cell death. Although apoptosis in HIV disease may be the result of chronic immunological activation, there are distinct means by which HIV (and/or HIV specific proteins) may enhance apoptosis (reviewed in Badley et al., 2000; Lum et al., 2001). Additionally, only a small fraction of physically infected lymphocytes undergo apoptosis indicating that apoptosis of lymphocytes from infected patients results from mechanisms other than direct infection (reviewed in Badley et al., 2000).

New insights on the physiological cell death program in mammalian cells has further confounded the central issue of T lymphocyte destruction during HIV infection. A disparity exists between apoptosis observed in cell culture systems and in *in vivo* models (Cavert et al., 1997; Haase, 1999; Perelson et al., 1996). Although infected and uninfected cells die following infection, recent evidence indicates that infected and uninfected cells may have unique pathways controlling death (Bolton et al., 2002; Lenardo et al., 2002). Two widely accepted models for apoptosis have been described,

namely the intrinsic and the extrinsic apoptotic pathway. In the former case, ligation of tumor necrosis factor (TNF) family of death ligands to their receptors causes oligomerization of the death receptors and recruitment of adaptor proteins typically involving caspase 8 activation (Fig 1 and Baumann et al., 2002; Peter et al., 1999; Sartorius et al., 2001; Schmitz et al., 2000; Schulze-Osthoff et al., 1998). In the latter model, apoptotic signals cause mitochondrial activation and subsequent release of cytochrome c, a key commitment step for caspase 9 activation through the formation of a multiprotein complex containing Apaf-1, cytochrome c, dATP and procaspase 9 (Fig 1 and (Budihardjo et al., 1999; Cardone et al., 1998; Green and Reed, 1998; Luo et al., 1998; Waterhouse et al., 2001; Waterhouse et al., 2002; Zimmermann et al., 2001). Expression of HIV proteins perturb these pathways resulting in enhanced levels of apoptosis. Although HIV infections results in T cell apoptosis, under some circumstances a small fraction of CD4 T cells and macrophages do not die following infection indicating this to be a critical step in the development of viral reservoirs (Chun et al., 1997b; Finzi et al., 1997). The establishment of these stable, antiretroviral therapy resistant cells represents the major obstacle in achieving complete sterile cure. However, this provides a unique opportunity to further understand the regulation of apoptosis and may facilitate development of novel immune based therapies aimed at modifying apoptosis in HIV disease.

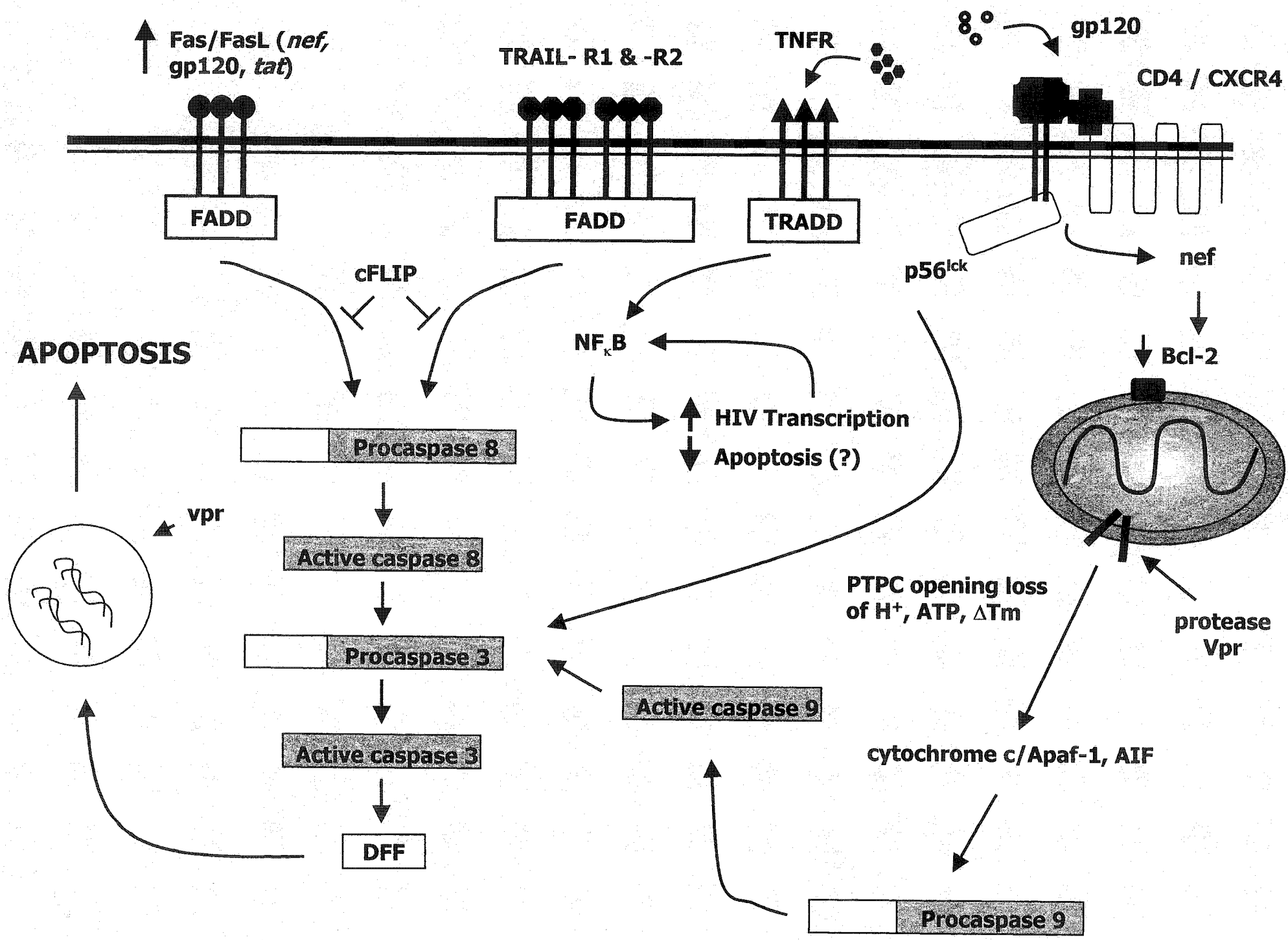
1.2 Evidence for Depletion of CD4 T Cells

In vitro infection of peripheral blood mononuclear cells (PBMCs) results in significant levels of apoptosis (Badley et al., 2000). In addition, accelerated lymphocyte apoptosis can be seen in cells obtained from HIV infected patients. However, comparison of the number of cells which undergo apoptosis during acute HIV infection in rapid progressors and long term non progressors (LTNPs) show a higher percentage of cell death in the former group of individuals (Badley et al., 1997). The lack of immune dysfunction and apoptosis of CD4 T cells in LTNPs are of particular interest because these individuals are not susceptible to the pathogenic effects of HIV infection. These asymptomatic individuals maintain high CD4 T cell counts and have low viral burdens without any anti-HIV intervention (Barker et al., 1998; Cohen et al., 1997a; Klein and Miedema, 1995). Unlike their LTNP counterparts, rapid progressors develop high viral load and CD4 T cell numbers are considerably lower (Barker et al., 1998; Cohen et al., 1997a; Klein and Miedema, 1995). Moreover, *ex vivo* cultures of cells from HIV infected individuals result in high levels of spontaneous apoptosis compared to uninfected controls (Badley et al., 1997), altogether suggesting a role for apoptosis in CD4 T cell depletion.

Further insight on the positive correlation of apoptosis during HIV infection can be obtained from several of the current animal models. In the best studied model, chimpanzees infected with HIV remain asymptomatic despite continuous viral burden and active viral replication in both lymphoid tissue and peripheral blood (Watanabe et al.,

1991). In this case, CD4 T cell death is virtually undetectable. However, simian immunodeficiency virus (SIV) infected macaques, cats infected with feline immunodeficiency virus and baboons infected with HIV-2 all develop disease profiles that closely approximate human HIV, whereby CD4 T cells undergo apoptotic cell death (Desrosiers, 1990a; Desrosiers, 1990b; Kestler et al., 1990). To date, there are no cases or animal models of lentivirus infection which exist that does not display CD4 T cell loss in the absence of immunodeficiency (Badley et al., 1997). In addition, only human T cells become activated following incubation with HIV proteins. These activated cells become primed to apoptosis following a second antigen or T cell receptor stimulus. In the most recent model, severe combined immunodeficiency (SCID) mouse, mice reconstituted with human PBLs followed by productive HIV infection results in preferential CD4 T cell depletion (Mosier et al., 1991). These collective animal studies correlating pathogenic lentivirus infection to induction of abnormal apoptosis are consistent with observations seen in human HIV infection, where circulating CD4 T cells have increased susceptibility to apoptosis compared to CD4 T cells in healthy uninfected individuals (Finkel et al., 1995; Muro-Cacho et al., 1995). Based on these findings, it was proposed that (a) HIV induced immunedeficiency and AIDS is associated with enhanced spontaneous apoptosis and (b) that apoptosis contributes to a major role in depletion of both infected and uninfected CD4 T cells.

Figure 1. Effects of HIV and HIV Specific Proteins on Apoptosis Regulatory Molecules. Ligation of death receptors through cognate ligands induces death receptor trimerization and recruitment of adapter proteins, FADD or TRADD. Both death receptor and membrane bound death ligand expression are increased by nef, gp120 and tat. Through the death effector domains of the adapter proteins, the inactive procaspase enzyme is recruited to the membrane leading to its autoactivation and cleavage of downstream effector caspases. This step can be inhibited by the cellular protein cFLIP. FLIP also contains a homologous death effector domain that can competitively prevent association of caspase 8 to FADD or TRADD. In some instances for example, TNFR engagement results in activation of the NFκB pathway which can led to upregulation of HIV transcription or protection from apoptosis. In addition to death receptors, engagement of CD4 and/or the chemokine coreceptor CXCR4 by gp120 causes activation of p56lck which in turn activates caspase-3. It has also been shown that nef can bind to p56lck and cause its activation. Nef also leads to a decrease in Bcl-2 levels and decreases the susceptibility of infected cells to death through the mitochondria.


Fas/FasL (*nef*, gp120, *tat*)
TRAIL- R1 & -R2
TNFR
gp120
CD4 / CXCR4
FADD
FADD
TRADD
p56lck
nef
cFLIP
NFκB
Bcl-2
HIV Transcription
Apoptosis (?)
APOPTOSIS
Procaspase 8
vpr
Active caspase 8
Procaspase 3
Active caspase 3
DFF
PTPC opening loss of H+, ATP, ΔTm
protease Vpr
Active caspase 9
cytochrome c/Apaf-1, AIF
Procaspase 9

1.3 Mechanisms of HIV Induced Apoptosis

Apoptosis is classically characterized by plasma membrane blebbing, nuclear condensation, DNA fragmentation and the formation of dense apoptotic bodies. Physiological apoptosis can occur through several different pathways. There are four well characterized cellular death receptors which upon ligation initiate receptor mediated apoptosis: Fas receptor, p55 TNF (Tumor Necrosis Factor) receptor and TRAIL/Apo2L (TNF related apoptosis inducing ligand) receptors 1 and 2 (Krammer, 2000; Medema et al., 1997; Peter, 2000; Peter et al., 1999; Schulze-Osthoff et al., 1998). Binding of these death receptors recruits the proteins FADD (Fas associated death domain) and TRADD (TNF receptor associated death domain) followed by sequential activation of cysteine proteases that function to cleave at aspartate residues (Krammer, 2000; Medema et al., 1997; Peter, 2000; Peter et al., 1999; Schulze-Osthoff et al., 1998). These proteins are collectively referred to as caspases (cysteine dependent aspartate specific proteases) which remain as inactive zymogen precursors and only become activated after proteolytic processing of the terminal prodomain (Krammer, 2000; Medema et al., 1997; Peter, 2000; Peter et al., 1999; Schulze-Osthoff et al., 1998). Upon activation, caspases can function to cleave other caspases or a variety of cellular proteases and endonucleases that degrade host DNA and structural proteins that are characteristic to apoptosis (Fig 1)

More recently, considerable attention has been focused at understanding the involvement of mitochondrial associated changes during apoptosis. In this model, disruption of mitochondrial function may result in opening of mitochondrial permeability

transition pore complexes (PTPCs) that leads to dissipation of mitochondrial transmembrane potential ($\Delta\psi_{Tm}$), loss of H^+ gradient, uncoupling of the electron transport chain (ETC) and oxidative phosphorylation collapse (reviewed in (Badley et al., 2000)). These changes are accompanied by the release of apoptosis regulating proteins (including cytochrome c and AIF) that further initiate downstream caspases (Daugas et al., 2000; Joza et al., 2001; Lorenzo et al., 1999; Susin et al., 1999). Bcl-2 can regulate this mitochondrial activity and may modulate other forms of apoptosis (Fig 1).

1.4 Contribution of HIV proteins to Direct and Indirect CD4 T Cell Death.

Binding of HIV envelope protein gp120 shed from infected cells or virions to the CD4 receptor has been shown to cause increased susceptibility to Fas mediated killing, down regulation of Bcl-2 expression and activation of caspase 3 (Accornero et al., 1997; Banda et al., 1992; Berndt et al., 1998; Groux et al., 1992; Laurent-Crawford et al., 1993). This effect can be inhibited through the use of soluble CD4 and anti-gp120 antibodies as well as mutations in the intracytoplasmic domains of CD4 that are important for signalling (Accornero et al., 1997; Banda et al., 1992; Berndt et al., 1998; Groux et al., 1992; Laurent-Crawford et al., 1993). Further, cross linking of CD4 and CXCR4 by gp120 results in an apoptotic death within several hours of stimulation that appears to be independent of $p56^{lck}$, G-protein coupled signalling, Fas or TNF receptors (see Fig 1 and Berndt et al., 1998). Because binding of gp120, soluble gp120 or immunocomplexed gp120 to CD4 and CXCR4 can occur without infection, these observed apoptotic effects may explain an indirect mechanism of uninfected CD4 T cell loss.

Several HIV proteins have demonstrated the ability to induce apoptosis *in vitro*. Ectopic expression of HIV Tat in uninfected cells causes a caspase 8 and FasL dependent apoptosis implicating that it is a potential mediator of CD4 T cell death (Bonavia et al., 2001; Ferri et al., 2000; Li-Weber et al., 2000; Park et al., 2001; Roshal et al., 2001; Selliah and Finkel, 2001). In humans with naturally occuring Nef deletions, CD4 T cell depletion is sharply less compared to strains with wildtype nef (Azad, 2000). Moreover,

Nef expressing T cells coexpress FasL and can synergistically activate the T cell receptor leading to increased sensitivity to apoptosis (Azad, 2000). Although the precise mechanism of Nef induced apoptosis is unknown, mutational analysis indicates that CD4 associates with both Nef and $p56^{lck}$ (Curtain et al., 1998; Curtain et al., 1994)

Of the numerous HIV gene products which influence viral pathogenesis, HIV-1 Vpr is a 96 amino acid accessory protein that is dispensable for viral replication in CD4 T cells lines but is required for infection of primary macrophages (Yao et al., 1998). Vpr assists nuclear localization of viral preintegration complexes and blocks activation of $p34^{cdc2}$ cyclin B complex resulting in G_2/M cell cycle arrest (Chen et al., 1999; Goh et al., 1998; He et al., 1995; Jowett et al., 1995; Shostak et al., 1999; Stewart et al., 1997; Zhou and Ratner, 2000). Both extracellular soluble Vpr as well as antibodies to Vpr are present in serum and cerebrospinal fluid from HIV infected patients (Levy et al., 1994; Levy et al., 1995; Patel et al., 2000; Piller et al., 1998). Soluble Vpr is cell permeable (Ferri et al., 2000; Jacotot et al., 2001; Vieira et al., 2000) and induces caspase dependent apoptosis in CD4 T cell lines (Stewart et al., 1997; Stewart et al., 2000), neurons (Patel et al., 2000), hepatocytes, fibroblasts and primary PBLs, a function which localizes to a c-terminal $(H(F/S)RIG)_2$ domain (Ferri et al., 2000; Jacotot et al., 2001; Vieira et al., 2000). Vpr has been postulated to play a causal role in depleting both HIV infected and uninfected CD4 T cells from infected patients. However, Vpr is capable of upregulating HIV LTR transcription (Ayyavoo et al., 1997), cellular activation and differentiation, suggesting a dual role as an apoptotic and anti-apoptotic protein (Conti et al., 2000). Vpr also induces apoptosis by binding to the adenine nucleotide translocator (ANT)

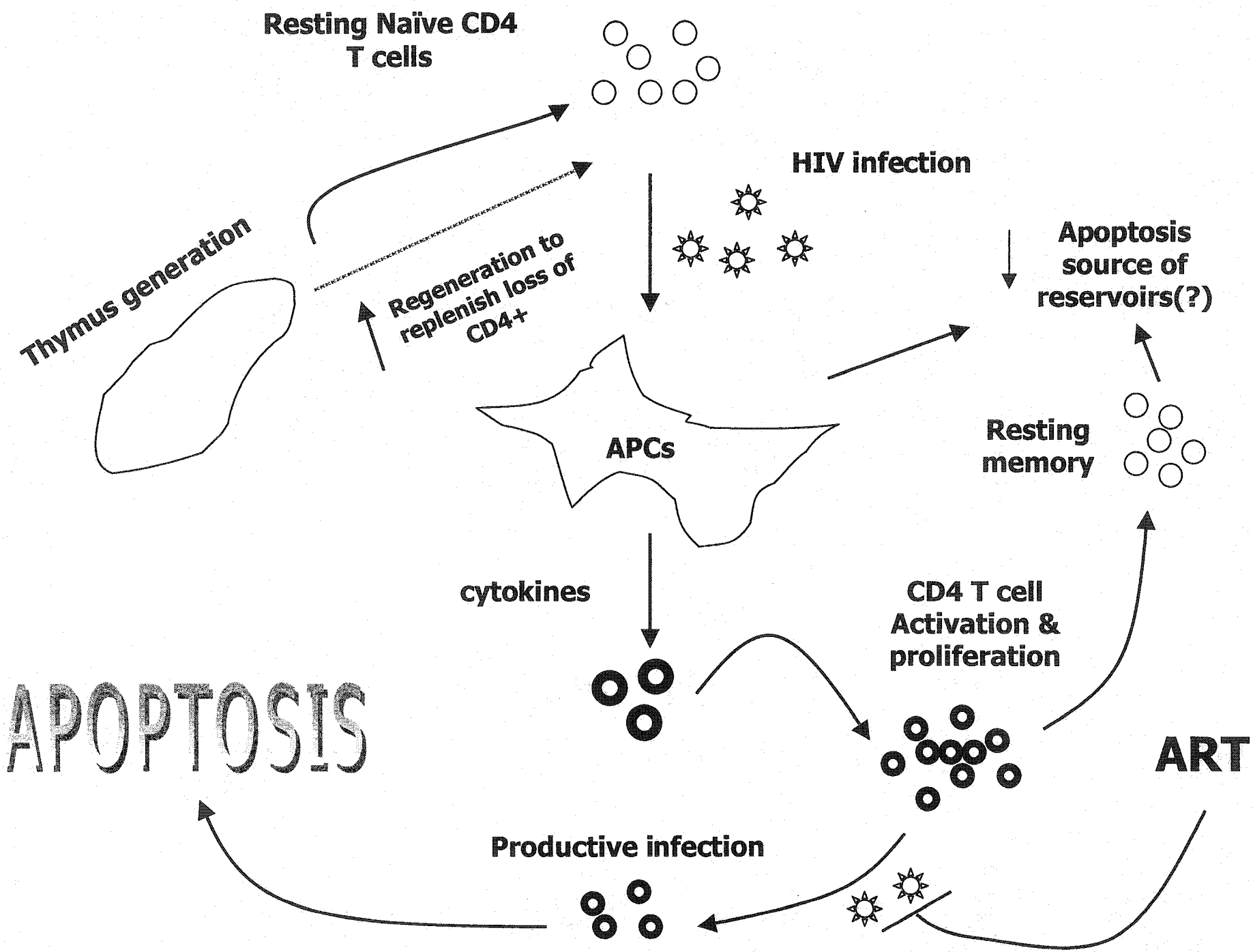
component of the PTPC, causing $\Delta_{\psi Tm}$, release of cytochrome c and AIF, uncoupling of the electron transport chain and oxidative phosphorylation collapse (Ferri et al., 2000; Jacotot et al., 2001; Vieira et al., 2000). The interaction of Vpr with ANT is specific, with an affinity in the nanomolar range, and is inhibited by Bcl-2.

TNF and the TNF receptor family of molecules display altered regulation during HIV infection. In contrast to asymptomatic patients, symptomatic HIV infected patients have elevated levels of serum TNF, increased lymphocyte production of TNF and increased TNF activity which in turn promotes activation of NFκB and HIV transcription (Chollet-Martin et al., 1994; Zangerle et al., 1994). This results in a TNF autocrine loop of increased TNF production and increased HIV transcription (Han et al., 1996; Osborn et al., 1989). Although the precise mechanism by which TNF induces apoptosis in HIV infected cells is not clear, killing of uninfected T cells by macrophages infected with HIV can be incompletely abrogated by soluble TNF receptor decoys (Srivastava et al., 1999). Evidence for HIV induced Fas/FasL apoptosis has been supported by the demonstration that soluble Fas receptor decoys block HIV cell death in the monocytic cell line U937 (Badley et al., 1996). In contrast, direct T cell apoptosis during acute HIV infection is independent of Fas/FasL interaction despite increased Fas expression (Badley et al., 1996).

1.5 HIV Reservoirs: Latently Infected Memory Cells and Macrophages

Although the use of antiretroviral therapy (ART) has been successful in reducing the mortality and morbidity of infected patients (Detels et al., 1998; Gulick et al., 1997) absolute viral eradiation has been elusive despite long term use of potent ART regimes. Recent evidence demonstrate that the main obstacle in the treatment of HIV are persistent drug resistant viral reservoirs (Chun and Fauci, 1999; Cooper and Emery, 1999; Pierson et al., 2000), including latently infected resting CD4 T cells capable of producing infectious HIV upon stimulation *in vitro*. While other potential cellular sites of persistent HIV exist (Pierson et al., 2000), the major reservoirs consist of latently infected resting memory CD4 T cells which carry integrated viral DNA (Pierson et al., 2000). These cells have a natural half life of greater than 44 months giving an estimate of 60 years to achieve complete eradication (Pierson et al., 2000), provided that complete viral suppression is maintained. The existence of these stably infected resting CD4 T cells can be considered in the context of normal establishment of physiological memory cells. Under normal circumstances naïve thymic T cells encounter antigen leading to blast transformation and activation of the cell (Fig 2 and Douek et al., 1998). This results in upregulation of activation markers and expression of cytokines required for survival. When the antigen is removed (i.e. clearance of pathogen), most cells undergo activation induced cell death, while a small number lose activation markers, exit the cell cycle and revert to a resting state with phenotypes of typical memory T cells (HLA-DR^{low}, $CD45RO^{high}$, $CD62L^{low}$) (Crabtree, 1989; Lenardo et al., 1999). These cells are capable of responding to subsequent exposure by the initiating antigen. Although HIV-1 cannot

Figure 2. Model for the Development of Reservoirs for HIV. Resting naïve CD4 T cells emerge from the thymus and enter the periphery where they may become infected by circulating plasma virus. Antigen presentating cells (APCs) are normally considered the first major target of HIV and later become the source of viral replication. APCs are highly resistant to the cytopathic effects of viral infection and may become a reservoir site. Secretion of cytokines and direct ligation of activating ligands/receptors by APCs activate naïve T cells to proliferate. Infection of T cells is most efficient following activation and the majority of infected T cells undergo apoptosis. A small population of infected cells escape death and revert to a resting memory state carrying integrated viral DNA, whereby they become the principal reservoir for HIV.


Resting Naïve CD4
T cells
HIV infection
Thymus generation
Regeneration to
replenish loss of
CD4+
Apoptosis
source of
reservoirs(?)
APCs
Resting
memory
cytokines
CD4 T cell
Activation &
proliferation
APOPTOSIS
ART
Productive infection

replicate in non-activated CD4 T cells, establishment of a state of latency in resting cells can occur through two ways, pre-integration and post-integration latency. In the first model, CD4 resting cells infected with HIV harbor non integrated viral DNA (Pierson et al., 2000). Earlier studies demonstrate that activation of an infected resting cell prior to integration leads to the production of replication competent viral particles (Bukrinsky et al., 1991; Zack et al., 1990). In the second model, latency is achieved following integration of HIV proviral DNA into the cellular genome (Chun et al., 1997a; Chun et al., 1995; Folks et al., 1989; Pomerantz et al., 1992; Pomerantz et al., 1990; Seshamma et al., 1992). However, post integration latency only occurs when an activated T cell carrying HIV DNA returns back to a resting state where viral transcription is absent. Thus, it has been postulated that post integrated latent CD4 cells arise when infection occurs in activated cells which are in the process of transitioning back to resting memory state or when antigen (i.e. plasma HIV) is removed during effective anti-HIV therapy. The importance of this reservoir is highlighted by three fundamental key points. First, viral transcription within these cells is absent to minimal, making the cells resistant to the cytopathic effects of the virus (Winslow and Trono, 1993). Second, the half life of memory CD4 T cells is estimated to be 5-6 months. However, this may be an underestimate as memory cells survive greater than 20 years as a consequence of intermittent cell divisions through cross reacting antigen or cytokines (Antia et al., 1998). This feature of long term survival is an essential component for immunization against pathogens. Several studies have established a link whereby patients who are on intensified ART do not have appreciable decreases in the size of the latent reservoirs because of ongoing but extremely low viral replication. This low level of viral

replication or 'blips' provided a new mechanism for reseeding the latent pool, in effect nullifying the positive affects of intensified ART. Therefore, the capacity of HIV to exploit these properties renders long term persistence of HIV. In addition to the extremely slow decay rate, cumulative toxicity of ART (Flexner, 1998) make eradication now improbable.

Cells of the myeloid lineage are susceptible to HIV infection, perhaps more importantly in mucosal transmission (Goulston et al., 1998; Mostad and Kreiss, 1996). Interestingly, during the progression of HIV, macrophages serve as a source of viral replication and are highly resistant to the cytopathic effects of the virus, thus macrophages have been implicated as another possible reservoir (reviewed in Ho et al., 1986).

1.6 Rationale and Statement of Objectives

The primary objective of the thesis is to further understand the mechanisms of apoptosis during HIV infection with the ultimate aim of developing novel therapies for the treatment of HIV in particular, targeting HIV reservoirs. Six sections form the basis of the current thesis. Given the recent data demonstrating the existence of stable HIV reservoirs the aims of this thesis are:

1) To determine whether TRAIL can eliminate HIV from resting memory CD4 T cells and HIV infected macrophages.

This would directly address one of the most pressing issues in the treatment of HIV – elimination of persistent reservoirs. These experiments will be performed *in vitro* using established protocols for the isolation of latently infected resting memory CD4 T cells (Chun et al., 1998; Chun et al., 1997b; Finzi et al., 1997; Lum et al., 2001) and infected macrophages. However, due to the current limitations of an appropriate and available animal model for HIV, the absence of *in vivo* trials using TRAIL will make the results difficult to interpret from a clinical perspective. The majority of *in vitro* and *in vivo* toxicity data has already been extensively examined through experiments evaluating the potential use of TRAIL as a therapy for selected human cancers (Ashkenazi et al., 1999; Griffith et al., 1998; Griffith and Lynch, 1998; Griffith et al., 1999b; Lawrence et al., 2001; Walczak et al., 1999). Both Genentech and Amgen (formerly Immunex

Corporation) share intellectual and commercial property rights to TRAIL, therefore these experiments were conducted with the collaboration of Dr. David H. Lynch (Amgen).

2) To evaluate approaches to enhance TRAIL expression using IL-15 stimulated human NK cells.

There is little information on ways to deliver TRAIL *in vivo* without the need for exogenous treatment using recombinant proteins. Recent work using NK cells from mice demonstrate a substantial enhancement of TRAIL specific NK cytolytic activity following stimulation with IL-15 (Cavazzana-Calvo et al., 1996; Kashii et al., 1999; Zamai et al., 1998). Interestingly, IL-15 possesses antiapoptotic activity and is currently under investigation as a potential adjuvant for HIV treatment (Bulfone-Paus et al., 1997; Chehimi et al., 1997; d'Ettorre et al., 2002; Mastroianni et al., 2000; Perera et al., 1999; Waldmann et al., 2001). Currently, few studies have examined the role of IL-15 on human NK cell activity (Cavazzana-Calvo et al., 1996). Therefore, this section evaluated the effect of IL-15 on TRAIL and TRAIL receptor function and determine if human NK cells treated with IL-15 can be used as a vehicle for TRAIL delivery.

3) To determine the molecular determinants of TRAIL sensitivity of cells infected with HIV.

Currently, it is not known how cells acquire sensitivity to TRAIL induced apoptosis, nor has any definitive answer been provided for the physiological role of TRAIL.

Considerable data has been generated to suggest TRAIL plays a normal role in tumor surveillance (Cretney et al., 2002b; Takeda et al., 2001) through liver NK cells. However, there do not appear to be any detectable phenotypes in TRAIL and TRAIL receptor deficient mice, nor do these mice have increased frequency of spontaneous tumor development (personal communication, Dr. David H. Lynch and (Sedger et al., 2002)). Therefore, we assessed the relative contribution of HIV proteins on sensitizing cells to TRAIL mediated apoptosis.

The cumulative data acquired from these experiments will help us understand the mechanism of TRAIL regulation and to test whether IL-15 has potential therapeutic value in the treatment of HIV infection.

Although the proceeding sections of the thesis is not directly related to the work on TRAIL, these studies were performed concurrently to the work in the first sections. This work is important since it probes and sheds light on the pathogenesis of HIV infection as well as the molecular pathways HIV uses to cause T cell depletion. Furthermore, it provides information on the basic cell death biology of immune cells which is applicable to all areas of research that involve inappropriate levels of apoptosis. Finally, it adds further support that apoptosis is one of the major pathways which HIV induces death of T cells and gives the opportunity to develop novel strategies for HIV treatment.

The latter sections will use genetic approaches to further understand the mechanisms whereby HIV proteins induced cell death. The aims will include:

4) To examine whether polymorphic determinants within Vpr correlate with an impairment of Vpr to induce apoptosis.

This section deals with the intrinsic pathway of mitochondrial induced apoptosis and will provide additional insight on mechanisms by which HIV activates this cell death machinery. Vpr has been shown to induce apoptosis in various cell culture models but whether naturally occurring vpr mutations impact cell death is not known. We used genetic analysis to identify vpr polymorphisms in patients who are long term non progressors and examined whether these mutations led to impaired ability to induce death of T lymphocytes.

5) To evaluate the antiapoptotic activity of a library of Vpr transdominant negative peptides

Several groups have shown that Vpr possesses antiapoptotic properties (Conti et al., 2000; Fukumori et al., 1998; Gaynor and Chen, 2001; Sawaya et al., 2000; Zhou and Ratner, 2001). Two groups have recently identified mutations within Vpr which have dominant negative activity, but have not tested whether cell death is inhibited using any established *in vitro* or *in vivo* models of apoptosis (Sawaya et al., 2000; Zhou and Ratner, 2001). Furthermore, Jacotot *et al.*, have localized the target of Vpr to ANT (Jacotot et al.,

2000). This aim examined whether transdominant Vpr peptides can block apoptosis induced via the mitochondria.

SECTION 2:
Regulation of TRAIL/TRAIL Receptors and the use of TRAIL against HIV Reservoirs

2.1 Introduction

Peripheral blood lymphocytes (PBL) isolated from patients infected with HIV, as well as cells infected with HIV *in vitro,* exhibit alterations in the physiological mechanisms controlling T cell apoptosis (Badley et al., 2000); Laurence, 1996 #88]. Although only a minority of CD4 T cells become infected by HIV, most that are infected undergo apoptotic cell death (Herbein et al., 1998a; Herbein et al., 1998b). Furthermore, a significant number of uninfected CD4 and CD8 T cells die by apoptosis induced either by immunological activation, by the effects of HIV proteins, or by elevated levels of death inducing ligands produced by infected cells (reviewed in Badley et al., 2000). In contrast to the usual fate of HIV infected T cells, some cells do not die following direct infection. As well, in a small fraction of CD4 T cells, infection with HIV does not result in apoptosis, but in a state of latent infection that appears to be critical for the persistence of HIV infection (Finzi et al., 1997; Finzi and Silliciano, 1998).

The development of post integration latency has been postulated to be a reversion of activated HIV infected CD4 T cells to resting memory cells in which viral transcription is absent (Finzi and Silliciano, 1998) and HIV is retained as a stably integrated provirus. These infected resting memory ($CD4^+$ $CD45RO^+$ $HLADR^-$) T cells have an estimated half life of greater than six months (Finzi and Silliciano, 1998), and the unique resistance of such latently infected T cells and HIV infected macrophages to HIV

induced apoptosis may be the critical step required for the development of viral reservoirs (Finzi et al., 1997). It is the presence of latently infected CD4 T cells and HIV infected macrophages that prevents complete virus eradication using standard antiretroviral therapies. Recent attempts to eradicate HIV reservoirs using agents including IL-2, anti CD45RO immunotoxin, anti-CD3 antibody and therapeutic HIV vaccination (Chun et al., 1999; Chun and Fauci, 1999; McCoig M. Van Praag, 1999), have so far been unsuccessful and highlight the need for novel therapeutic approaches.

TNF-related apoptosis inducing ligand (TRAIL/Apo2L) is a member of the tumor necrosis factor (TNF) superfamily and was identified by sequence homology with Fas Ligand (FasL) and TNF (Ashkenazi et al., 1999). There are five cognate receptors for TRAIL/Apo2L yet only two (TRAIL-R1 and TRAIL-R2) contain death domains that trigger the apoptotic caspase cascade (Griffith and Lynch, 1998). In contrast, TRAIL-R3, TRAIL-R4 and osteoprotegerin lack functional death signaling domains (Degli-Esposti, 1999; Emery et al., 1998; Zhang et al., 1999b). TRAIL/Apo2L can induce apoptosis in tumor cells and a number of virally infected cells including CMV (Sedger et al., 1999), but it is not cytotoxic to normal cells. Despite one recent report indicating that TRAIL induces apoptosis of freshly isolated hepatocytes (Jo et al., 2000), subsequent cumulative *in vitro* and *in vivo* data clearly indicate that TRAIL/Apo2L does not induce tissue or cell injury following injection in murine and non human primate models (Ashkenazi et al., 1999; Griffith et al., 1998; Griffith and Lynch, 1998; Vidalain et al., 2000; Zhang et al., 1999b). The ability of TRAIL/Apo2L to kill transformed cells, as well as the resistance

of normal cells to TRAIL/Apo2L, has led to its preclinical evaluation as potential therapy for selected human malignancies.

There have also been reports that TRAIL may prevent cellular proliferation (Lunemann et al., 2002). These and other correlative studies show that in TRAIL deficient animals, experimentally induced autoimmunity is exascerbated indicating that TRAIL may prevent unwanted proliferation, whereas other groups demonstrate that TRAIL enhances proliferation of T cells in response to anti-CD3 stimulation (Lunemann et al., 2002; Hillard B et., 2001). In these studies, only one third of the cells treated with TRAIL were refractory to proliferation suggesting other mechanisms other than through TRAIL play a role in stimulating growth of T cells. In experiments using antibodies to block TRAIL signaling, only a partial inhibition of cellular proliferation could be achieved (Song et al., 2000). Furthermore, it is not clear what governs this inhibitory action, although one group proposed that TRAIL prevented cell cycle entry (Lunemann et al., 2002). Much of the discrepancy arising from these studies culminates from the different experimental systems. In some cases human cells or cell lines were used while in other primary murine cells were used. The activation stimuli also varied amongst experiments where some groups used anti-CD3/anti-CD28 while other used plate bound TRAIL. Therefore, it is clear that further studies are required to substantiate the hypothesis that TRAIL effects cellular proliferation.

The regulation of TRAIL/Apo2L and TRAIL receptors in HIV infection is undefined. Reports that TRAIL/Apo2L may contribute to HIV associated activation

induced cell death (Jeremias et al., 1998; Katsikis et al., 1997) suggests that the regulation of TRAIL and its receptors may be altered in patients with HIV infection. Furthermore it has recently been proposed that TRAIL may be involved in CD4 T cell depletion in a Hu-PBL-SCID model (Miura et al., 2001). In this study, we demonstrate altered regulation of TRAIL/Apo2L and TRAIL receptor expression in T cells infected with HIV *in vitro* as well as in T cells from HIV infected patients. Further, TRAIL/Apo2L treatment *in vitro* induces apoptosis, significantly reduces the amount of replication competent HIV and significantly decreases the levels of HIV integrated provirus in resting memory cells, in some cases to undetectable levels. Thus, TRAIL/Apo2L may offer a new therapeutic approach towards eradication of HIV in infected patients.

2.2 Materials and Methods:

Study Patients. HIV infected patients and HIV negative healthy donors were recruited from the Ottawa Hospital, General Campus following informed consent. The study protocol was reviewed and approved by Ottawa Hospital Research Ethics Board. For experiments assessing TRAIL sensitivity, a random selection of HIV patients was chosen; some on therapy and some who had suppressed levels of viral replication. For coculture experiments, only patients with suppressed levels of viral replication (<50 copies/mL) for >12 months were chosen.

***In Vitro* Jurkat HIV infection**. Jurkat T cells (ATCC) were maintained in RPMI 1640 containing 10% heat inactivated fetal bovine serum, 2mM L-Glutamine and 100U/mL each of penicillin and streptomycin. All cell culture products were purchased from Canadian Life Technology, Montreal Canada, unless otherwise stated. Cells were infected with either 100ng/mL HIV_{IIIB} (Badley et al., 1996) (NIH AIDS Research and Reference Reagent Program) or mock infected using culture supernants from uninfected Jurkat T cells, in the presence of 10ng/mL polybrene for 4 hours, washed twice in complete media and incubated at 37°C, 5.0% CO_2 in a humidified environment.

Cell Culture. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation in Ficoll-Hypaque (Pharmacia, Toronto Canada), washed once with PBS and resuspended in media containing RPMI 1640 supplemented with 10% heat inactivated human AB serum (Sigma, Grand Island New York), 2mM L-Glutamine and

100U/mL each of penicillin and streptomycin. To obtain PBLs, monocytes were depleted by adherence for 1 hour. Cells were kept at 37°C in humidified 5% CO_2 environment.

Detection of TRAIL receptor by RT-PCR and Flow Cytometry. Total mRNA was isolated using RNeasy mini prep (Qiagen, Toronto Canada) and quantified by UV spectrophotometry (Becton Dickinson, Toronto, Ontario). Complementary DNA (cDNA) synthesis was performed using Superscript RT-PCR (Canadian Life Technology, Montreal Quebec) using conditions and primers previously described for estimation of message intensity (Griffith et al., 1999b; Sedger et al., 1999). Prior to experiments, we confirmed that these conditions allow detection of the PCR product within the linear range of the assay (Griffith et al., 1999b; Sedger et al., 1999). Samples were resolved on a 1.0% agarose gel and visualized by ethidium bromide staining. Surface expression of TRAIL receptors was determined by flow cytometry analyzed using 1.0μg of mouse monoclonal antibodies to TRAIL-R1 (clone M271, IgG2a, Immunex Corporation), TRAIL-R2 (clone M412, IgG1, Immunex Corporation), TRAIL-R3 (clone M430, IgG1, Immunex Corporation) and TRAIL-R4 (clone 445, IgG1, Immunex Corporation) (Griffith et al., 1998). $1x10^6$ cells were incubated with primary mAbs in PBS/1% BSA for 1 hour on ice, washed and stained sequentially with 1:100 biotinylated goat-anti-mouse IgG1 or IgG2a (Immunotech, Toronto Canada) and then 1:500 streptavidin-PE (Pharmingen, Toronto Canada). For each sample, isotype IgG1 or IgG2a (Immunotech, Toronto Canada) matched controls were used. For detection of TRAIL receptors on macrophages, culture media was removed, followed by the addition of 10mL ice cold PBS. Macrophages were scraped from T75 flasks and 1×10^6 cells were used for

isolation of RNA or flow cytometry. RT-PCR assessment of macrophage expression of TRAIL receptors was performed as described above for T cells, and flow cytometry of TRAIL receptor expression was performed as described above with the following modification: prior to primary antibody staining, MDM were incubated in PBS plus 10% human AB serum for 30 minutes at 4°C. For macaque studies, PBMCs were obtained from Dr. Erling Rud (Health Canada) and stained using identical procedures as human samples with one modification. PBMCs from macaques were fixed (Cedarlane Products) following staining with antibodies.

LZhuTRAIL Treatment. MDM (see below), human PBL or macaque PBMCs were treated for 12 hours with LZhuTRAIL using 1μg/mL unless otherwise stated (Immunex Corp.). LZhuTRAIL is a recombinant preparation of human TRAIL that forms trimers due to an N-terminal leucine zipper motif (Walczak et al., 1999). For studies using agonistic monoclonal antibodies to induce apoptosis, 1.0μg of mAb or isotype control (Immunotech, Toronto Canada) was incubated in 24 well culture dishes in PBS for 1 hour at 4°C and washed twice with PBS before addition of cells.

Preparation of monocyte derived macrophages (MDM). PBMC′ s were isolated from healthy donors and monocytes were isolated by adherence in T-125 flasks for 2 hours in RPMI 1640 containing 10% heat inactivated human AB serum. Cells were scraped, counted and 1 x 10^6 cells were then plated on microslides (Nalge, Naperville, IL). Every 3 days, 50% of the media was changed . On day 6, cells were mock infected or infected with 100pg/mL HIV_{Bal} (NIH AIDS Reference Reagent Program), and on day 16, where

appropriate, 100ng/mL of GM-CSF (R&D Systems) was added to each culture. 1μg LZhuTRAIL was added on day 17 and apoptosis was measured 12 hours following treatment using TUNEL (Intergen, Purchase New York) as described by the manufacturers instructions. Three hundred cells from each condition were counted individually by 3 different laboratory personnel all of whom were unaware of the treatment conditions, and the average scores were used for data analysis.

Detection of Apoptosis. Apoptotic cell death was determined using Hoechst 33342 (Molecular Probes, Eugene Oregon) or TUNEL (Intergen, Purchase New York) staining. For Hoechst staining, cells were washed in ice cold PBS, resuspended in PBS/1% BSA and stained for 20 minutes at 4°C in 5μl of various antibody combinations: unless otherwise stated all antibodies were purchased from Becton Dickinson, Oakville Ontario: anti-CD4-ECD (Coulter), anti-CD8-PE (Immunotech), anti-CD45RO-APC, anti-CD62L-FITC, anti-CD45RA-FITC and anti-HLA-DR-PE. Stained cells were washed with ice cold PBS and incubated with 1μg of Hoescht for 7 minutes (Badley et al., 1996). After 2 washes with ice cold PBS, cells were analyzed by flow cytometry (Coulter Epics Altra). For apoptosis detection using TUNEL (ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit, Intergen) all assays were performed following manufacturers instruction with the following modifications: cells were fixed and permeabilized using fix and perm reagent (Cedarlane Products) according to specifications provided by the manufacture. Data is expressed as median TRAIL specific apoptosis. TRAIL specific apoptosis (TSA) was calculated as follows:

TSA = % apoptosis following TRAIL treatment minus % apoptosis in control sample.

Isolation of latently infected CD4 T cells. Resting memory $CD4^+$/HLA-DR^-/$CD45RO^+$ T cells were isolated from PBMCs using magnetic bead separation (Miltenyli Biotec) as described (Finzi et al., 1997). All antibodies for magnetic bead separation were purchased from Mitenyli Biotec. A second step purification was performed by sorting flow cytometry using anti-CD4-ECD (Coulter) and anti-HLA-DR-PE (Becton Dickinson, Oakville Canada). The purity of final $CD4^+$/HLA-DR^-/$CD45RO^+$ cell suspensions was ≥98%.

Quantitative Micro-coculture Assay. To determine the frequency of latently infected cells, highly purified resting memory cells were subjected to high input quantitative micro-coculture assay as previously described (Chun et al., 1999). We used the maximum available number of cells in each coculture for each patient. The total starting cell concentrations ranged from 3.4×10^6 cells to 5.0×10^7 cells. Following overnight LZhuTRAIL (or mock) treatments, cells were washed extensively to remove all traces of LZhuTRAIL, and coincubated with irradiated feeder cells (as described in (Chun and Fauci, 1999)) for 14 days. For the macrophage coculture, we isolated MDMs from healthy donors and mock or HIV_{Bal} infected samples on day 6 following isolation. The following day cells were treated with 1μg LZhuTRAIL overnight, washed three times and resuspended in culture media. On day 14, p24 and viral RNA (see below) was measured. Viral replication was measured in duplicate using a commercial p24 ELISA assay (NEN, Life Sciences Products, Belgium). In independent experiments we

determined that LZhuTRAIL treatment does not impair the ability of resting T cells to become activated (data not shown).

HIV Viral Load Testing. Tissue culture supernatants were processed and stored at -80°C until time of testing using either the Amplicor assay for studies involving the MDM, or the Quantiplex bDNA assay for studies using PBL or sorted cells from HIV infected patients.

(i) The Amplicor HIV monitor 1.5 assay (Roche Diagnostics, Laval, QC) was performed according to the manufacturer's instructions and all samples were run singly following a three-step workflow: (i) specimen preparation (including viral lysis, RNA precipitation, RNA washing, and suspension of purified RNA in buffer), (ii) amplification, and (iii) detection. This assay has a range of sensitivity from 400 to 750,000 HIV RNA copies/ml.

(ii) The Quantiplex bDNA 3.0 assay (Bayer Diagnostics, Markham, Ontario) was performed in conjunction with the semi-automated Quantiplex 340 system according to manufacturer's instructions and all samples were run singly. Samples with values between 50 and 500,000 RNA copies/ml were within the limit of quantitation for this assay.

Detection of Proviral HIV DNA. (i) DNA Extraction from Cell Pellets. DNA was extracted from cell pellets using the extraction reagent from the Amplicor Whole Blood Specimen Preparation Kit (Roche Diagnostics, Laval, Quebec). Two hundred and fifty microliters of extraction reagent was added to each pellet, and the pellets were incubated

in a dry heat block for 30 minutes at 60°C and 100°C respectively. The samples were vortexed briefly and stored at –20°C until further use.

(ii) DQγ Gene Amplification. The presence and quality of the DNA was determined by PCR amplification of the human DQ-γ gene. Briefly, the PCR reactions contained 5µl of 10X PCR buffer (Perkin Elmer, Mississauga, ON), 200µM each dNTP (Perkin Elmer, Mississauga, ON), 1µM of each primer GH26 (5'-GTGCTGCAGGTGTAAACTTGTACCAG-3') and GH27 (5'-CACGGATCCGGTAGCAGCGGTAGAGTTG-3'), 1 unit of AmpliTaq DNA Polymerase (Perkin Elmer, Mississauga, ON), and 12.5µl of sample per 50µl reaction. The samples were amplified for 35 cycles (96°C - 30s, 60°C - 30s, 72°C - 30s). The amplified PCR products (243 bp) were separated by 1% agarose gel electrophoresis for 30 minutes at 100 V and visualized by ethidium bromide staining and ultraviolet transillumination of the gel. Amplification and detection of HIV-1 specific samples was analysed using the Amplicor HIV-1 Amplification and Detection kits (Roche Diagnostics, Laval, QC) according to the instructions from the manufacturer. To eliminate the possibility of false negative results, all negative results from the Roche assay were subjected to a nested PCR using primers specific to the "pol" region. The PCR reactions contained 5µl of 10X PCR buffer (Perkin Elmer), 200µM of each dNTP (Perkin Elmer), 1µM of each primer, 1U of AmpliTaq DNA Polymerase (Perkin Elmer) and 2.25mM $MgCl_2$ (Perkin Elmer) per 50µl reaction. The outer primer pair was HPOL4235 (5'-CCCTACAATCCCCAAAGTCAAGG-3') and HPOL4538 (5'-TACTGCCCCTTCACCTTTCCA-3'), and 12.5µl of sample was used in the first round reaction. Two µl of the first round products were added to the second round PCR

using the inner primers HPOL4327 (5'- TAAGACAGCAGTACAAATGGCAG-3') and HPOL4481 (5'GCTGTCCCTGTAATAAACCCG-3'). Both first and second rounds were amplified for 35 cycles (96°C - 30s, 65°C - 30s, 72°C - 30s). The second round PCR products (175 bp) were separated by 1% agarose gel electrophoresis for 30 minutes at 100 V, followed by ethidium bromide staining and ultraviolet transillumination of the gel. Limiting dilution analysis revealed a sensitivity of 1 copy of the 8E5 gag DNA, as previously described (Janssens et al., 1995).

Statistics. Estimation of infectious units per million (IUPM) were done using data from limiting dilution co-cultures, according to maximum likelihood methods (Myers et al., 1994). The 95% confidence intervals for individual determinations spanned 1.1 logarithms. Statistical comparisons between treatment groups and control groups were performed using standard Student's T test.

2.3 Results

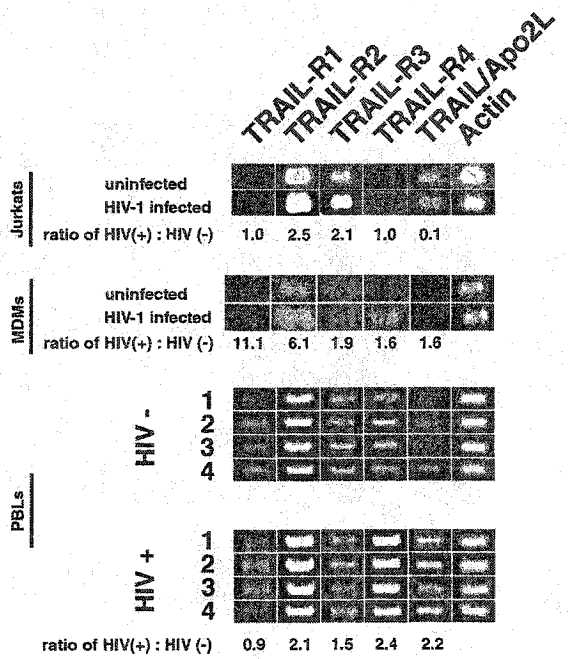
TRAIL/Apo2L and TRAIL receptor expression are altered following HIV infection in vitro and in vivo.

To evaluate whether HIV infected T cells demonstrate changes in TRAIL/Apo2L and/or TRAIL receptor expression, Jurkat T cells were infected with HIV_{IIIB} (Badley et al., 1996) and expression of mRNA specific for TRAIL receptors 1, 2, 3, 4 and TRAIL/Apo2L was determined by RT-PCR (Griffith et al., 1999b; Sedger et al., 1999). The relative band intensity of amplified products for each receptor and TRAIL was measured and normalized to the intensity of amplified β-actin message. Relative band intensity of TRAIL-R2 ($p < 0.04$, $n = 3$) and -R3 ($p < 0.04$, $n = 3$) mRNA was significantly increased (by 2.5 to 2.1 fold) in infected cells compared to mock infected cells, while mRNA for TRAIL-R1 ($p = 0.1$, $n = 3$) and TRAIL-R4 ($p < 0.1$, $n = 3$) was unchanged (Fig 3A- top panel). No differences were observed in TRAIL/Apo2L mRNA expression between infected and uninfected cells.

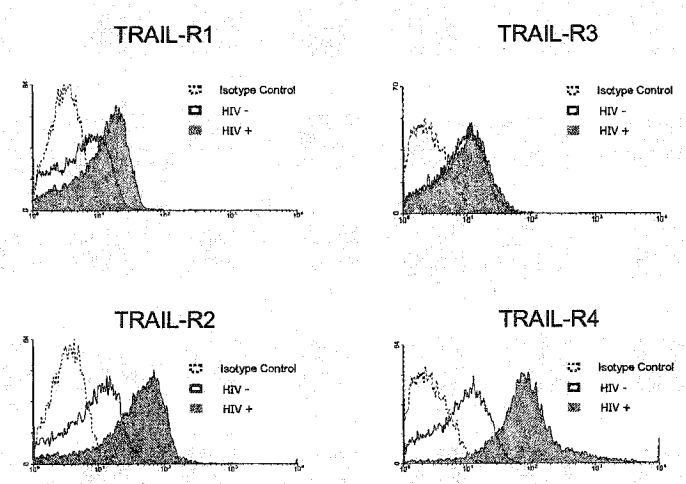
We also analyzed mRNA expression in mock or HIV_{Bal} *in vitro* infected monocyte derived macrophages (MDM). In comparison to mock infected MDM, HIV infected MDM had increased expression of all four TRAIL receptors and TRAIL ligand ($n = 3$, $p < 0.01$ respectively; Fig 3A- middle panel). We next examined TRAIL/Apo2L and TRAIL receptor expression in PBL from patients infected with HIV using RT-PCR. RNA for all four receptors as well as TRAIL were detected and significant differences

Figure 3. Altered TRAIL/TRAIL Receptor Expression by HIV Infection. (A) Analysis of TRAIL Receptor mRNA expression by RT-PCR. (Top) HIV- infected Jurkat T cells have increased transcripts for TRAIL-R2,-R3 and TRAIL compare to mock infected controls. (Middle) HIV-1_{Bal}-infected MDM have increased transcripts for all TRAIL receptors and TRAIL compared to mock infected controls. *In vitro* infections were performed at least 3 times and all band intensities were normalized to β-actin. (Bottom) PBL from four HIV-1 positive individuals have increased transcripts for TRAIL-R2, -R3, -R4 and TRAIL compared to PBL from four HIV-1 negative individuals. The increase in expression is denoted by relative ratios greater than 1. (B) CD4 T cell surface expression of TRAIL receptors from HIV infected patients and healthy controls. TRAIL-R1, -R2 and –R4 expression is increased in CD4 T cells from HIV infected patients.

A


TRAIL-R1
TRAIL-R2
TRAIL-R3
TRAIL-R4
TRAIL/Apo2L
Actin
Jurkats
uninfected
HIV-1 infected
ratio of HIV(+) : HIV (-) 1.0 2.5 2.1 1.0 0.1
MDMs
uninfected
HIV-1 infected
ratio of HIV(+) : HIV (-) 11.1 6.1 1.9 1.6 1.6
PBLs
HIV -
1
2
3
4
HIV +
1
2
3
4
ratio of HIV(+) : HIV (-) 0.9 2.1 1.5 2.4 2.2

B


TRAIL-R1
Isotype Control
HIV -
HIV +
TRAIL-R3
Isotype Control
HIV -
HIV +
TRAIL-R2
Isotype Control
HIV -
HIV +
TRAIL-R4
Isotype Control
HIV -
HIV +

were found between HIV positive and HIV negative samples. Densitometric analysis of the PCR products from PBL of 4 HIV infected donors compared to 4 uninfected individuals revealed a consistent 2.0-2.4 fold increase in the level of mRNA expression of TRAIL-R2 ($p = 0.001$), TRAIL-R4 ($p = 0.01$) and TRAIL/Apo2L ($p = 0.02$; Fig 3A-bottom panel). However, levels of TRAIL-R1 ($p = 0.690$) and TRAIL-R3 ($p = 0.286$) mRNA in HIV infected and uninfected individuals were similar.

Cell surface expression of all four TRAIL receptors was also evaluated by flow cytometry using TRAIL receptor specific antibodies (Griffith and Lynch, 1998). In accordance with the RT-PCR results, cell surface expression of TRAIL-R2 (CD4 MCF uninfected 3.0, infected 9.6 $p < 0.001$, CD8 MCF uninfected 0.6, infected 1.8 $p < 0.001$) and TRAIL-R4 (CD4 MCF uninfected 1.7, infected 11.2 $p < 0.001$, CD8 MCF uninfected 0.2, infected 2.1 $p < 0.001$) was increased on both CD4 T cells (Fig 3B) and CD8 T cells (data not shown) from 7 HIV infected patients compared to 19 HIV uninfected donors tested, while the levels of TRAIL-R1 increased by less than 2 fold while TRAIL-R3 was not significantly altered. In mock or HIV_{Bal} infected MDM (data not shown), *in vitro* infection was associated with increased levels of TRAIL-R1 ($n = 3$ MCF uninfected 2.4, infected 5.8, $p = 0.01$), TRAIL-R2 ($n = 3$, MCF uninfected 4.1, infected 13.2, $p < 0.004$), TRAIL-R4 ($n = 3$, MCF uninfected 2.7, infected 9.6, $p = 0.006$) while TRAIL-R3 expression was not significantly changed ($n = 3$, $p < 0.1$).

These data demonstrate significant dysregulation of TRAIL receptor expression in cells from patients infected with HIV and following HIV infection *in vitro* at both the

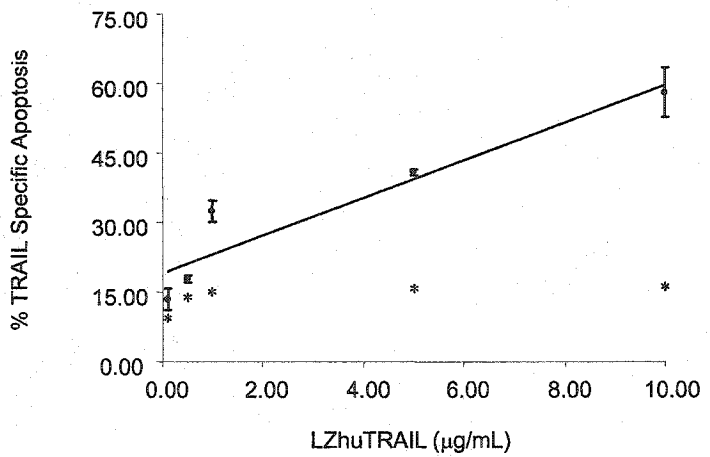
mRNA and protein level, and suggest that cells from both uninfected and HIV infected patients may be sensitive to TRAIL receptor ligation.

Cells from HIV (+) patients undergo cell death following in vitro treatment with LZhuTRAIL and agonistic TRAIL receptor antibodies

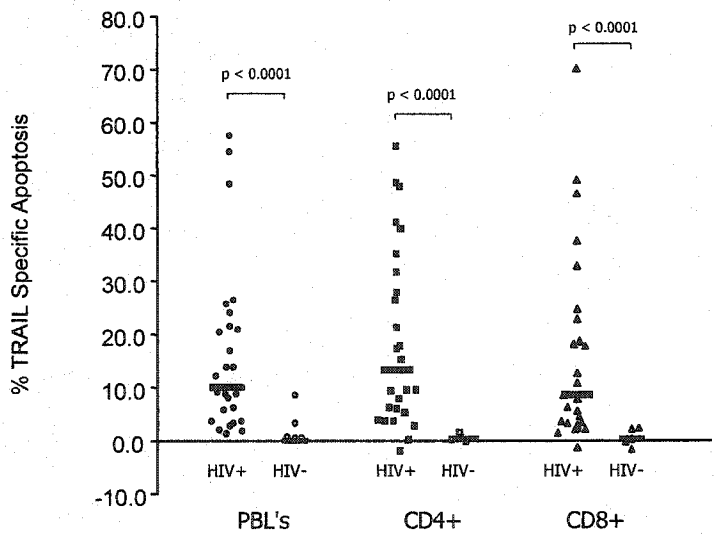
Previous studies have shown that surface expression of TRAIL receptors is insufficient to predict the sensitivity of cells to TRAIL induced killing (Griffith et al., 1998; Irmler et al., 1997). Therefore, PBL from HIV infected individuals with varying treatment histories and viral loads were cultured *in vitro* with LZhuTRAIL and analyzed for TRAIL mediated killing. A dose dependent death of PBL (Fig 4A) and CD4 T cells (data not shown) was observed in cells from HIV positive patients treated with increasing amounts of LZhuTRAIL but not in cells from HIV negative donors (indicated by asterisks). By Hoechst staining, the median TRAIL/Apo2L specific apoptosis (TSA) in PBL from HIV infected patients was 10.0% ($n = 26$) compared to 0.70% ($n = 5$) in healthy donors ($p < 0.0001$; Fig 4B). The median TSA observed in CD4 T cells from HIV infected patients was 13.3% ($n = 26$) compared to 0.3% for healthy donors, ($n = 5$, $p = 0.0001$; Fig 4B), while median TSA of CD8+ T cells was 8.5% ($n = 26$) compared to 0.3% ($n=5$, $p < 0.0001$; Fig 4B) for controls. To confirm the results obtained by Hoechst staining, we also analyzed apoptosis following TRAIL/Apo2L treatment in specimens obtained from an additional 10 HIV infected and 5 uninfected patients using TUNEL. In confirmation of the results obtained by Hoechst staining, the median TSA in HIV positive PBL was 4.65% ($n = 10$, data not shown), whereas the median TSA for HIV negative

Figure 4. Sensivitity of PBL from HIV infected donors to titrated doses of LZhuTRAIL. (A) Cell death was measured by Hoechst staining. Data is representative of three independent experiments. Spontaneous levels of cell death are indicated by asterisks. (B) PBLs from HIV infected patients undergo cell death following stimulaton with LZhuTRAIL. PBLs from 26 patients with varying clinical histories or 5 uninfected controls were treated with LZhuTRAIL and 3 colour flow cytometry was used to analyze for cell death (by Hoescht staining) in CD4+ and CD8+ T cell subsets.

A


75.00
60.00
45.00
30.00
15.00
0.00
% TRAIL Specific Apoptosis
0.00
2.00
4.00
6.00
8.00
10.00
LZhuTRAIL (μg/mL)

B


80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
-10.0
% TRAIL Specific Apoptosis
p < 0.0001
p < 0.0001
p < 0.0001
HIV+
HIV-
HIV+
HIV-
HIV+
HIV-
PBL's
CD4+
CD8+

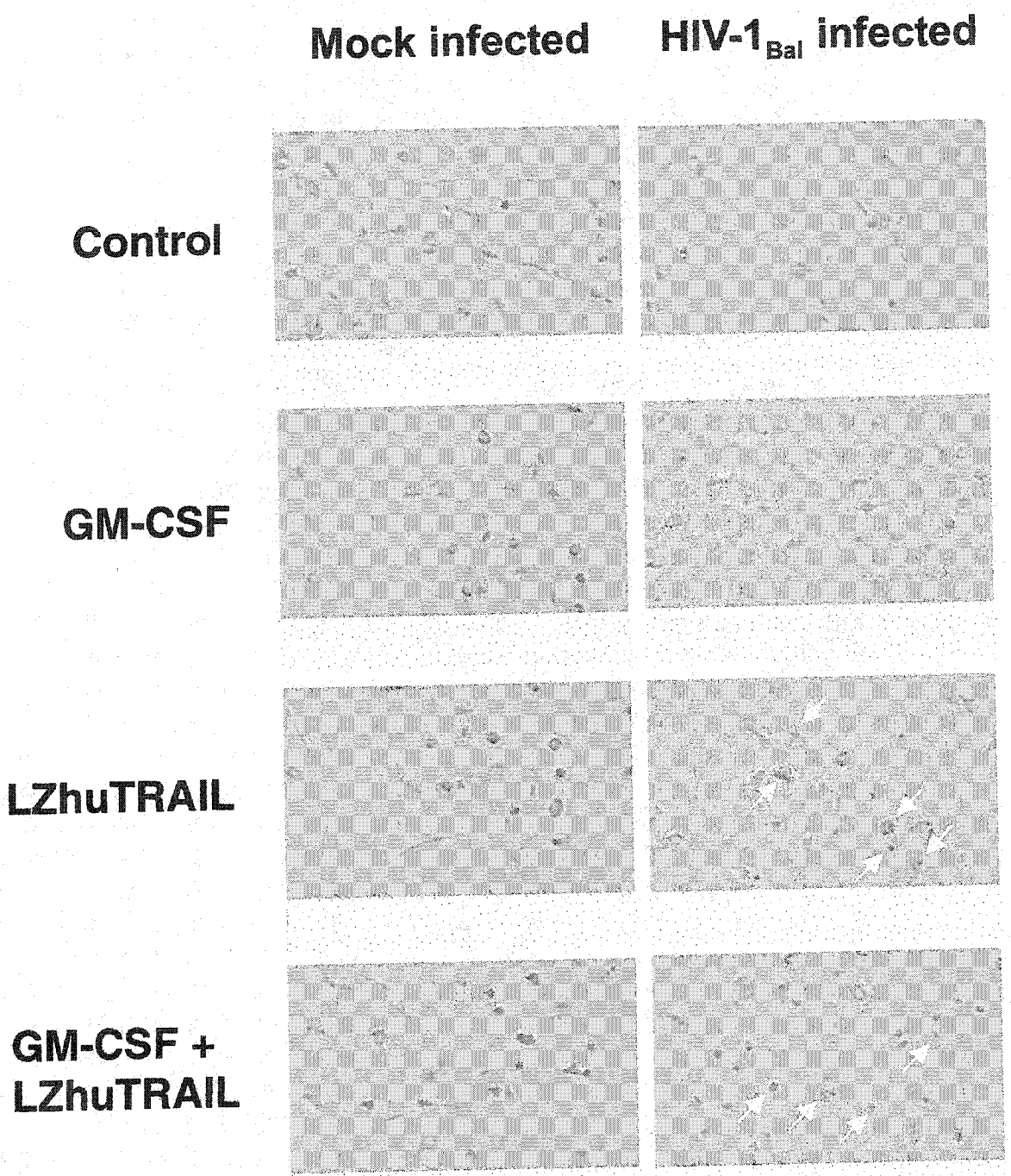
donor was only 0.7%, ($n = 5$, $p < 0.0001$, data not shown). The median TSA in CD4 T cells was 8.3% ($n = 5$, data not shown) while the median TSA of healthy controls was 0.3%, ($n = 5$, $p = 0.001$); median TSA in HIV positive CD8+ cells was 4.7% ($n = 5$, data not shown) in contrast to controls where median TSA was 0.3%, ($n = 5$, $p = 0.004$, data not shown). Further, agonistic mAbs directed against TRAIL-R1 (clone M271) and TRAIL-R2 (clones M412 and M413) (Griffith et al., 1999a) induced apoptosis in PBLs, CD4 and CD8 T cell subsets (data not shown), similar to that observed with LZhuTRAIL.

Together, these data indicate that LZhuTRAIL and agonistic mAbs to TRAIL-R1 and -R2 specifically and selectively induce apoptosis of CD4 and CD8 T cells from HIV infected patients, but not in cells from uninfected controls.

LZhuTRAIL induces selective apoptosis of MDM infected with $HIV_{Bal.}$

In view of the important role played by macrophages in the pathophysiology of HIV disease (Meltzer et al., 1990; Poli and Fauci, 1992), we investigated whether TRAIL/Apo2L induces apoptosis in macrophages. MDM from 11 donors were mock or HIV_{Bal} infected and 14 days later were treated with 1μg/mL LZhuTRAIL for 12 hours and assessed for apoptosis by TUNEL staining. LZhuTRAIL induced significant apoptosis in HIV_{Bal} cultures (TSA was 20.47% versus 7.96% in mock infected cultures ($p < 0.001$), indicating that TRAIL/Apo2L triggers cell death in *in vitro* infected macrophages (Fig 5). As it has been previously reported that granulocyte macrophage colony stimulating factor (GM-CSF) increases the sensitivity of MDMs to TRAIL/Apo2L

Figure 5. LZhuTRAIL mediates death in HIV infected monocyte derived macrophages (MDM). MDM were mock treated or HIV-1 infected and on day 16, 100ng GM-CSF was added to the indicated cultures. 14 days following infection cells were treated with LZhuTRAIL and analyzed by TUNEL. Arrows indicate TUNEL + (apoptotic) cells. Micrographs represents 1 of 11 patients enumerated.


Mock infected
HIV-1Bal infected
Control
GM-CSF
LZhuTRAIL
GM-CSF +
LZhuTRAIL

induced cell death (Sedger et al., 1999), we also treated MDMs with GM-CSF and assessed for apoptosis induced by TRAIL/Apo2L. The addition of GM-CSF did not significantly alter the sensitivity of HIV positive or HIV negative MDM to TRAIL/Apo2L (Fig 5).

LZhuTRAIL induces apoptosis of resting memory CD4 T cells from patients infected with HIV

To determine whether TRAIL/Apo2L has cytotoxic effects on those cells that represent the principle HIV reservoir *in vivo*, we treated PBL from 14 HIV positive patients receiving highly active antiretroviral therapy (ART) who had suppressed viral replication (<50 copies viral RNA/mL for greater than 12 months) with 1μg/mL LZhuTRAIL and assessed cell death in CD4 T cells with phenotypic markers which predict the latently infected CD4 T cell pool. Several groups have established that latently infected T cells express $CD4^+$/HLA-DR^-/$CD45RO^+$ and $CD4^+$/HLA-DR^-/$CD62L^+$, however, only a small fraction of these cells is latently infected (Chun et al., 1998; Chun et al., 1997b; Finzi et al., 1999; Finzi and Silliciano, 1998; Ostrowski et al., 1999; Ostrowski et al., 1998). The median TSA for cells with $CD4^+$/HLA-DR^-/$CD62L^+$ phenotype treated with LZhuTRAIL was 3.6% compared to untreated cell cultures where median TSA was 1.2% ($n = 8$, $p = 0.01$, Table 1). CD4+/HLA-DR-/CD45RO+ cells from HIV infected patients had a median TSA of 3.6% ($n = 8$) in cultures treated with LZhuTRAIL, in contrast to untreated cultures where median TSA was 1.2% ($n = 8$, $p =$

Table 1.0. CD4 resting memory cells undergo death following LZhuTRAIL treatment. Cells from HIV infected patients were isolated and treated with control of TRAIL for 18 hours followed by staining with the indicated antibodies. Five colour FACS analysis was used to measure the % of apoptotic cells.

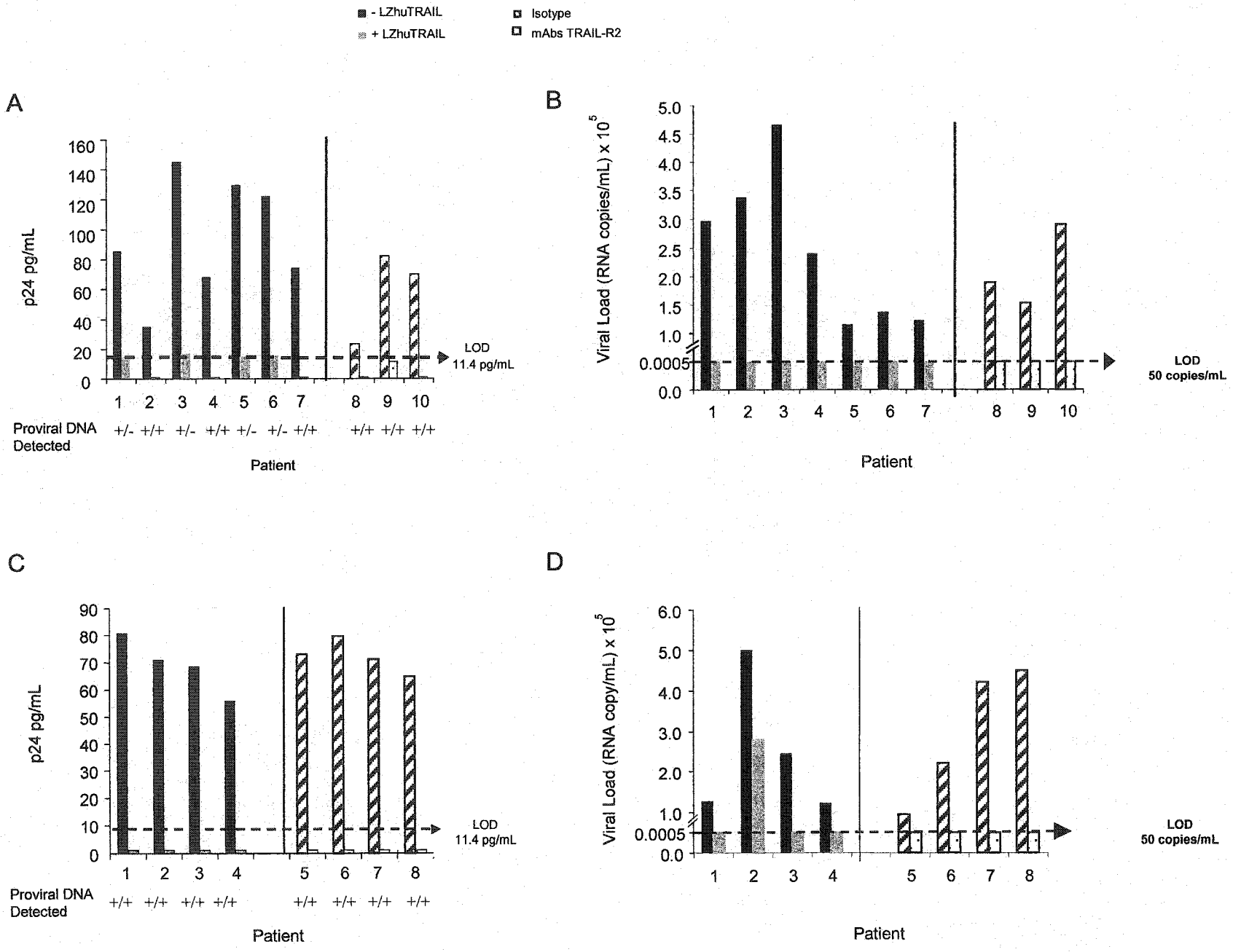
	CD4+/HLA-DR-/CD62L+		CD4+/HLA-DR-/CD45RO+	
Patient	control	TRAIL	control	TRAIL
1	1.2	1.7	6.5	11.9
2	1.6	4.3	1.3	0.9
3	4.1	11.2	6.8	16.0
4	1	2.5	7.5	9.0
5	0.7	2.9	1.1	1.9
6	11.6	12.8	6.7	11.0
7	0.4	4.2	15.0	18.7
8	1.2	1.4	6.7	10.8
Median	1.2	3.6	6.5	10.0

0.005). These findings suggest that LZhuTRAIL induces apoptosis in a variety of cell types including latently HIV infected CD4 T cells.

In vitro treatment of cells from HIV infected patients with LZhuTRAIL reduces HIV production and reduces the proportion of provirus containing cells.

Isolated $CD4^+$/HLA-DR^-/$CD45RO^+$ cells from 7 HIV infected patients receiving ART, whose plasma viral load remained below the level of detection (<50 copies/mL) for more than 12 months, were assessed for detectable HIV, following treatment with TRAIL. By microculture, untreated cultures produced a mean of 94.35 pg/mL (SD = 39.28) of p24 antigen. In contrast, we could not detect p24 antigen production from 6 of 7 cultures treated with LZhuTRAIL ($p < 0.001$), where the limit of detection in this assay is 11.4 pg/mL (Fig 6A, patients 1-7). We also measured viral RNA in the culture supernatants and found similar results: untreated cultures had significant levels of HIV RNA (mean = 2.45×10^5 copies/mL) whereas there were undetectable levels of HIV RNA in all 7 culture supernatants from cells treated with LZhuTRAIL ($p = < 0.002$) (Fig 6B, patients 1-7). Amplification of HIV provirus was also performed, using a commercial assay specific for the "gag" region of HIV. By this measure, HIV provirus could not be detected in 4 of 7 treated samples. To eliminate the possibility of a false negative result from the commercial assay, the four samples which tested negative were retested using an in house nested PCR with a different set of primers specific for the "*pol*" region of HIV (Fransen et al., 1994; Janssens et al., 1994). This assay has a detection limit of 1 copy of DNA (Janssens et al., 1995). These samples also tested consistently negative for HIV

Figure 6. Reduction in HIV gene expression in infected cells by LZhuTRAIL. $CD4^+/HLA\text{-}DR^-/CD45RO^+$ cells were isolated from 7 HIV infected patients with suppressed viremia for more than 12 months and treated with LZhuTRAIL or agonistic TRAIL-R2 (or isotype control) antibodies. Following 14 day of limiting dilution quantitative microculture, culture supernants were analyzed for (A) p24 antigen production and the presence of integrated viral DNA or (B) viral RNA. Unfractionated cells from 4 HIV infected patients were treated with or without LZhuTRAIL or agonistic TRAIL-R2 (or isotype controls) and tested for (C) p24 antigen or (D) viral RNA in culture supernants and presence of viral DNA following microculture.


- LZhuTRAIL
+ LZhuTRAIL
Isotype
mAbs TRAIL-R2
A
p24 pg/mL
160
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
LOD
11.4 pg/mL
Proviral DNA
Detected
+/- +/+ +/- +/+ +/- +/- +/+ +/+ +/+ +/+
Patient
B
Viral Load (RNA copies/mL) x 10^5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.0005
0.0
1 2 3 4 5 6 7 8 9 10
LOD
50 copies/mL
Patient
C
p24 pg/mL
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8
LOD
11.4 pg/mL
Proviral DNA
Detected
+/+ +/+ +/+ +/+ +/+ +/+ +/+ +/+
Patient
D
Viral Load (RNA copy/mL) x 10^5
6.0
5.0
4.0
3.0
2.0
1.0
0.0005
0.0
1 2 3 4 5 6 7 8
LOD
50 copies/mL
Patient

DNA (Fig 6A). Treatment and control groups were also compared using maximum likelihood estimates to determine the infectious units per million (IUPM). By this estimate LZhuTRAIL significantly reduced HIV burden of resting memory cells ($p = 0.03$, Table 2). Similar experiments were conducted using agonistic mAbs to TRAIL-R2. All three PBL cultures treated with TRAIL R2 specific mAb had undetectable levels of viral p24 antigen production ($p = 0.04$; Fig 6A patients 8-10) and undetectable levels (<50 copies/mL) of supernatant HIV specific RNA ($p < 0.001$; Fig 6B, patients 8-10), whereas untreated cultures produced high levels of virus as measured by p24 antigen (mean = 58.15, SD = 4.97pg/mL) and supernatant viral RNA (mean = 2.05×10^5 copies/mL, SD = 0.58×10^5, $p < 0.001$). In contrast to treatments with LZhuTRAIL (where provirus was undetectable in 4 of 7 samples) provirus was detected in 3 of 3 samples treated with agonistic TRAIL-R2 antibodies, and IUPM was not significantly different between treated and untreated cells (Table 2).

Finally, to determine whether all cell types capable of producing virus that were present in peripheral blood of infected patients were killed by TRAIL/Apo2L treatment, we subjected unfractionated PBL to microculture following treatment with either LZhuTRAIL or mAbs to TRAIL-R2. In PBL cultures treated with LZhuTRAIL, p24 antigen production was undetectable in 4 of 4 samples, whereas untreated samples produced a mean of 69.13 pg/mL of p24 antigen (SD = 10.17 pg/mL, $p < 0.001$, Fig 6C patients 1-4). In these same samples supernatant viral RNA was 2.65×10^5 copies/mL in untreated versus 280 copies/mL in LZhuTRAIL treated samples ($p = <0.001$). Although proviral DNA was still detectable in all samples, IUPM was significantly reduced in

LZhuTRAIL treated samples ($p = 0.02$) (Table 2). Agonistic TRAIL-R2 antibodies had a similar effect in PBL cultures: untreated cultures produce significant amounts of p24 antigen (mean 72.31, SD 6.06 pg/mL, $n = 4$; Fig 6C patients 5-8) and of viral RNA (mean 2.97×10^5 copies/mL, SD = 1.7×10^5; Fig 6D patients 5-8) whereas treated samples had undetectable levels of p24 antigen production ($p < 0.001$) and mean viral load of <50 copies/mL ($p = 0.01$), but provirus DNA was still detectable. In these assays using agonistic anti-TRAIL R2 antibody IUPM was not different between treatment groups (Table 2).

Table 2.0. Effects of LZhuTRAIL or agonistic TRAIL-R2 treatments on IUPM. IUPM values were calculated based on p24 results obtained following 14 days micrococulture using maximum likelihood analysis. In 4 of 7 cases where cells were treated with TRAIL, the IUPM was below the limit of detection.

Treatment and cell type	Patient	IUPM in:	
		Control cells	Treated cells
LZhuTRAIL, resting memory cells[a]	1	0.41	0.02
	2	2.29	0.02
	3	0.08	0.01
	4	2.29	0.08
	5	0.41	0.01
	6	0.41	0.01
	7	0.41	0.01
LZhuTRAIL, PBL[b]	1	0.41	0.41
	2	0.41	0.01
	3	0.41	0.01
	4	0.41	0.01
Anti-TRAIL-R2, resting memory cells[c]	1	2.29	2.29
	2	2.29	2.29
	3	7.18	2.29
	4	7.18	2.29
Anti-TRAIL-R2, PBL[d]	1	2.29	0.41
	2	0.41	0.01
	3	2.29	2.29
	4	2.29	2.29

[a] $P = 0.03$ for the difference between control and treated cells.
[b] $P = 0.02$ for the difference between control and treated cells.
[c] $P = 0.13$ for the difference between control and treated cells.
[d] $P = 0.49$ for the difference between control and treated cells.

2.4 Discussion

Despite successful control of HIV replication in patients receiving ART, the prolonged life span and slow rate of decay of latently infected CD4 T cells provides a long lasting cellular reservoir for HIV (Chun and Fauci, 1999; Cooper and Emery, 1999; Finzi et al., 1999; Saag and Kilby, 1999). Indeed, independent projections estimate the time required to fully eliminate HIV in patients on completely suppressive ART alone to be from 10-60 years (Chun and Fauci, 1999; Finzi et al., 1999; Finzi et al., 1997; Zhang et al., 1999a). Latently infected resting memory CD4 T cells contain integrated DNA provirus yet are transcriptionally inactive and may therefore escape both immune recognition and the antiviral effects of ART regimes which affect only viral RNA species (Cooper and Emery, 1999). Since current therapies for HIV are ineffective in eradicating latently infected cell populations, control of these populations depends upon the interplay of cellular half life, and the ability to suppress viral replication in order to prevent repopulation of the latent reservoir (Grossman et al., 1999). The importance of this reservoir (Finzi and Silliciano, 1998) is underscored by the observations of viral rebound following withdrawal of ART (Davey et al., 1999). Thus, in order to eradicate HIV, it is critical to develop strategies to eliminate latently infected cells. Here we demonstrate that TRAIL/Apo2L treatment *in vitro* induces death of cells including the relevant latently infected cell populations that are principle HIV reservoirs *in vivo*.

While both FasL and TNF have been shown to induce apoptosis of cells from HIV infected patients (Badley et al., 2000; Laurence et al., 1996), the non selective

induction of apoptosis and toxicity related to activation limit their clinical utility as a potential therapy for HIV infection. By contrast, systemic administration of TRAIL/Apo2L in healthy mice and nonhuman primates has been shown to be safe and lack cytotoxic effects (Ashkenazi et al., 1999; Chinnaiyan et al., 2000; Giovarelli et al., 1999; Gliniak and Le, 1999; Griffith et al., 1999a; Nagane et al., 2000; Walczak et al., 1999). In models utilizing animals engrafted with human tumors, treatment with TRAIL/Apo2L induces significant tumor specific apoptosis, tumor regression and improved survival (Ashkenazi et al., 1999; Walczak et al., 1999), with no identifiable toxicity.

The first goal of this study was to determine whether TRAIL receptor expression changes during HIV infection. *In vitro* infection of Jurkat T cells and MDM infected with HIV *in vitro* and both CD4 and CD8 T cells from HIV positive patients are associated with dysregulation of TRAIL and TRAIL receptor expression. In addition, LZhuTRAIL kills HIV infected cells as well as bulk PBL, CD4 T cells, CD8 T cells, $CD4^+/CD62L^+/HLADR^-$, and $CD4^+/HLADR^-/CD45RO^+$ cells from HIV infected patients. Together these data lay the foundation for the hypothesis that TRAIL/Apo2L may induce apoptosis of a variety of cells (including latently infected cells) from HIV infected patients and may therefore be of clinical benefit.

In order to explore this hypothesis, we evaluated the ability of TRAIL/Apo2L to eradicate cells capable of producing HIV by analyzing (i) p24 antigen production, (ii) viral RNA production and (iii) HIV provirus in cells treated with LZhuTRAIL and (iv)

IUPM. In 6 of the 7 cultures of $CD4^+/HLADR^-/CD45RO^+$ cells treated with LZhuTRAIL and in 3 of 3 cultures treated with agonistic mAbs to TRAIL-R2, p24 antigen production was not detected. In all cultures of $CD4^+/HLADR^-/CD45RO^+$ cells treated with LZhuTRAIL or with agonistic antibody, no viral RNA was detected, thus demonstrating the anti-viral effects of TRAIL/Apo2L on these cells. Of particular interest, HIV proviral DNA was not detected in 4 of 7 cultures of $CD4^+/HLADR^-/CD45RO^+$ cells treated with LZhuTRAIL, suggesting an ability to eradicate latently infected cells. Further, in these experiments LZhuTRAIL reduced IUPM in both sorted resting memory cells and bulk PBL's. In subsequent experiments, we evaluated whether 2 or 3 rounds of TRAIL or agnostic TRAIL-R2 mAbs would further reduce IUPM and note that in all cases tested, 3 rounds of treatments significantly reduced IUPM, in some cases up to 2 logs (data not shown). Together these findings indicate that TRAIL/Apo2L *in vitro* can induce significant apoptosis in cells from HIV patients including latently infected CD4 T cells and HIV infected macrophages.

It is noteworthly that our cumulative data suggest that both infected and uninfected cells die following TRAIL receptor stimulation. In bulk assays, up to 20% of cells die following LZhuTRAIL treatment, which is significantly more cells than are physically infected by the virus. Further, limiting-dilution coculture assays demonstrate that infected cells are killed by such treatments. Thus, uninfected cells and latently infected cells (which do not express HIV proteins) die following treatment.

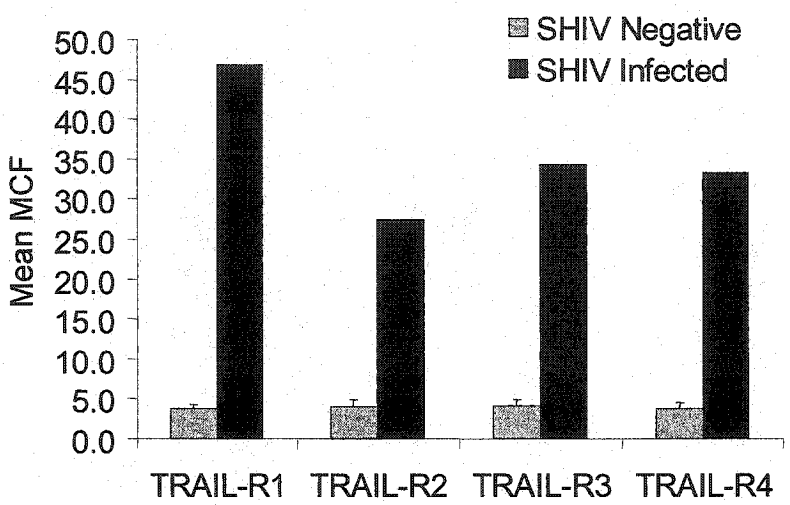
Thus, in addition to the evidence that TRAIL/Apo2L is cytotoxic to transformed or CMV infected human cells, we have shown that cells from HIV infected patients are similarly sensitive to TRAIL mediated apoptosis. These data provide a basis for future evaluation of TRAIL/Apo2L as therapy in humans particularly in view of the encouraging safety results of *in vivo* administration of TRAIL/Apo2L in mice and non human primates. A note of caution has been raised by a recent report using primary human hepatocytes from livers demonstrated TRAIL induced apoptosis in these cells (Jo et al., 2000). However, different TRAIL/Apo2L preparations or agonists do not all possess this activity (Lawrence et al., 2001), and whether TRAIL induces apoptosis of freshly isolated hepatocytes, or hepatocytes *in vivo* remains to be determined.

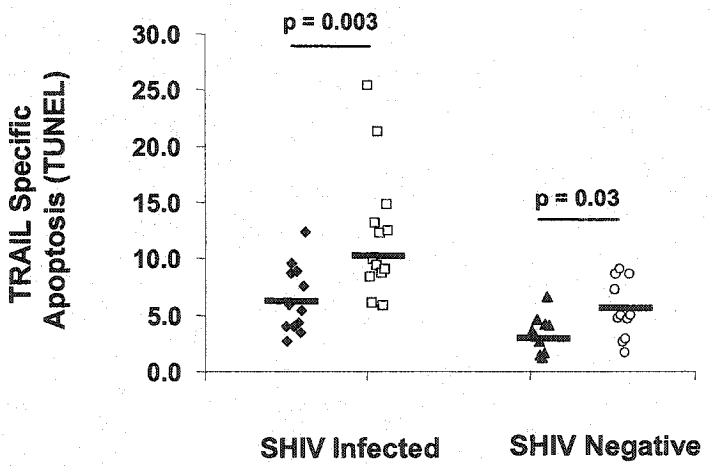
Our findings are the first to demonstrate that TRAIL/Apo2L selectively induces death in cells from HIV patients including latently infected CD4 T cells and macrophages without deleterious effects on cells from uninfected patients. Based on these findings, we propose that patients with prolonged viral suppression be treated with TRAIL agonists. In such patients, prolonged viral suppression has been associated with eradication of >99.9% of lymphoid (and thus macrophage associated) virus (Cavert et al., 1997; Gunthard et al., 1998; Haase et al., 1996; Orenstein et al., 1999; Ruiz et al., 1999) and persistence of HIV chiefly within CD4+/CD45RO+/HLA-DR- T cells (Chun et al., 1998; Chun and Fauci, 1999; Chun et al., 1997b; Finzi et al., 1997; Wong et al., 1997; Zhang et al., 1999a) some of which are latently infected. Further, as a single dose *in vitro* therapy with LZhuTRAIL can eradicate provirus and inducible virus production in slightly more than half of the cultures tested, multiple cycles of therapy need to be considered.

2.5 Preclinical Evaluation of TRAIL in Cells from SHIV infected macaques

To advance preclinical studies on TRAIL as a possible therapy for HIV, a small cohort of cells from SHIV infected macaques were obtained in a collaboration with Health Canada (Dr. Erling Rud). In these preliminary studies, it was observed that cells from infected macaques have significantly higher expression of all four TRAIL receptors (Fig 7A). In addition, cells from infected macaques have enhanced sensitivity to TRAIL killing when compared to controls (Fig 7B). Due to costs, availability of macaques and patent issues, no further work has been conducted to evaluate these results.

Figure 7. Cells from SHIV infected macaques express high levels of TRAIL receptors. (A) 1 x 10^6 PBLs from SHIV infected or control macaques were stained using TRAIL receptor specific monoclonal antibodies (see materials and methods) followed by analysis by flow cytometry. Black squares represents the average MCF of 14 individual SHIV infected macaques. White squares represents the average of MCF of 10 individual control. (B) Enhanced Sensitivity of SHIV Infected Macaques to TRAIL Induced T Cell Death. PBLs from SHIV infected (n=14) or negative (n=10) were untreated or treated with 1μg/mL LZhuTRAIL for 16 hours. Cell death was measured by TUNEL.


SHIV Negative
SHIV Infected
Mean MCF
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
TRAIL-R1
TRAIL-R2
TRAIL-R3
TRAIL-R4


p = 0.003
p = 0.03
TRAIL Specific Apoptosis (TUNEL)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
SHIV Infected
SHIV Negative

SECTION 3 :
Modulation of TRAIL in NK Cells by IL-15

3.1 Introduction

The nature of latent HIV reservoirs continues to be a problematic hurdle in complete elimination of quiescently HIV infected cells. Our findings that TRAIL dramatically reduces the size of the latently infected resting memory pool harboring integrated HIV DNA suggested that identifying potential ways to enhance TRAIL expression and activity may be clinically beneficial. A number of cells including T cells, neurons, hepatocytes and fibroblasts express TRAIL. However, cells which function as CTLs or killer cells are attractive candidates to enhance expression. In this regard, murine NK cells stimulated with IL-2 have enhanced FasL expression that results in increased FasL specific killing of murine NK targets (Kayagaki et al., 1999). In contrast, IL-15 stimulation specifically upregulated TRAIL expression which increased NK cytolytic activity (Kayagaki et al., 1999; Zamai et al., 1998). Therefore, we determined whether IL-15 could enhance TRAIL expression on human NK cells and what effect this may have on NK mediated killing of HIV reservoirs.

Natural killer (NK) cells comprise a subset of lymphocyte effector cells of the innate immune response that can be activated and exert antiviral (Atedzoe et al., 1997; Biron, 1997; Biron et al., 1996; Orange et al., 1995, Flamand, 1996 #35) and antitumor (Cretney et al., 2002b; Takeda et al., 2001) host defenses. Lysis by mature NK cells is mediated by two predominant mechanisms: (i) Ca2+ dependent release of perforin and

granzymes (Zamai et al., 1998) and (ii) induction of membrane bound or secreted TNF family of death ligands, FasL (Lowin et al., 1995; Perez et al., 1990) and TNF-α (Zamai et al., 1998). Recently, TRAIL has been shown to contribute to both human (Johnsen et al., 1999; Kashii et al., 1999; Zamai et al., 1998) and mouse (Cretney et al., 2002b; Kashii et al., 1999; Kayagaki et al., 1999; Smyth et al., 2001; Takeda et al., 2001) NK cell protection from tumor metastasis and provides evidence for the central role of death receptor mediated apoptosis in tumor rejection.

IL-15 shares two receptor subunits with the IL-2Rβ/γ_c but combines with a unique α chain to form the IL-15 receptor (Carson et al., 1997; Waldmann et al., 2001). IL-2 and IL-15 have a number of overlapping and contrasting biological activities. *Ex vivo* functional studies and knockout mice demonstrate that IL-2 is important in the growth and survival of Fas induced AICD of CD4 T cells (Van Parijs et al., 1999). In contrast, mice overexpressing IL-15 exhibit high frequencies of memory CD8 T cells as well as inhibits IL-2 mediated AICD through the maintenance of cells in G_0/G_1 phase and downregulation of Fas. IL-15 has also been shown to protect mice from lethal apoptosis induced by anti-Fas (Bulfone-Paus et al., 1997). These results have led to the suggestion that IL-15 acts to stimulate memory phenotypes in CD8 and CD4 cells by inhibiting apoptotic death (Zhang et al., 1998). An additional role of IL-15 in the development, survival and activation of NK cells has been suggested in a number of experimental models (Carson et al., 1997; Cavazzana-Calvo et al., 1996; Suzuki et al., 1997). Mice deficient for IL-15 or IL-15 signalling molecules lack NK cells (Ogasawara et al., 1998; Ohteki et al., 1998). The addition of IL-15 to immature fetal thymic cultures leads to

specific expansion of $CD3^-$, $CD56^-$, $CD94^+$ NK cells (Mingari et al., 1997)). These data suggest that IL-15 regulates the development of both NK cells and CD8 T cells (Kennedy et al., 2000; Lodolce et al., 1998; Zhang et al., 1998). The IL-7 receptor also uses a common β/γc chain and has profound impact on early T cell development (Fry et al., 2001). Emerging data indicates that IL-7 may also have effects on extrathymic T cell differentiation (Fry et al., 2001) in athymic T cell depleted hosts (Fry et al., 2001). *In vivo* administration of IL-7 into mice increased CD8 T cell numbers and enhanced T cell function in the absence of phenotypic changes (Geiselhart et al., 2001). These effects are independent of IL-2 and appear to induce CdK2 kinase activity and Rb phosphorylation, thereby allowing cells to undergo cell cycle progression (Barata et al., 2001). In addition, IL-7 has been shown to upregulate Bcl-2 expression resulting in prolonged cell survival and regenerative capacity (Guillemard et al., 2001). HIV infection is accompanied by a decrease in thymic function (Douek et al., 1998) that is reversed by IL-7 through expansion of CD4 and CD8 HIV specific T cells.

Infection with HIV leads to impairment of IL-2 production and loss of NK cell mediated immunity that is characteristic of progression to AIDS (Lucey et al., 1997; Meyaard et al., 1994). Moreover, HIV infection is accompanied by a significant decrease in activation and proliferative capacity of LPS stimulated PBMC (d'Ettorre et al., 2002) as well as depressed levels of CTL and NK cell activity (Chehimi et al., 1997). A number of cytokines have been shown to enhance NK effector killing. IL-2 has a critical role in modulating NK cell activity but incompletely restores NK function in cells from HIV infected individuals (Kovacs et al., 1995; Loubeau et al., 1997). In contrast,

IL-15 enhances proliferative responses to mitogen, tetanus or HIV specific antigens in PBMC from HIV infected individuals (Seder et al., 1995). Moreover, PBMC from HIV infected patients display lower cytotoxicity against K562 target cells than PBMC from HIV uninfected individuals (Chehimi et al., 1997). However, treatment with IL-15 restored depressed NK activity in PBMC from these patients (Chehimi et al., 1997). Unlike IL-2, IL-15 alone or in combination with TNF-α and PMA does not activate viral expression (d'Ettorre et al., 2002; Lucey et al., 1997) in the latently infected cell lines ACH-2 and U1. Recent studies also suggest a role of IL-7 in restoring CTL activity in HIV infected patients (Barata et al., 2001; Geiselhart et al., 2001; Guillemard et al., 2001; Leong et al., 1997). However, the mechanism of action by these cytokines is not clearly defined. Thus, we examined the role of IL-15 on TRAIL expression in human $CD56^{+}CD16^{+}$ NK cells and determined whether IL-15 stimulated NK cells could be used to target cells harboring HIV.

3.2 Materials and Methods

Cell lines, cultures and cytokines. All cell lines were purchased from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 2mM L-Glutamine and 100U/mL each of penicillin and streptomycin (Canadian Life Technologies Products, Montreal, Canada) unless otherwise stated. IL-2, IL-7 and IL-15 were purchased from R&D Systems (Minneapolis, MN) reconstituted in PBS and frozen at -20°C until use.

Detection of TRAIL and TRAIL receptors by RT-PCR and Flow Cytometry. As described in section 2.2. For detection of surface expression of FasL (NOK-1, Immunex Corporation) and TRAIL/TRAIL receptors, 5 x 10^5 cells were stained with monoclonal primary antibodies as previously described (Lum et al., 2001).

Isolation of primary human NK cells. PBMCs were isolated by Ficoll Hypaque from healthy or HIV infected donors. All subjects provided informed consent according to Ottawa Hospital Ethic Guidelines. Monocyte depleted PBLs (plastic adherance for 3 hrs) were incubated using anti-CD3 labelled beads (Miltenyi Biotec. Aurbun, CA) to remove T cells, followed by positive NK selection using anti-CD16 and anti-CD56 monoclonal antibodies (Miltenyi Biotec. Auburn, CA) according to the manufacturer's instructions. NK cell purity was consistently ≥90% as determined by flow cytometry.

Cytotoxicity and Cell Death Assay. Cell mediated cytotoxicity was assessed using a flow cytometry based assay (Kane et al., 1996). 1 x 10^6 NK effector cells were stimulated for 72 hrs with 10ng/mL of IL-2, IL-7 or IL-15 (R&D Systems, Minneapolis, MN.) in complete media and washed extensively with PBS to ensure no residual cytokine was present at the time of coculture. K562 target cells were grown as described above, collected and washed twice in PBS. Cells were resuspended in phenol red free RPMI to a final density of 10^6 cells/mL. K562 targets were labelled with 10mM of 3,3-Dioctadecyloxacarbocyanine, DiO (Molecular Probes, OR) for 20 minutes at 37°C and washed three times with PBS. Target and effector cells were mixed together at (E/T) ratios of 50:1, 25:1, 12.5:1 and 6.25:1 (Chang et al., 1993). Cells were incubated for 3 hours and 1μg/mL propidium iodide (Sigma, St. Louis, MO) was added and cells were incubated for an additional 20 minutes. Where indicated, target cells were PBMCs from autologous donors and were prepared as with K562 cells. To block ligand / receptor induced cell death, antibodies specific for TRAIL-R or FasL (NOK-1) were used at 1μg/mL. The granzyme B inhibitor, Z-Ala-Ala-Asp(Ome)-CK (ZAAD-cmk) was reconstituted in DMSO and used at 10μM working concentrations. Cells were incubated with ZAAD-cmk for 30 minutes and washed twice before use. Percent specific cytotoxicity was determined by DiO positive / PI positive cells ≥ 5.0%; background was determined for each assay and was ≤ 3.0%.

Isolation of HIV by Limiting Dilution Quantitative Microculture in PBMC from infected patients. NK cells were isolated from HIV infected donors using magnetic bead separation as described above. NK cells were stimulated with IL-2, IL-7 or IL-15 for 72

hours. NK cells were washed extensively and cocultured with autologous PBLs for 16 hours. After coculture, NK cells were depleted from PBL coculture by magnetic bead separation by positive selection using anti-CD16 and anti-CD56. Resulting PBLs were subjected to quantitative microculture (Chun et al., 1997b; Finzi et al., 1997; Lum et al., 2001) to determine the frequency of latently infected cells. The amount of viral replication was quantitated using a) p24 ELISA (Perkin Elmer, Boston, MA) and b) viral RNA (Bayer Diagnostics, Markham, ON), while proviral DNA was used to quantify the amount of integrated HIV DNA, all as previously described (Lum et al., 2001).

Statistical Analysis. Assays with K562 targets using control NK cells or NK cells from HIV infected patients were all performed using the paired t test. For comparison of cytotoxicity between cytokine treated NK cells, the Wilcoxon signed-rank test was used. Maximum likelihood analysis was used to determine the frequency of replication competent CD4 resting memory cells as described previously (Chun et al., 1997b; Finzi et al., 1997; Lum et al., 2001).

3.3 Results

Differential Modulation of TRAIL and FasL Expression by IL-7 and IL-15 activated NK Cells.

Murine NK cells stimulated with IL-15 exhibit enhanced TRAIL expression and TRAIL dependent cytolytic activity against L929 target cells (Kayagaki et al., 1999). In order to characterize the effects of IL-15 on expression of TRAIL/TRAIL receptors in human NK cells, NK cells from healthy or HIV infected donors were isolated and stimulated for 72 hours with 10ng/mL of IL-2, IL-7 or IL-15. No significant changes in TRAIL receptors 1, 2, 3 or 4 mRNA was detected in NK cells stimulated with IL-2, IL-7 or IL-15 when compared to untreated NK cells (Fig 8A). However, the amount of TRAIL transcripts was increased 4.9 fold in NK cells treated with IL-15 (Fig 8A – top). Interestingly, FasL mRNA doubled with IL-7 treatment whereas TRAIL mRNA was unaffected by IL-7 (Fig 8A). When identical experiments in NK cells from HIV infected individuals were performed, in every subject IL-2, IL-7 and IL-15 had no effect on TRAIL receptor 1, 2, 3 and 4 mRNA (Fig 8B). IL-15 increased the level of TRAIL transcripts by 4.5 fold, while transcripts for FasL were unchanged (Fig 8B). In contrast, IL-7 treatment increased the amount of FasL mRNA nearly 3.2 fold (Fig 8B). To determine whether the changes in mRNA could be observed at the protein level, flow cytometry was used to analyze receptor expression at the cell surface. Consistent with RT-PCR data, IL-2, IL-7 and IL-15 did not affect surface expression of TRAIL receptor 1, 2, 3, 4 (data not shown). As seen in figure 9, TRAIL and FasL were differentially

Figure 8. RT-PCR Analysis of TRAIL Receptor, TRAIL and FasL/CD95L mRNA in NK cells Isolated from PBMCs of (A) uninfected and (B) HIV infected donors. $CD3^-/CD16^+/CD56^+$ NK cells were stimulated for 72 hours with control, or 10ng of IL-2, IL-7 or IL-15. Total RNA was extracted and was analyzed for the presence of TRAIL receptor, TRAIL and FasL transcripts. Band intensity was normalized to levels of actin. Data are representative of 3 independent experiments.

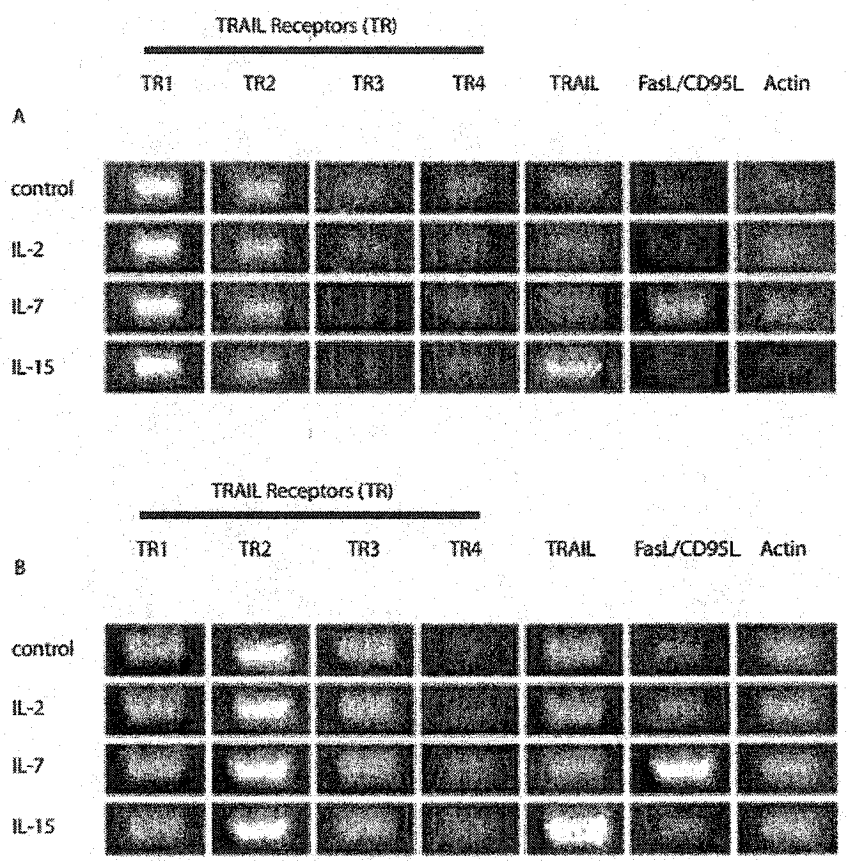
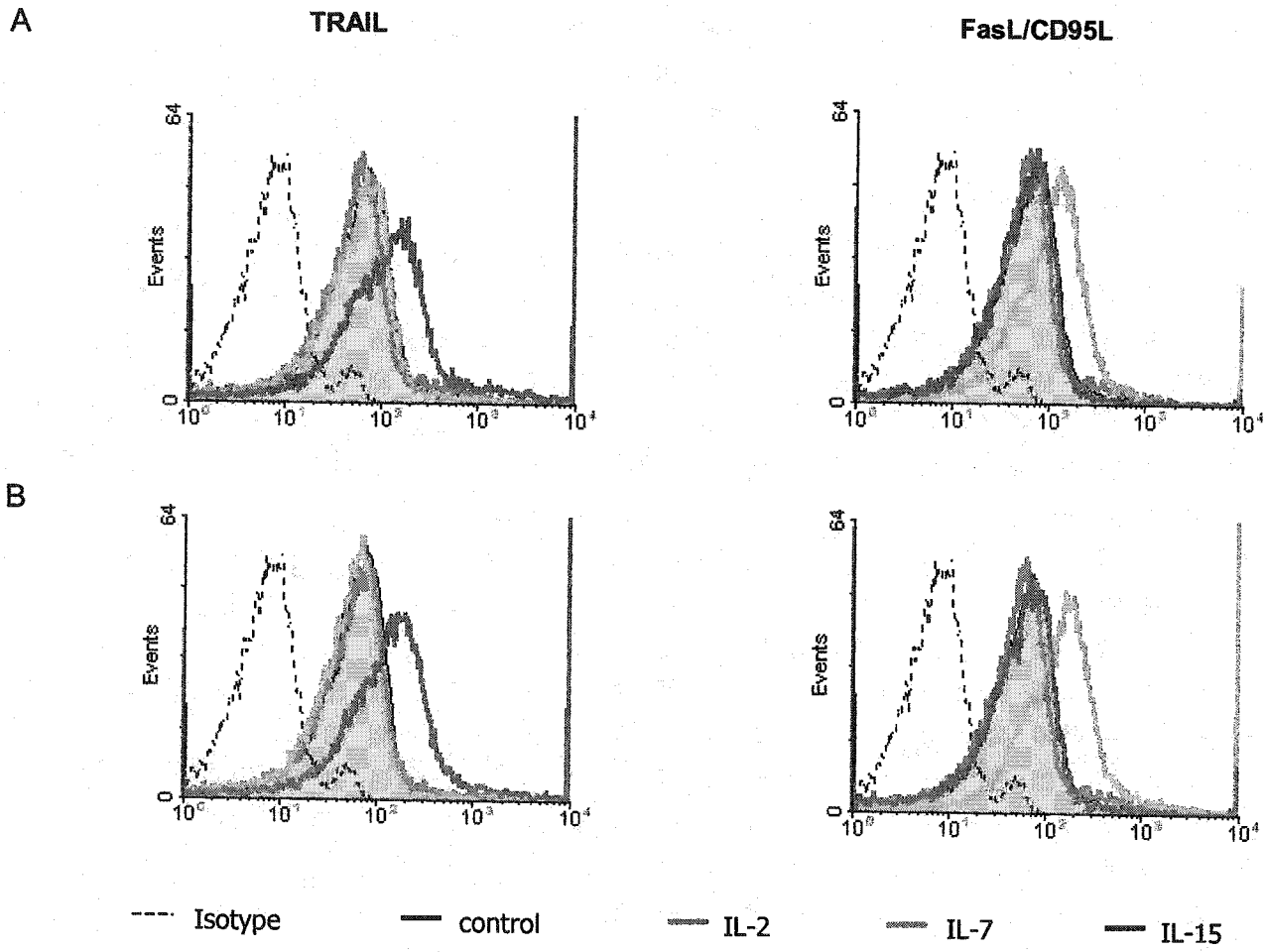

TRAIL Receptors (TR)
TR1
TR2
TR3
TR4
TRAIL
FasL/CD95L
Actin
A
control
IL-2
IL-7
IL-15
TRAIL Receptors (TR)
TR1
TR2
TR3
TR4
TRAIL
FasL/CD95L
Actin
B
control
IL-2
IL-7
IL-15

Figure 9. Phenotypic surface analysis of TRAIL and FasL/CD95L following stimulation of isolated NK Cells. (A – top panel) $CD56^{+}/CD16^{+}$ NK cells from uninfected donors have increased TRAIL expression following 72 hour exposure to 10ng/mL IL-15, but not with IL-2 or IL-7 treatments. Whereas, the amount of TRAIL expression increased by 1.5 fold (MCF = 9.4 vs IL-15 = 14.1, p = 0.03) with IL-15, FasL/CD95L increased by 3.4 fold with IL-7 (MCF = 10.2 vs IL-7 = 35.1, p = 0.03) but not IL-15 treatments. (B – bottom panel) $CD56^{+}/CD16^{+}$ NK cells from HIV infected patients have similar changes in TRAIL and FasL/CD95L expression. While a 2.8 increased (MCF = 7.4 vs IL-15 = 20.4, p = 0.03) was observed in NK cells from infected patients, no effect was seen with IL-7. In contrast, IL-7 significantly increased FasL/CD95L expression by 2.0 fold (MCF = 7.7 vs IL-7 = 15, p = 0.04). All histograms are representative of 4 independent analysis (n=4) and background MCF was normalized using isotype control antibodies.

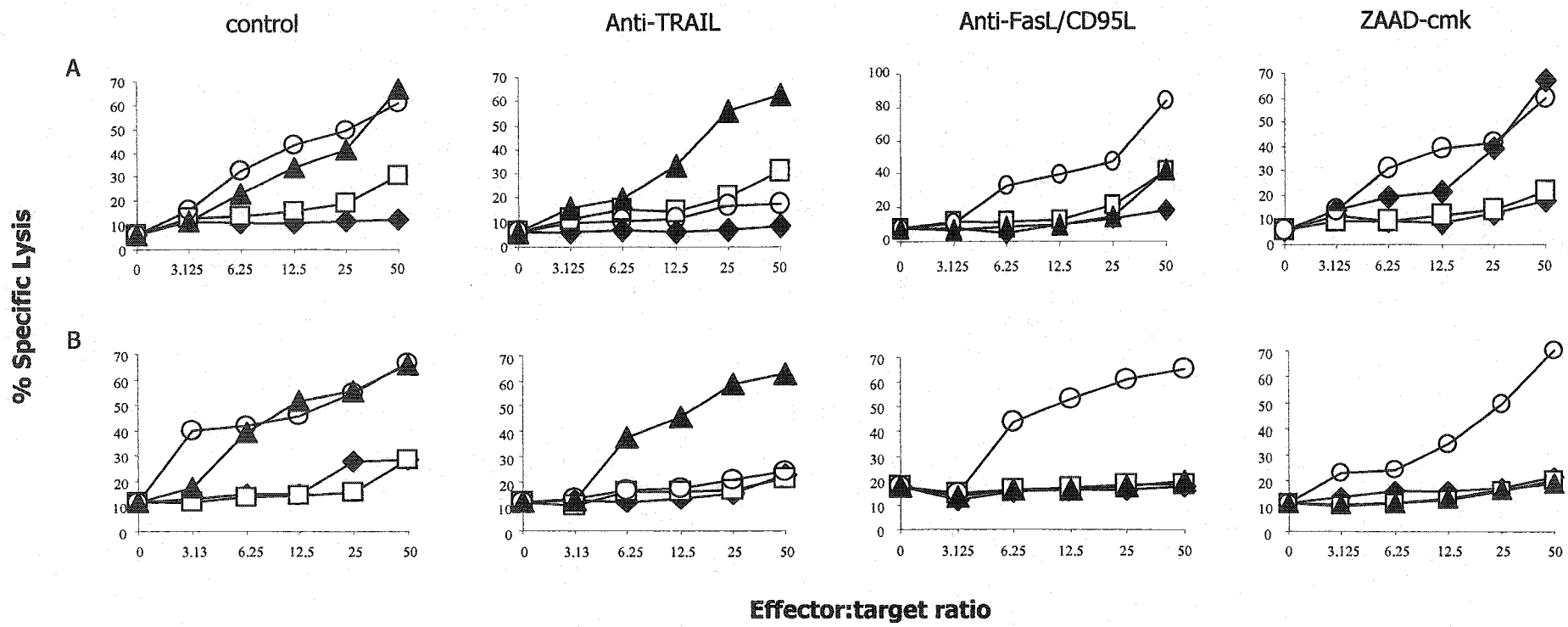

A
TRAIL
FasL/CD95L
B
Events
64
0
Isotype
control
IL-2
IL-7
IL-15

upregulated by IL-15 and IL-7 on NK cells of uninfected (Fig 9A) and HIV infected individuals (Fig 9B) respectively. In HIV uninfected subjects, TRAIL expression was increased by 1.5 fold following stimulation with IL-15 compared with control. Conversely, treatment with IL-7 but not IL-15 resulted in 3.4 fold increase in FasL expression. Similar changes were observed in NK cells from HIV infected patients. IL-15 treatment resulted in a 2.8 fold upregulation of TRAIL expression. However, FasL surface expression was doubled with IL-7 treatment. Thus, IL-15 but not IL-7 or IL-2, is a potent inducer of TRAIL expression in NK cells, while IL-7 and not IL-15 or IL-2 induces FasL expression. This suggests that IL-7 and IL-15 can have distinct effects on the expression of the death ligands FasL and TRAIL.

Enhanced cytolytic activity of NK cells by IL-7 and IL-15.

To directly show that IL-15 induction of TRAIL expression is associated with enhanced NK cytolytic activity, cytotoxic assays were performed using flow cytometry (Kane et al., 1996). Consistent with observations by other investigators (Atedzoe et al., 1997; Loubeau et al., 1997; Lucey et al., 1997), IL-15 treatment of NK cells from HIV uninfected individuals resulted in significant cell death of K562 target cells ($p=0.02$) when compared to untreated cultures (Fig 10A). The addition of TRAIL neutralizing Abs completely abolished IL-15 mediated killing ($p=0.02$) (Kashii et al., 1999; Kayagaki et al., 1999; Zamai et al., 1998). In contrast, neutralizing FasL Abs had no effect on the cytolytic activity of IL-15 ($p=0.14$) stimulated NK cells. Unexpectedly, treatment of NK cells from uninfected donors with IL-7 increased the amount of K562 cell death ($p=0.05$).

Figure 10. Differential effects of IL-7 and IL-15 NK cytolytic activity *in vitro*. (A – top panel) $CD16^{+}/CD56^{+}$ NK cells from uninfected donors or (B – bottom panel) HIV infected patients were stimulated with 10ng/mL of each, control (♦), IL-2 (□), IL-7 (▲) or IL-15 (○) for 72 hours. NK cytolytic activity was performed for 3 hours in the presence or absence of 1.0ug/mL neutralizing TRAIL or FasL antibodies, or 10uM ZAAD-cmk as indicated. K562 target cells were labeled with 10uM DiO for 20 minutes and 1ug/mL propidium iodide prior to FACS analysis. Enhanced killing of target cells was seen in IL-15 stimulated NK cells which was completely inhibited by anti-TRAIL antibodies, but neither anti-FasL/CD95L or ZAAD-cmk had any observable effects. IL-7 lysis was blocked completely by anti-FasL/CD95L antibodies. ZAAD-cmk partially inhibited death of IL-7 treated NK cells in HIV infected cultures.


control
Anti-TRAIL
Anti-FasL/CD95L
ZAAD-cmk
A
B
% Specific Lysis
Effector:target ratio
0
3.125
3.13
6.25
12.5
25
50

Whereas IL-7 induced killing was significantly reduced by neutralizing FasL mAbs ($p=0.04$), little change in IL-7 stimulated NK cells was observed with neutralizing TRAIL Abs ($p=0.9$). The granzyme B inhibitor, ZAAD-cmk had no effect on IL-15 killing on NK cells ($p=0.8$) from uninfected donors. This effect was distinct from the results with IL-7 where FasL was the predominant pathway of enhanced NK killing of K562 targets. These results indicate that IL-15 upregulates TRAIL expression and enhances TRAIL specific cytolytic activity of NK cells from uninfected donors.

IL-15 enhances TRAIL cytolytic activity of NK cells from HIV infected individuals.

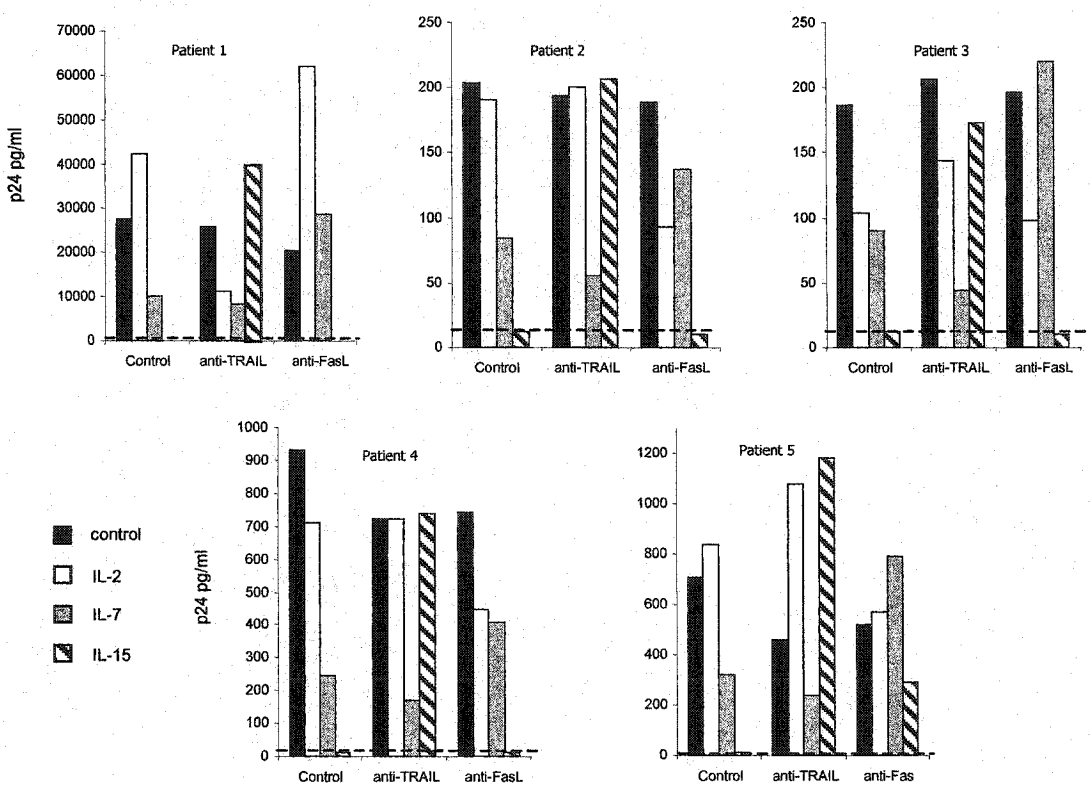
Numerous groups have shown that IL-15 has profound effects on inducing strong proliferative and cytolytic responses in NK cells from HIV infected patients (Atedzoe et al., 1997; Chehimi et al., 1997; Lucey et al., 1997). Therefore, to demonstrate that increased TRAIL expression by IL-15 results in enhanced functional TRAIL activity in NK cells from HIV infected individuals, similar experiments were performed as described above. Consistent with published results, NK cells treated with either IL-7 ($p=0.04$) or IL-15 ($p=0.01$) displayed significant cytolytic activity against K562 target cells (Fig 10B). IL-7 mediated killing was FasL dependent as anti-FasL antibodies blocked death of K562 targets ($p=0.03$). In contrast, antibodies to TRAIL completely blocked IL-15 induction of cell death in K562 cells ($p=0.006$). Interestingly, ZAAD-cmk completely inhibited IL-7 mediated killing ($p=0.01$) which is in contrast to NK cells from uninfected individuals where IL-7 induced killing was unaffected by ZAAD-cmk. These results suggest that IL-15 enhances NK cytolytic activity in HIV infected patients and

this activity is mediated by the death ligand TRAIL. In contrast, IL-7 treated NK cells mediated killing of K562 cells through FasL and granzyme B.

Enhanced Killing of IL-7 and IL-15 stimulated NK cells against autologous resting memory CD4 T cells.

Previous studies have suggested that IL-15 stimulates expansion of HIV specific CTLs (Kanai et al., 1996). Additionally, treatment of PBMC from HIV infected patients with IL-15 increases NK cell cytotoxicity (Chehimi et al., 1997). Thus, we investigated whether increased functional TRAIL expression on IL-15 activated NK cells can kill latent HIV reservoirs. NK cells were isolated from HIV patients who were on combination ART and who had undetectable levels of viral RNA for greater than 12 months. The cells were stimulated with IL-2, IL-7 or IL-15 for 72 hrs. PBLs from the same donors were used as target cells in a 16 hr coculture. NK cells were subsequently depleted from the coculture and the resulting PBLs were subjected to limiting dilution quantitative microculture (Chun et al., 1997b; Finzi et al., 1997). In all 5 subjects, replication competent virus as measured by p24 antigen production could not be isolated in PBL cultures that had been subjected to IL-15 stimulated NK cells (Fig 11). The addition of neutralizing TRAIL antibodies abrogated the effects of IL-15 stimulated NK cell cytolytic activity. Although virus was detectable in cells stimulated with IL-7, the level of virus present was substantially lower than untreated cultures (Fig 11, $p = 0.01$). However, the addition of anti-FasL antibodies to IL-7 treated NK cells augmented viral p24 levels indicating that anti-FasL blocked IL-7 mediated cytolytic activity of NK cells

Figure 11. Undetectable levels of p24 antigen (pg/mL) in PBLs coculture with IL-15 NK cells *in vitro*. NK cells from HIV infected patients were incubated with cytokines for 72 hours followed by coculture with autologous PBL as target cells. The resulting assay was performed for 3 hours in the presence or absence of neutralizing TRAIL or FasL/CD95L antibodies. $CD56^{+}/CD16^{+}$ NK cells were subsequently depleted and the remaining PBLs were subjected to limiting dilution quantitative microculture as previously described. Replication competent virus was not isolated in 5 of 5 subjects with IL-15 treatment of NK cells. The addition of anti-TRAIL antibodies inhibited IL-15 killing resulting in detectable levels of replication competent virus. Dashed line indicates a 11.3 pg/ml p24 antigen limit of detection.


Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
p24 pg/ml
Control
anti-TRAIL
anti-FasL
anti-Fas
control
IL-2
IL-7
IL-15

(Fig 11). We confirmed this data using an ultrasensitive viral RNA detection system which has a limit of detection of 50 viral RNA copies. In all cases using this analysis, viral RNA was not detectable in samples treated with IL-15 (data not shown). Using maximum likelihood analysis to estimate the number of IUPM, the average estimate of virus obtained in 5 untreated cultures was 6.79 IUPM, while treatment with IL-15 resulted in 1.01 IUPM (Table 3.0). The addition of anti-TRAIL in IL-15 treated NK cell cultures abrogated this reduction where IUPM increased to 4.20. Interestingly, the IUPM of IL-7 treated NK cells was significantly higher than controls. When we analyzed samples for cellular integration of viral DNA, 3 of 5 cultures revealed undetectable levels of integrated HIV DNA measured using semiquantitative PCR (data not shown, Table 3.0 denoted by asterisks). However, in cocultures which were performed in the presence of anti-TRAIL antibodies, the amount of viral DNA recovered was similar to controls. These results demonstrate that IL-15 treated NK cells exert TRAIL mediated killing of cells and dramatically reduces the number of cells containing stably integrated HIV DNA. Moreover, IL-7 treated NK cells resulted in enhanced killing via FasL as demonstrated by the inhibition of IL-7 killing using anti-FasL antibodies.

Table 3.0. Reduction in IUPM of HIV Infected PBMCs Following Cytokine Treatment of NK cells. IUPM was analyzed by maximum likelihood method following micrococulture. NK cells were stimulated with cytokines followed by coculture using autologous PBLs as target cells. Asterisks indicates cultures where viral DNA was undetectable by semiquantitative PCR.

Patient ID		IUPM			
		Control	IL-2	IL-7	IL-15
1	Control	8.08	8.08	236.17	1.01*
	Anti-TRAIL	1.61	1.61	236.17	8.08
	Anti-FasL	8.08	1.61	236.17	1.61
		Control	IL-2	IL-7	IL-15
2	Control	1.61	1.61	236.17	1.01
	Anti-TRAIL	1.61	1.61	236.17	1.61
	Anti-FasL	1.61	1.61	236.17	1.01
		Control	IL-2	IL-7	IL-15
3	Control	8.08	1.61	236.17	1.01*
	Anti-TRAIL	1.61	1.61	236.17	1.61
	Anti-FasL	1.61	1.61	236.17	1.01
		Control	IL-2	IL-7	IL-15
4	Control	8.08	8.08	236.17	1.01*
	Anti-TRAIL	8.08	8.08	236.17	8.08
	Anti-FasL	8.08	8.08	236.17	1.01
		Control	IL-2	IL-7	IL-15
5	Control	8.08	8.08	236.17	1.01
	Anti-TRAIL	1.61	1.61	236.17	8.08
	Anti-FasL	8.08	8.08	236.17	1.01

* undetectable viral DNA levels

3.4 Discussion

Because of the safety issues discussed in section 2.4, recombinant preparations of TRAIL will require further *in vivo* safety assessment. Therefore, we have examined ways to modulate TRAIL expression, in particular through the use of cytokines to enhance TRAIL expression in human NK cells. NK cells represent a major innate effector mechanism in response to viral infections. Previous studies using FasL and perforin deficient mice indicate that these effector molecules constitute the major pathway of NK cytotoxicity (Kayagaki et al., 1999). Recent evidence supports the role of TRAIL in tumor surveillance, suggesting that FasL and TRAIL may have unique *in vitro* physiological functions and are differentially regulated (Cretney et al., 2002b; Screpanti et al., 2001; Takeda et al., 2001). In this regard, Zamai et al., report that immature and mature human NK cells have distinct usage of TRAIL and FasL (Zamai et al., 1998). Indeed TRAIL expression is preferentially induced on NK cells after stimulation with IL-2 and IL-15 (Kayagaki et al., 1999). However, the expression of functional TRAIL in these mice models was restricted to $CD3^-/NK1.1/DX5^+$ NK cells from the liver (Takeda et al., 2001). In this report, we demonstrate that human $CD3^-/CD56^+/CD16^+$ NK cells from PBMCs express all TRAIL receptors, TRAIL and FasL. The expression of TRAIL receptors was unchanged following treatments with IL-2, IL-7 and IL-15. However, induction of TRAIL expression was observed in NK cells cultured with IL-15 (Kayagaki et al., 1999; Takeda et al., 2001; Zamai et al., 1998) but neither IL-2 or IL-7 had any significant effect on TRAIL expression. Unexpectedly, FasL expression increased in response to IL-7. In contrast, IL-15 and IL-2 had no effect on FasL expression.

Although we could not detect any increase in TRAIL expression with IL-2 treatment as previously reported, this may reflect the differences in human and mouse NK cells as well as the source of NK cells (i.e. liver or PBMC NK cells). These results indicate distinct regulation of TRAIL and FasL by IL-15 and IL-7.

A recent finding that TRAIL is involved with HIV infected T cell depletion in hu-SCID-PBL models (Miura et al., 2001) suggested that TRAIL participates in the pathogenesis of HIV infection. Moreover, in contrast to cells from uninfected human donors, cells from HIV infected individuals undergo apoptosis following treatment with TRAIL (Jeremias et al., 1998; Katsikis et al., 1997; Lum et al., 2001). We previously showed that latently infected resting memory cells may be the target of TRAIL killing. It is unclear how to modulate TRAIL expression although type 1 cytokines such as IFNγ have been shown to enhance TRAIL in monocytes (Sedger et al., 1999). Recent reports showing the contribution of IL-15 in enhancing the cytolytic activity of NK cells suggested that IL-15 may have similar effects on NK cells from HIV infected patients. In all cases tested, NK cells cultured with IL-15 substantially enhanced TRAIL mediated killing of target cells, whereas IL-7 treatments resulted in FasL dependent cytotoxicity. The findings that a) NK cells mediate apoptosis through the death ligand TRAIL (Biron, 1997; Kashii et al., 1999; Kayagaki et al., 1999; Oliva et al., 1998; Screpanti et al., 2001; Smyth et al., 2001; Zamai et al., 1998) and b) IL-15 potentiates NK cytolytic activity (Azimi et al., 2000; Carson et al., 1997; Chehimi et al., 1997; d'Ettorre et al., 2002; Katz et al., 1987; Loubeau et al., 1997; Mastroianni et al., 2000) suggested that IL-15

stimulated NK cells from HIV patients may exert targeted killing of cells considered to be reservoirs for HIV.

The underlying NK cytolytic dysfunction seen during HIV infection is not well understood. Several groups demonstrated that IL-2 therapy both *in vitro* and *in vivo* may have little impact on viral reservoirs and could possibly serve to induce further viral replication (Chun et al., 1999; Dybul et al., 2002). IL-2 incompletely restores NK function in HIV infected patients, however the reasons for this are not known. One possible explanation may be that IL-2 has no effect on the death ligands TRAIL and FasL. In cytolytic assays performed using IL-15 stimulated NK cells, we observed a dramatic increase in NK activity against K562 target cells. These effects were completely inhibited by neutralizing TRAIL specific mAb indicating that IL-15 drives TRAIL restricted cytolytic killing which is consistent with published data in murine NK cells where inhibition of TRAIL blocked lysis of murine NK target cells (Zamai et al., 1998). In contrast to IL-2 and partially IL-7, IL-15 restores cytolytic activity of NK cells even in patients infected with HIV. Interestingly, in patients infected with HIV we observed an increase in IL-7 mediated NK cell activity which was completely blocked by the granzyme B inhibitor, ZAAD-cmk, suggesting that IL-7 mediated death involves both FasL and granzyme B pathways. Additionally, we found that ZAAD-cmk had no effect on IL-7 killing in NK cells from uninfected subjects. This suggests that NK cells may have additional effector mechanisms in the granzyme B pathway which is activated during HIV infection and is not apparent in uninfected NK cells. The differential use of FasL and TRAIL in the NK model, may also shed important information regarding

selection of thymocytes in early T cell development. During T cell development in the thymus, $CD4^+CD8^+$ T cells undergo selection resulting in apoptosis of a large number of double positive non-reactive T cells (Kishimoto and Sprent, 2000a; Kishimoto and Sprent, 2000b). Although IL-7 plays a role in protection of thymocyte apoptosis during HIV infection, it is unclear whether FasL expression by IL-7 has similar effects on central and peripheral T cell homeostasis. A recent report suggests that thymic development was independent of TRAIL (Simon et al., 2001). Therefore, our observations of differential use of IL-7 and IL-15 inducing FasL and TRAIL may have physiological implications on central and peripheral T cell development.

One critical unresolved issue is the maintenance of TRAIL death receptor expression and TRAIL sensitivity in the resting memory CD4 T cell subset despite low viral replication in patients on ART. Both studies performed by our group and others suggest that Tat (Zhang et al., 1999b) and gp120/gp120 complexes upregulate TRAIL and TRAIL receptor expression (Lum et al., 2001 , unpublished and see section 4.3) in T cells and macrophages. It remains a formal possibility that gp120 or whole viral particle interactions with dendritic cells via DC-SIGN may provide additional signals to maintain TRAIL and TRAIL receptor expression in the absence of active viral transcription. Studies are currently underway in our laboratory to examine this phenomenon.

We have now expanded the findings and determined that IL-15 activated NK cells could target cells which harbour stably integrated HIV DNA using a modified NK cytolytic coculture technique. In these experiments, we chose to use unfractionated bulk

PBLs as opposed to highly enriched CD4 resting memory cells as autologous targets for the following reason. Although latent HIV reservoirs have uniformly been considered to be CD4 resting memory cells, we cannot exclude the formal possibility that other unidentified cell subsets within the PBL fraction may also harbour latent HIV. Therefore, we chose to use bulk unfractionated PBL as NK target cells to test whether any PBL subset harbouring latent HIV could be removed by IL-15 treatments. In all 5 subjects, IL-15 stimulated NK cells cocultured with autologous PBL did not result in the detection replication competent virus. IUPM assessment revealed a significant reduction in the pool size of cells harboring HIV, in 5 of 5 cases. A somewhat unexpected observation is that IL-7 stimulated NK cells increase the amount of virus present in autologous co-cultured PBL. Although potential reasons for this increase are speculative, these data are consistent with the known ability of FasL to induce proliferation, particularly in resting cells (such as latently HIV infected resting memory T cells) (Alderson et al, 1993). Most notably, in 3 of 5 IL-15 stimulated NK cultures, HIV proviral DNA could no longer be detected. When TRAIL blocking antibodies were used to inhibit NK killing, cells harboring replication competent virus and viral DNA were recovered. Our results indicate that IL-15 activation of NK cells preferentially mediates TRAIL specific killing of cells containing HIV. In addition, we report for the first time enhanced expression and lytic activity of FasL on NK cells following IL-7 stimulation. Collectively, this provides further support for the role of IL-15 as an immunotherapy against HIV.

SECTION 4:
Upregulation of TRAIL Expression and Sensitization of Cells to Apoptosis Through Ligation of the HIV Chemokine Coreceptors

4.1 Introduction

The two studies in the last sections have shed important information on the regulation of TRAIL/TRAIL receptor expression during HIV infection. However, one of the outstanding questions in TRAIL biology is the issue of how a normal cell which is resistant to TRAIL induced cell death acquires a sensitive phenotype following viral infection or tumor transformation. Two models exist to explain in part this phenomena. The first involves the relative abundance of the R1/R2 death receptors versus R3/R4 non signaling decoy receptors. While the expression of TRAIL-R1/R2 death receptors is important in some cell lines, a number of melanoma cells express high levels of death receptors despite being exceedingly resistant to TRAIL killing (Griffith and Lynch, 1998). Therefore, this model is insufficient to account for the acquisition of TRAIL sensitivity. The second model infers that cellular inhibitors of apoptosis, namely high levels of cellular FLICE inhibitor protein (cFLIP) expression, block TRAIL induced death (Irmler et al., 1997; Kreuz et al., 2001; Lens et al., 2002; Siegmund et al., 2001). Recent evidence by Zhang et al. suggested HIV Tat modulates TRAIL and TRAIL receptor expression in HIV infected macrophages (Zhang et al., 2001). Therefore, to examine the molecular pathway whereby cells gain sensitivity to TRAIL killing, we studied the effect of HIV proteins on TRAIL receptor expression and TRAIL sensitivity.

The interaction of gp120 with T cells bearing the CD4 receptor is the initial entry event requiring one of several chemokine receptors belonging to the G-coupled seven transmembrane protein family (Baggiolini et al., 1997). Chemokine receptor usage depends on the tropism of HIV. M-tropic viruses primarily infect myeloid cells through CCR5 whereas T-tropic viruses primarily infect lymphocytes through CXCR4 (Deng et al., 1996; Deng et al., 1997; Dragic et al., 1996). X-ray crystallographic structural analysis demonstrate that binding of HIV gp120 to CD4 induces a conformation change that results in an interaction with the coreceptors (Deng et al., 1996; Deng et al., 1997; Dragic et al., 1996). It has also been shown that HIV can infect by a CD4 independent pathway (Endres et al., 1996). HIV infection is associated with enhanced apoptosis in both infected an uninfected CD4 T cells (Badley et al., 1997; Finkel et al., 1995; Laurence et al., 1996; Lenardo et al., 2002; Muro-Cacho et al., 1995; Roshal et al., 2001; Selliah and Finkel, 2001). It has been proposed that cross linking of CD4 T cells by gp120 causes Fas mediated apoptosis (Banda et al., 1992), downregulation of Bcl-2 (Hashimoto et al., 1997) and activation of caspases (Cicala et al., 2000). Yet more recently, it has been demonstrated that killing of CD4 T cells is more characteristic of necrotic death (Bolton et al., 2002) which is independent of gp120 (Lenardo et al., 2002). However, in the latter studies the necrotic death observed occurred predominantly in the infected population whereas in the former studies, apoptotic death occurred in uninfected cells. It has also been proposed that death receptor mediated activated induced cell death may play a role in T cell depletion during HIV infection. Both *in vitro* and *in vivo*, HIV infection is associated with an activated T cell phenotype (Gougeon et al., 1996), increased Fas expression and susceptibility to Fas mediated death (Boudet et al., 1996;

Katsikis et al., 1997; Katsikis et al., 1995; Westendorp et al., 1995). Administration of Fas, TRAIL or TNF antagonists reduces AICD supporting the casual role of death receptor/ligand interactions in T cell death induced by HIV (Dockrell et al., 1999; Katsikis et al., 1997). Thus, we examined the role of gp120 on TRAIL/TRAIL receptor regulation.

To date, stromal derived factor 1 (SDF-1) is the only known ligand for the CXCR4 receptor and its expression increases following bacterial, viral, allergic or autoimmune stimuli (Baggiolini et al., 1997; D'Apuzzo et al., 1997; Gonzalo et al., 2000). Although not considered an inflammatory chemokine, SDF-1 is a potent chemoattractant for T cells, B cells, and leukocytes. Apoptosis induced by gp120 has been shown to involve CXCR4 independently of other HIV receptors (Berndt et al., 1998). This suggested that CXCR4 and SDF-1 may play a role in physiological death processes which are independent from HIV infection. Therefore, SDF-1 may cause AICD in susceptible cells expressing CXCR4, such as neutrophils in a manner analogous to AICD of T cells. Furthermore, we postulated that SDF-1 may induce TRAIL expression and cause TRAIL specific AICD. This would indicate an alternative role for TRAIL irrespective of its role as a molecule of tumor surveillance.

4.2 Materials and Methods

Cell Lines and Reagents. Jurkat T cells were obtained from ATCC (Rockville, MD) and maintained in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 2mM L-Glutamine and 100U/mL of each penicillin and streptomycin (Canadian Life Technologies Products, Montreal, Canada) unless otherwise stated. HOS cell lines (gift from Dr. Karen Copeland), HL60 (ATCC) and HT29 (ATCC) were maintained in DMEM, 10% FBS, 100U/mL penicillin and streptomycin. PBLs from healthy subjects were isolated by Ficoll Hypaque following informed consent according to Guidelines of the Ottawa Hospital and prepared as follows: PBMCs were depleted of monocytes by plastic adherence for 16-18 hrs (in 10% human AB serum) followed by two washes in PBS and resuspension in media containing AB serum. Cells were seeded in a 96 well round bottom plate (Costar) followed by 16-18 hrs incubation with monomeric recombinant gp120 (Immunodiagnostics), recombinant SDF-1 (R&D Systems), anti-CD4 (Leu3A), anti-CXCR4 (12G5), anti-CD3, anti-CCR5 or PHA (Sigma, St. Louis) at the indicated concentrations (all antibodies from BD Pharmingen). After 16-18 hours, LZhuTRAIL was added for an additional 8-16 hours and apoptosis was measured by annexin V-FITC/PI staining. Pertussus toxin (PT; R&D Systems) was added directly to cultures at 5ng/mL for 3-4 hours followed by three washes and resuspension of cells in culture media. Brefeldin A (BA; Sigma) was reconstituted in DMSO and stored at -20°C. BA was added directly to cultures at 10ng/mL for 2 hours followed by extensive washes in PBS.

Detection of TRAIL/TRAIL receptors. This analysis was performed identical to that described in section 2.0.

Isolation of Neutrophils. Whole blood from healthy donors was mixed thoroughly in ACD (3% w/v citric acid, 6% w/v sodium citrate and 4% w/v dextrose in ddH_20 stored at 4°C) in a 5:1 ratio. ACD/blood mixture was overlaid on a 5% Dextran/6%NaCl solution and inverted 20 times to ensure adequate mixing. Tubes were left standing at room temperature for 45 minutes. Supernants were collected and spun at 1150 rpm for 12 minutes with low brake. Pellets were resuspended in 12mL ice cold ddH_20. 4 mL of 0.6M KCl was added and mixed several times. The mixture was diluted to 50 mL with PBS and spun at 1300 rpm for 6 minutes. This was repeated 3-4 times or until no erythrocytes remained. Final cell pellets were resuspended 2.5mL PBS, overlaid on 3 mL Ficoll Hypaque (Amersham Pharmacia, Uppsula, Sweden) and spun for 30 minutes at 1500 rpm. Supernants were gently removed using a transfer pipette and pellets containing neutrophils were resuspended in 2mL HBSS (Canadian Life Technologies). Flow cytometry was used to verify purity of neutrophils using anti-CD16-PE (gift from Dr. Ruth Wilkins).

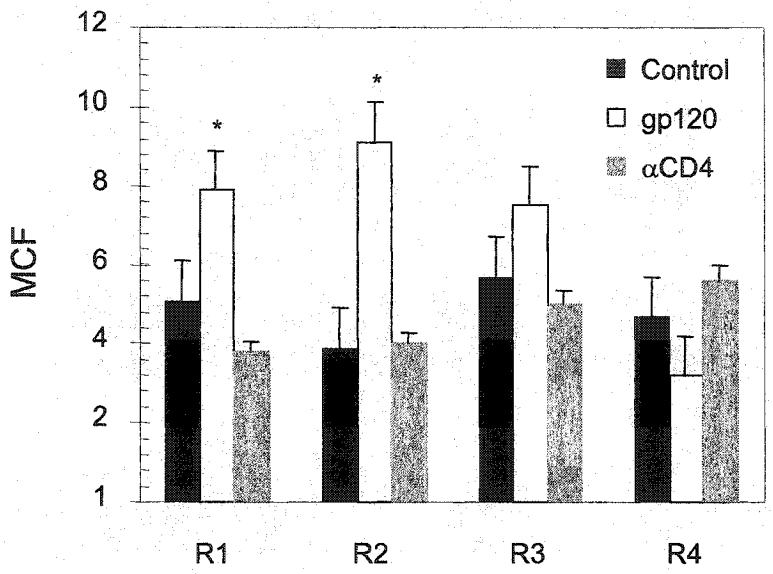
4.2 Results

Upregulation of TRAIL Death Receptors and TRAIL by gp120.

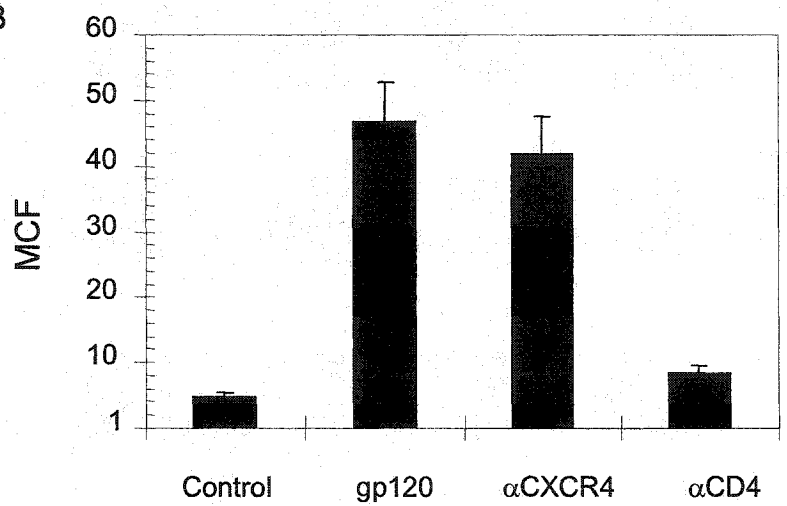
Using TRAIL and TRAIL receptor specific monoclonal antibodies, all four TRAIL receptors and ligand were detected by flow cytometry on the surface of Jurkat T cells. Jurkats treated with rgp120, but not anti-CD4 or control had a 1.5 fold increase in TRAIL-R1 expression ($n=3$, $p < 0.03$) and a 2.3 fold increase in TRAIL-R2 expression ($n=3$, $p < 0.002$). No significant changes were observed in TRAIL-R3 or R4 expression (Fig 12A). When Jurkats were stimulated with anti-CXCR4 ($p < 0.005$ versus control) or rgp120 ($p < 0.003$ versus control), a dramatic increase in TRAIL expression was observed compared to anti-CD4 or control (Fig 12B). The increase in TRAIL-R2 was dose responsive and could be mimicked using anti-CXCR4 ($n=3$, $p < 0.03$, Fig 12C). Activation with PHA or anti-CD3 did not cause changes in TRAIL receptor expression (data not shown). In primary PBLs, TRAIL-R2 expression increased 2.3 times while TRAIL expression increased 1.7 times following stimulation with rgp120 (Fig 13). Using anti-CXCR4 treatments, a 3.3 fold upregulation of TRAIL-R2 was observed (Fig 13). Control or anti-CD4 treatments had no effect on either TRAIL-R2 or TRAIL expression (Fig 13) nor could any changes be detected in TRAIL R1, R3 or R4 expression following stimulation (data not shown).

Figure 12. Jurkat T cell expression of TRAIL Death Receptors and TRAIL is upregulated by HIV gp120 and anti-CXCR4. (A) Jurkat T cells were stimulated with control, anti-CD4 or $rgp120_{IIIB}$ for 12-16 hours. Cell surface expression of TRAIL/TRAIL receptor was monitored by flow cytometry. TRAIL-R1 and –R2 is increased following rgp120 treatments but not by anti-CD4 or control, * $p < 0.03$. No change in TRAIL-R3 or –R4 was observed. (B) TRAIL expression is upregulated by rgp120 and anti-CXCR4, $p < 0.02$. (C) Dose dependent increase in R2 and TRAIL expression following rgp120 and anti-CXCR4 treatments. Data represents 5 independent experiments.

A


12
10
8
6
4
2
1
MCF
*
*
Control
gp120
αCD4
R1
R2
R3
R4

B


60
50
40
30
20
10
1
MCF
Control
gp120
αCXCR4
αCD4

C

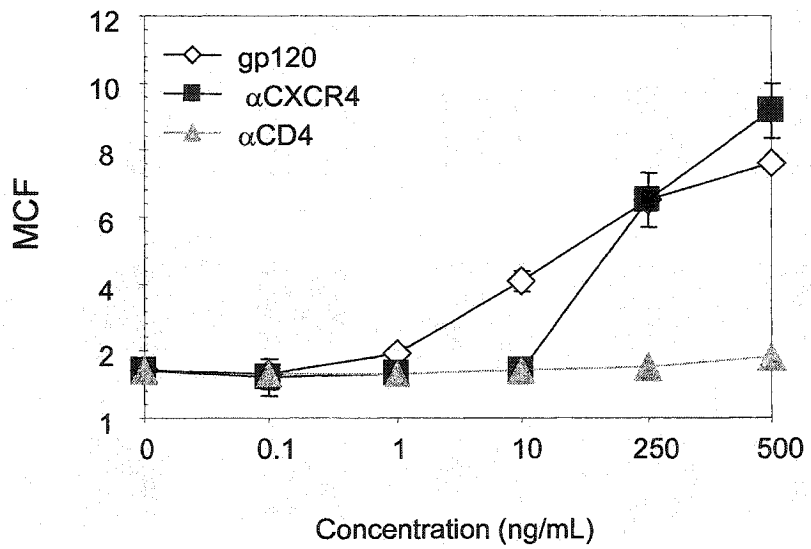
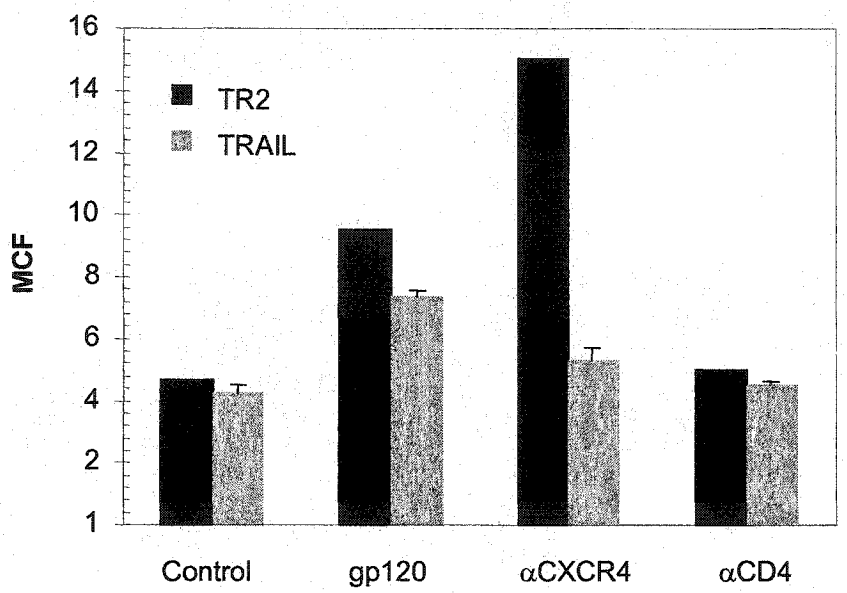

12
10
8
6
4
2
1
MCF
gp120
αCXCR4
αCD4
0
0.1
1
10
250
500
Concentration (ng/mL)

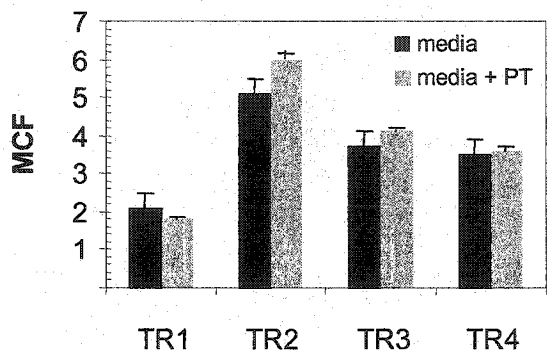
Figure 13. PBL expression of TRAIL-R2 and TRAIL is upregulated by HIV gp120 and anti-CXCR4. PBLs from healthy controls were treated in the presence or absence of rgp120, anti-CXCR4 or anti-CD4 and analyzed for TRAIL/TRAIL receptor expression by flow cytometry. Only increases in (Top) TRAIL-R2 and (Bottom) TRAIL expression were observed ($p < 0.04$ in each case, n=5).

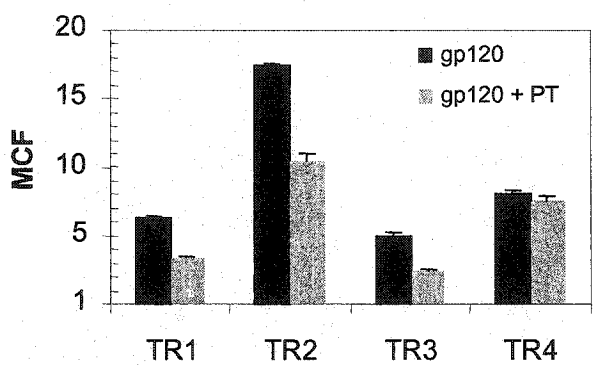

16
14
12
10
8
6
4
2
1
MCF
TR2
TRAIL
Control
gp120
αCXCR4
αCD4

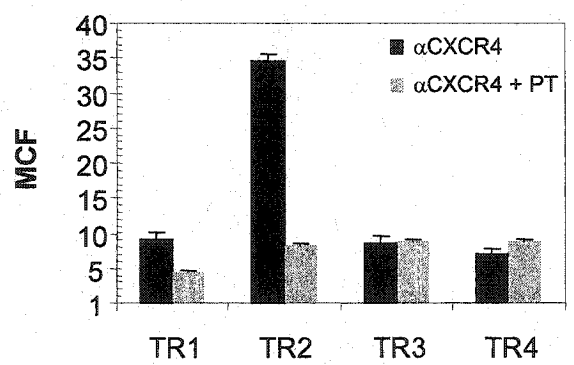
Regulation of TRAIL R2 and TRAIL requires G-coupled Protein Signaling.

Analysis of Jurkat cells pretreated with the protein synthesis inhibitor cycloheximide revealed an independence of *de novo* protein synthesis for upregulation of TRAIL and TRAIL receptors by gp120 (data not shown). The chemokine receptors belong to the G-coupled protein seven transmembrane family. Pharmacological inhibition of G-coupled protein activity can be achieved using pertususs toxin (PT) which binds to Gi_{α} subunit and prevents activation of G protein signaling through adenylate cyclase (Codina et al., 1983; Hildebrandt et al., 1983). PT alone did not affect the levels of TRAIL receptors expression (Fig 14, left figure). The mean channel fluorescence intensity (MCF) in TRAIL-R1 induced by gp120 was reduced from 6.3 to 3.3 by PT, while the MCF increase in TRAIL-R2 by gp120 was also reduced from 17.4 to 10.5 (Fig 14, middle figure). As expected, the effect of PT was more pronounced in TRAIL-R2 expression induced by anti-CXCR4 where the MCF with gp120 alone was 34.6 and decreased to 8.4 with PT treatments (Fig 14, right figure). These data indicate PT blocks upregulation of TRAIL and TRAIL receptors by gp120 and anti-CXCR4. Brefeldin A is an inhibitor of protein translocation from the ER to the golgi apparatus (Pelham, 1991). To determine whether increased expression of TRAIL/TRAIL receptor was dependent on protein translocation, Jurkat T cells were treated for 2 hours with BA followed by stimulation with rgp120, anti-CXCR4 or both. In all three cases, BA substantially reduced the upregulation of TRAIL-R2 (data not shown). Collectively, these data show that the regulation of TRAIL by gp120 and anti-CXCR4 is independent of *de novo*

Figure 14. Upregulation of TRAIL Death Receptors by HIV gp120 is blocked by a G-coupled Protein Inhibitor. (Left) PT does not alter TRAIL receptor expression. (Middle) PT inhibits rgp120 induction of TRAIL death receptor expression. Jurkats were pretreated with PT for 4-5 hours followed by 12-16 hours stimulation with rgp120. (Right) Upregulation of TRAIL-R1 and –R2 by anti-CXCR4 is blocked by PT. Cell surface expression was analyzed by flow cytometry using TRAIL receptor antibodies normalized to isotype control background level staining. Each bar represents average of 4 independent experiments.


MCF
7
6
5
4
3
2
1
media
media + PT
TR1
TR2
TR3
TR4


MCF
20
15
10
5
1
gp120
gp120 + PT
TR1
TR2
TR3
TR4

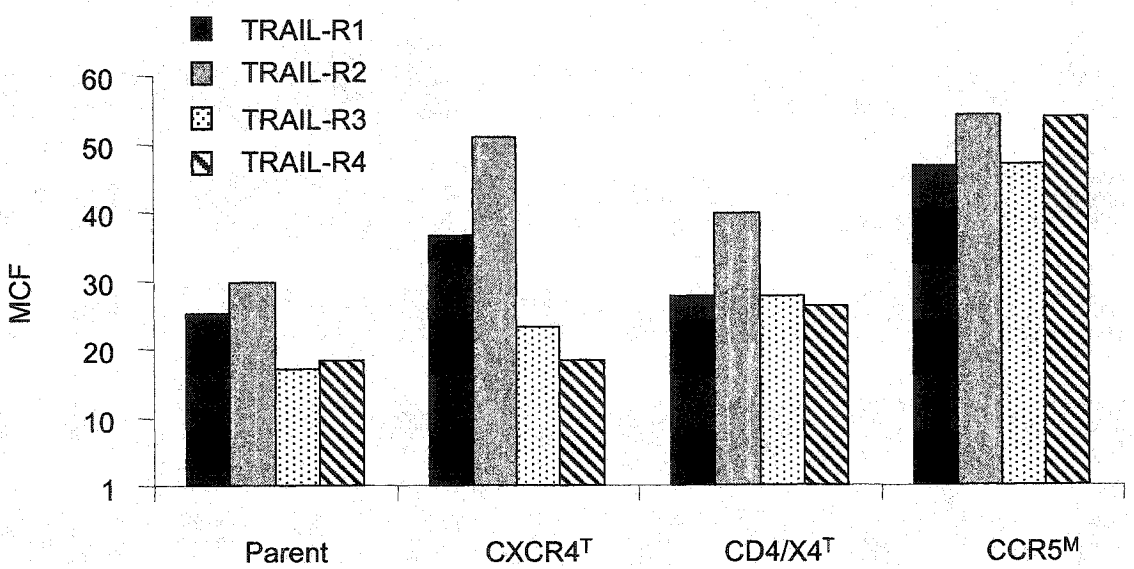

MCF
40
35
30
25
20
15
10
5
1
αCXCR4
αCXCR4 + PT
TR1
TR2
TR3
TR4

protein synthesis, but requires G-coupled protein signaling and the upregulation likely involves preformed proteins.

Enhanced TRAIL and TRAIL Receptor Expression Requires Chemokine Receptors but is Independent of CD4.

To determine the receptor requirements for TRAIL/TRAIL receptor expression, HOS cell lines which are stably transfected with CD4, CXCR4, CCR5 or in combinations were used. In this analysis, parental cells deficient in all three receptors expressed all four TRAIL receptors. Stimulation of parental cells and CD4 expressing HOS cells with rgp120, anti-CXCR4 and anti-CCR5 did not affect TRAIL/TRAIL receptor expression (data not shown). However, when HOS-CXCR4 expressing cells were used, rgp120 caused a 1.4 fold increase in R1 and a 1.7 fold increase in R2 (Fig 15). There was no statistically significant change in either TRAIL R3 or R4. Interestingly, M tropic forms of rgp120 induced all four TRAIL receptors in CCR5 bearing HOS cells, but not with T tropic forms of rgp120 compared to parental cells (Fig 15 and data not shown). Similar changes were observed when heat inactivated whole viral particles were used (data not shown). We also performed control experiments where M-tropic rgp120 was incubated with CXCR4 bearing cells and T-tropic rgp120 was incubated with CCR5 bearing cells. In both cases, we did not observe any changes in TRAIL receptor expression (data not shown) indicating that the interaction with rgp120 and its respective chemokine receptor was specific.

Figure 15. Effects of chemokine receptors on TRAIL/TRAIL receptor expression. Parental HOS cells which are deficient in CD4 and CXCR4 or stably transfected with either CXCR4, CD4/CXCR4 or CCR5 were stimulated with either M-tropic (^{M}) or T-tropic (^{T}) rgp120 for 16 hours. Cells were collected and stained for TRAIL receptors. When T-tropic forms of rgp120 were incubated with CXCR4 bearing cells, TRAIL-R1 and –R2 expression increased ($n=3$, $p<0.02$). Conversely, when M-tropic forms of rgp120 were incubated with CCR5 bearing cells, similar increases in TRAIL receptors were observed ($n=3$, $p<0.01$). Data represents 1 of 3 independent experiments. All MCF values were normalized to isotype control antibodies.

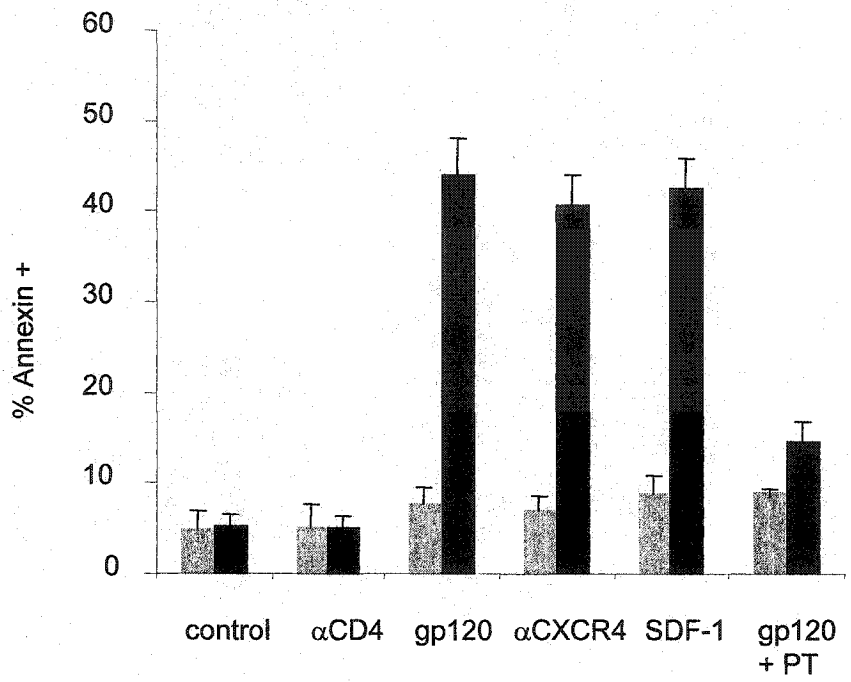

TRAIL-R1
TRAIL-R2
TRAIL-R3
TRAIL-R4
MCF
60
50
40
30
20
10
1
Parent
CXCR4T
CD4/X4T
CCR5M

Ligation of CXCR4 with rgp120 or rSDF-1 Sensitizes Cells to TRAIL Mediated Apoptosis.

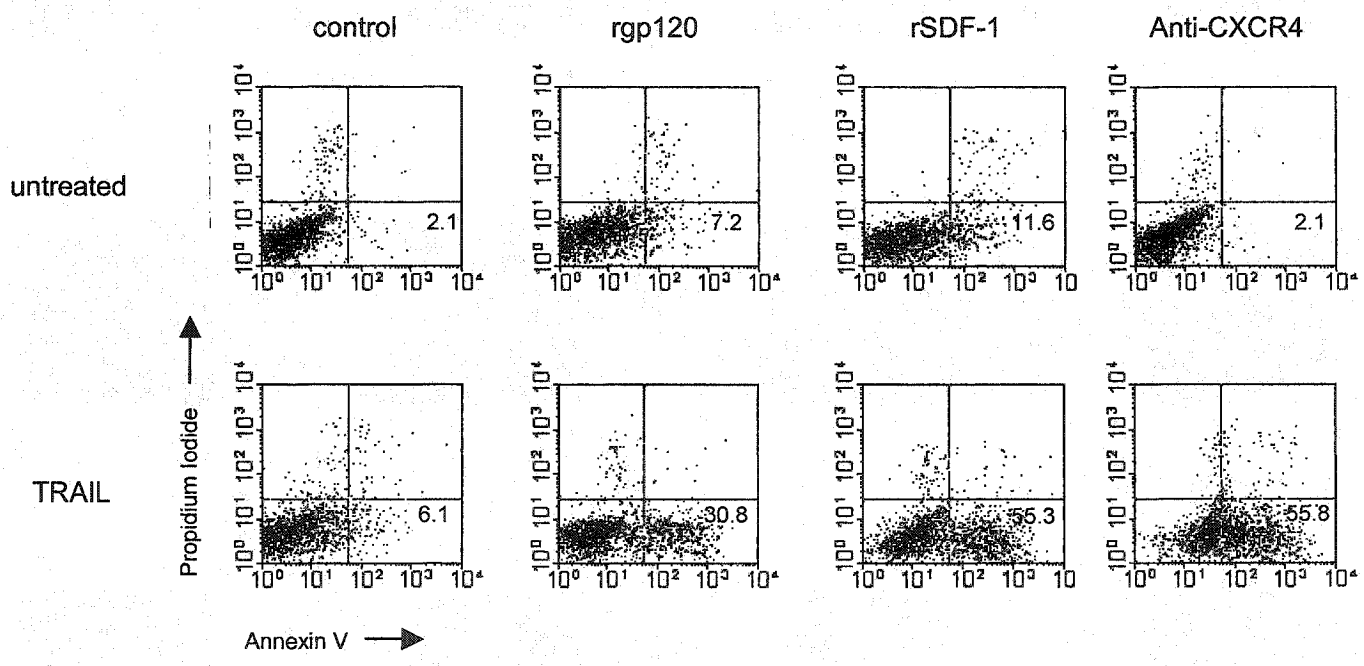
To date, the only identified ligand for CXCR4 is SDF-1. Since activation of PBLs with anti-CXCR4 upregulates TRAIL/TRAIL receptors, we determined whether treatment of PBLs with rSDF-1α has the same effect. Stimulation of PBLs with rSDF-1 had no effect on TRAIL-R1, R3, R4 or TRAIL expression, whereas a 1.2 fold increase was detected for TRAIL R2 ($n=3$, $p < 0.02$, data not shown). When PBLs were pretreated with rgp120 followed by ligation with LZhuTRAIL (black bars), 43% of cells became annexin + ($p<0.03$) when compared to control or αCD4 treatment. Similar results were seen when PBLs were stimulated with rSDF-1 or αCXCR4 ($n=3$, $p<0.03$ in all cases; Fig 16A). This indicates that these treatments provide activation signals for PBLs to acquire sensitivity to TRAIL induced apoptosis. To further confirm these findings, we used the TRAIL resistant cell line HL60 which expresses CXCR4 (data not shown) and HT29 which are CXCR4 deficient (data not shown) to test whether rgp120 and rSDF-1 could render cells sensitive to TRAIL killing. Following a 16 hour stimulation with rgp120 or rSDF-1, cells were treated with 300ng/mL of LZhuTRAIL and cultured for an addition 6-8 hours. HL60 cells (Fig 16B) pretreated with rgp120 ($p <0.02$), rSDF-1 ($p <0.01$) and anti-CXCR4 ($p<0.04$) acquired a TRAIL sensitive phenotype with greater than 30% of cells in all cases staining positive for annexin V ($n=5$). In contrast, HT29 cells remained insensitive to TRAIL killing (data not shown). This indicates that priming with rSDF-1 and rgp120 enhance sensitivity to TRAIL mediated apoptosis through CXCR4.

Figure 16. Ligation of CXCR4 primes cells for TRAIL induced cell death. (A) Primary PBLs from healthy donors were stimulated with rgp120, rSDF-1 or anti-CXCR4 for 12-16 hours followed by incubating with LZhuTRAIL. Cell death was assessed by annexin V staining and the average of 5 experiments were plotted. (B) HL60 were stimulated in the presence or absence of rSDF-1, gp120 or anti-CXCR4 for 12-16 hours followed by 400ng LZhuTRAIL treatments. After 6-8 hours, cells were analyzed for apoptosis by annexin V / PI staining. Values denote % of cells staining annexin V+ / PI-. Dot plots represent 1 of 3 independent experiments.

A


60
50
40
30
20
10
0
% Annexin +
control
αCD4
gp120
αCXCR4
SDF-1
gp120 + PT

B


control
rgp120
rSDF-1
Anti-CXCR4
untreated
TRAIL
2.1
7.2
11.6
2.1
6.1
30.8
55.3
55.8
Propidium Iodide
Annexin V

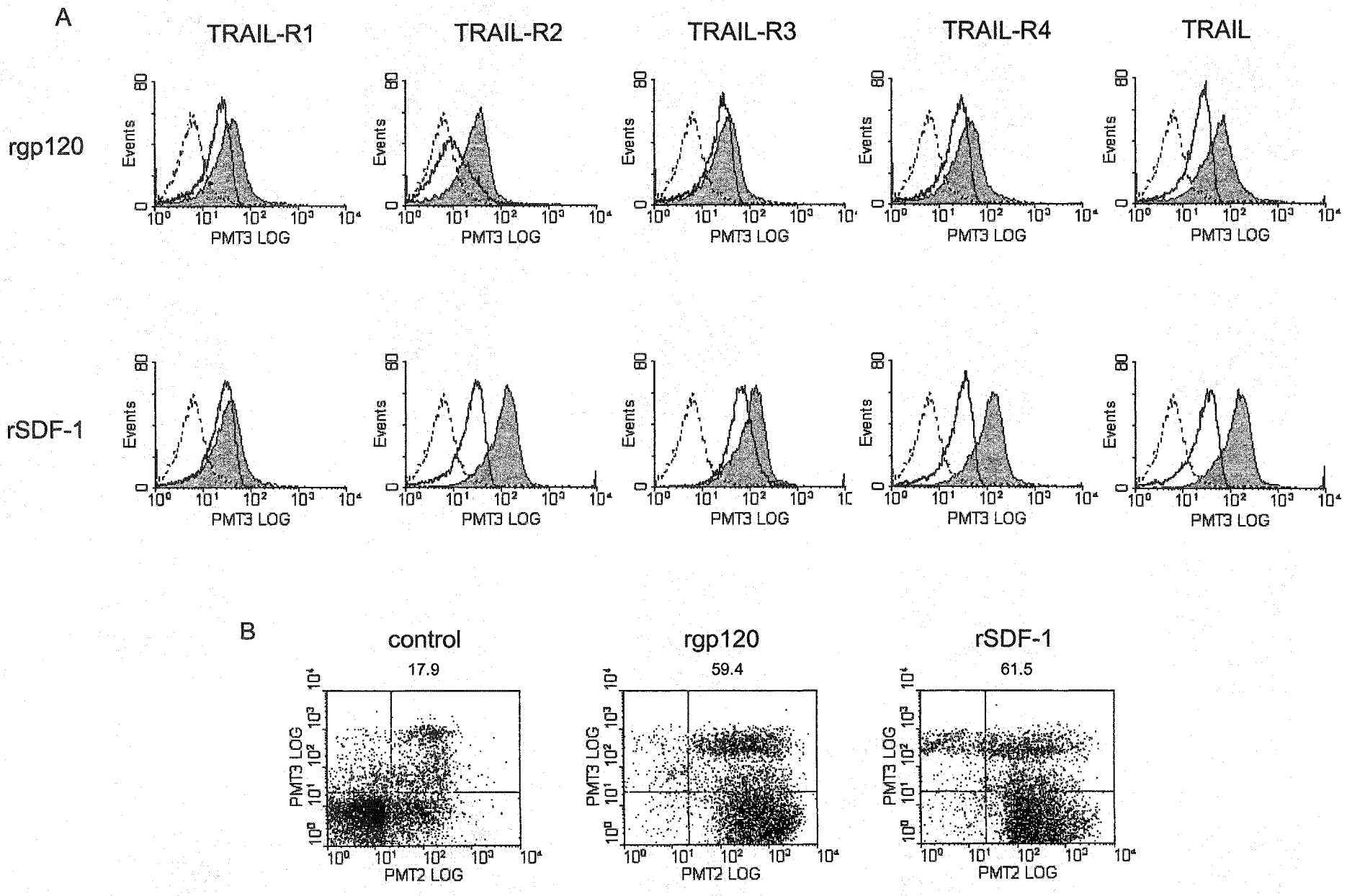
Neutrophil Expression of TRAIL/TRAIL Receptors is Increased by rSDF-1 and rgp120.

The observation that rSDF-1 could render cells sensitive to TRAIL mediated apoptosis prompted us to investigate whether TRAIL plays a role in rSDF-1 mediated inflammatory responses. We used human primary neutrophils because they express high levels of CXCR4 (data not shown) and undergo chemotaxis following stimulation with rSDF-1. All four TRAIL receptors and TRAIL could be detected by flow cytometry on the surface of neutrophils (Daigle and Simon, 2001). Both rgp120 and rSDF-1 upregulated all four receptors and ligand on the surface of neutrophils (n=3, Fig 17A). Following 16 hours of stimulation with rgp120 or rSDF-1, neutrophils were stimulated with 100ng/ml LZhuTRAIL. Both rgp120 and rSDF-1 rendered neutrophils sensitive to TRAIL mediated apoptosis (n=3, Fig 17B). Interestingly, 3 day stimulation with rSDF-1 alone induced greater than 43% annexin V positive cells, indicating that rSDF-1 activates neutrophils and causes AICD similar to activated T cells (Fig 18). No change in neutrophil expression of CXCR4 by rSDF-1 was detected (data not shown).

Blockage of TRAIL-R1 Signaling Rescues Neutrophil AICD.

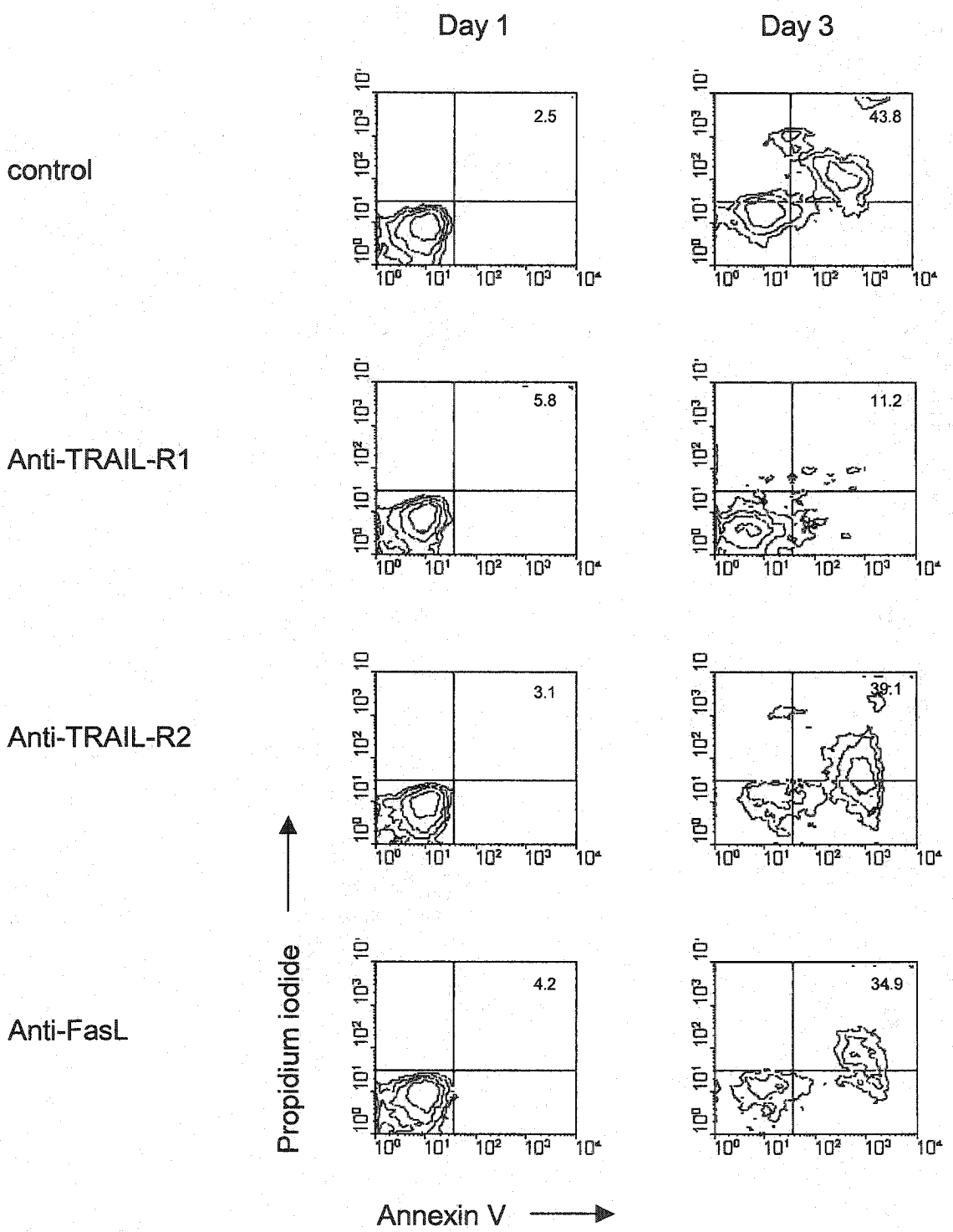
The observation that 72 hour treatment with rSDF-1 induces death of neutrophils suggested that TRAIL may play a role in death receptor mediated AICD. Isolated neutrophils were seeded in 96 well round bottom plates and treated with rgp120, rSDF-1 or anti-CXCR4 in the continuous presence of 1μg/mL anti-TRAIL-R1, R2 or anti-FasL for 72 hours. Cells were collected and apoptosis was determined by annexin V staining.

Figure 17. Effect of rgp120 and rSDF-1 on neutrophils. (A) Expression of TRAIL/TRAIL receptor on neutrophils. 12-16 hour treatment of neutrophils with rgp120 (top) and rSDF-1 (bottom) increase TRAIL/TRAIL receptor expression. Dash histogram: isotype control, open histogram: control treatment, closed histogram: rgp120 or rSDF-1 treatment. (B) Increased cell death in neutrophils by rgp120 and rSDF-1 following 12-16 hour treatment. % Apoptosis is denoted on top of dot plot and was counted by annexin V + / PI + cells. Each experiment was performed at least 2 times.


A
TRAIL-R1
TRAIL-R2
TRAIL-R3
TRAIL-R4
TRAIL
rgp120
rSDF-1
Events
PMT3 LOG
B
control
17.9
rgp120
59.4
rSDF-1
61.5
PMT3 LOG
PMT2 LOG

On day 1, few apoptotic cells were detected by flow cytometry in all cases (Fig 18). However, as expected, on day 3, the number of apoptotic neutrophils in control treatments was 43.8% (Fig 18). In the presence of anti-TRAIL-R2 and anti-FasL, there was no inhibition of AICD observed when compared to control samples (Fig 18). However, in cultures treated with anti-TRAIL-R1, apoptosis was reduced from 43.8% to 11.2% ($n=3$, $p<0.01$; Fig 18). Anti-TRAIL-R1 treatments also reduced rgp120 induced death of neutrophils from 60.2% to 30.5% ($n=3$ $p < 0.03$, data not shown). Finally, cell death induced by anti-CXCR4 was inhibited by anti-TRAIL-R1 (59% versus 36.2%, data not shown). These experiments show that TRAIL-R1 plays a role in providing neutrophils with death signals following activation through CXCR4 with rgp120, rSDF-1 and anti-CXCR4.

Figure 18. TRAIL-R1 agonist inhibit rSDF-1 mediated death of neutrophils. Neutrophils were seeded in 96 well plates and activated with rSDF-1 in the continuous presence of 1μg/mL TRAIL-R1, -R2 or FasL neutralizing antibodies. On day 1 and 3, cells were collected and stained with annexin V. rSDF-1 activated induced death of neutrophils is blocked by anti-TRAIL-R1, but not –R2 or FasL antibodies. Dot plots represent 1 of 3 independent experiments and values denote the % annexin + / PI + cells.


Day 1
Day 3
control
Anti-TRAIL-R1
Anti-TRAIL-R2
Anti-FasL
2.5
43.8
5.8
11.2
3.1
39.1
4.2
34.9
Propidium iodide
Annexin V

4.4 Discussion

The molecular signals governing the acquisition of TRAIL sensitivity following HIV infection are not known. Here we describe one possible mechanism whereby uninfected cells can become sensitive to TRAIL mediated killing through upregulation of the death receptors TRAIL-R1 and R2. Recently, HIV Tat has been shown to increase TRAIL receptor expression in HIV infected macrophages (Zhang et al., 2001). Previous experiments by our group using HIV infected Jurkats showed an increase in TRAIL receptor expression within 16 hours of infection (unpublished data). Thus, it is likely an early viral event results in TRAIL receptor alterations leading to T cell susceptibility to apoptosis. Therefore, we examined whether gp120 could modulate TRAIL/TRAIL receptor expression and sensitivity to TRAIL mediated death. As expected, gp120 caused an increase in TRAIL R1 and R2 expression, but no increase in the level of decoy receptor expression was observed. This upregulation did not require *de novo* protein synthesis as experiments using cycloheximide did not affect the increase of TRAIL/TRAIL receptors (data not shown). However, pretreatment of Jurkats with pertussus toxin inhibited TRAIL/TRAIL receptor increases, suggesting the involvement of chemokine receptor signaling. The monoclonal antibodies anti-CD4 and anti-CD3 and the mitogen PHA did not cause alterations in TRAIL or TRAIL receptors. This indicates that general activation stimuli are not responsible for the increase in TRAIL/TRAIL receptors. However, similar to gp120 treatments, anti-CXCR4 resulted in TRAIL-R1 and R2 upregulation. These observations were confirmed using a second approach whereby cell lines stably transfected with CD4 alone or in combination with one of CXCR4 or

CCR5 were stimulated with gp120. In this case, cell lines expressing only CD4 did not exhibit changes in TRAIL/TRAIL receptor expression. However, cell lines deficient in CD4 but expressing either CXCR4 (in the case of T tropic gp120) or CCR5 (in the case of M tropic gp120) had increases in TRAIL receptors following gp120 treatments, providing evidence for the requirement of chemokine receptors but not CD4 in the regulation of TRAIL and TRAIL receptor expression. Since SDF-1 is the only known ligand for CXCR4, we determined whether stimulation of PBLs with SDF-1 could enhance TRAIL/TRAIL receptors. Surprisingly, treatment of PBLs with SDF-1 upregulated TRAIL-R2 expression suggesting that TRAIL may be involved in physiological processes independent of HIV infection. It is not clear from these experiments why rgp120 caused an increase in both death receptors, R1 and R2, while rSDF-1 only caused an increase in R2. Although we performed experiments using whole killed virus, (as a source for gp120) most preparations of recombinant gp120 are monomeric and therefore may explain the difference we observed. When we tested the sensitivity of PBLs treated with rgp120, anti-CXCR4 or rSDF-1, we found that all three treatments significantly enhanced cell death induced by TRAIL.

Unlike the involvement of Fas/FasL pathway in deletion of immature thymocytes during clonal selection and development, the relevance of TRAIL/TRAIL receptor mediated apoptotic pathway has not been clearly defined. Studies indicate that central and peripheral sensitivity to TRAIL is differentially controlled (Simon et al., 2001) and TRAIL has a limited role in antigen induced deletion of thymocytes (Simon et al., 2001). Recent studies using gene targeted deletion of TRAIL suggest that TRAIL participates in

the surveillance of tumor growth and metastasis with TRAIL deficient mice demonstrating decreased ability to clear lethally transplanted tumors (Cretney et al., 2002a; Sedger et al., 2002; Takeda et al., 2002). In contrast to the documented anti-tumoricidal activity of TRAIL, it was recently demonstrated that injection of antagonist TRAIL-R2 antibodies into mice have exacerbated experimental autoimmune encephalomyelitis (EAE) when compared to mice injected with control antibodies. This provided evidence for the role of TRAIL in downregulating inflammatory responses and suggests an *in vivo* function for TRAIL (Hilliard et al., 2001; Huang et al., 2000; Song et al., 2000; Wendling et al., 2000). Therefore, we used neutrophils to study the role of SDF-1 and its ability to modulate TRAIL sensitivity. We found high levels of TRAIL/TRAIL receptor expression on neutrophils which was upregulated by SDF-1. In addition, we observed neutrophil death following SDF-1 treatments. This form of death was blocked by anti-TRAIL R1 indicating the involvement of TRAIL in neutrophil AICD similar to AICD in T cells. Collectively, this implicates TRAIL as a regulator of neutrophil mediated inflammatory responses.

SECTION 5:
Vpr Polymorphism in Long Term Non Progressors is Associated with Decreased Apoptosis

5.1 Introduction

Some HIV infected individuals have detectable HIV replication yet do not experience progressive immunosuppression even in the absence of ART (Buchbinder et al., 1994; Zhang et al., 1997). In such long term non progressors (LNTPs) as many as 25 - 30% may have specific homozygous mutations in the HIV-1 co-receptors CCR5 and CCR2, which are associated with an impairment of viral attachment and thus infectivity, explaining in part their slow disease progression (Cohen et al., 1997b). Other LTNPs may have undetectable levels of viral replication, potentially due to mutations in viral genes which influence replication, such as nef (Deacon et al., 1995) . Other factors may modify HIV disease progression. Certain MHC haplotypes (B27, B57 and B51) are strongly associated with long term non progression, perhaps due to an efficient presentation of immunodominant epitopes for cell mediated immune responses (Kaslow et al., 1996). Finally, accumulating evidence suggest that HIV specific CD8 CTLs may impact disease progression and are associated with long term non-progression (Chun et al., 2001). As these mechanisms do not fully account for all cases of LTNP, other mechanisms of LTNP must exist, yet they remain undefined and likely include both host and viral factors.

Viral protein R (Vpr), an HIV-1 accessory protein, is a 14-kDa protein of 96 amino acid in length. Vpr is expressed in infected cells and incorporated into virions by

interacting with the p6 region of the Gag precursor (Paxton et al., 1993). *In vivo*, Vpr enhances viral replication in non-dividing macrophages although it has little effect on replication of actively dividing T cells (Matsuda et al., 1993). Moreover, Vpr facilitates nuclear import of preintegration complexes (PIC) (Jenkins et al., 1998; Popov et al., 1998), induces latent virus replication and prevents establishment of chronic infection (Rogel et al., 1995). Structural studies of synthetic Vpr peptides reveal two critical helical domains, one at 17-34 and the second at 53-74, spaced by an arginine-rich c-terminus (Schuler et al., 1999). The N-terminus of Vpr is important in virion incorporation and stability of the protein, while the C-terminus (HFRIGCRHSRIG) is implicated in cell cycle arrest (Goh et al., 1998) and initiation of apoptosis (Stewart et al., 2000). However, the mechanism whereby Vpr exerts these effects are not clear. Three possible modes of action explain in part the biochemical actions of Vpr. First, wildtype Vpr induces transient and dynamic herniations in the nuclear envelope resulting in dislocalization of cell cycle kinases and cyclins (de Noronha et al., 2001). Although not directly evaluated by de Noronha, this effect may provide sufficient nuclear pore size to allow translocation of PICs across the nuclear membrane, thus enabling viral integration into the host genome. In the second model, Vpr forms cation selection ion channels across lipid bilayers which cause large inward cation currents and depolarization of neurons (Piller et al., 1998). On intact neurons, this causes perturbations in the plasma membrane integrity and eventual cell death. The third model predicts that synthetic Vpr crosses the outer membrane of the mitochondria and directly interacts with the adenine nucleotide translocator (ANT) in the inner membrane of mammalian and yeast cells (Macreadie et al., 1996; Macreadie et al., 1995; Macreadie et al., 1997; Nishizawa et al., 2000). The

contact causes cooperative channel formation via direct protein-protein interactions of Vpr and ANT and induces rapid uncoupling of the respiratory chain (Jacotot et al., 2001; Jacotot et al., 2000) resulting in the release of apoptotic inducing factors, an effect inhibited by overexpression of Bcl-2 (Jacotot et al., 2001; Jacotot et al., 2000). In this study, the use of R73A, R77A or R80A peptide had minimal effect on the viability of intact cells or isolated mitochondria when directly compared to WT Vpr treatments suggesting these mutants have decreased channel forming activity as a result of reduced binding affinity for ANT (Jacotot et al., 2001; Jacotot et al., 2000). In addition, a synthetic peptide corresponding to the binding domain of Bcl-2 and ANT blocked Vpr-ANT interaction (Jacotot et al., 2001; Jacotot et al., 2000). Interestingly, the effects described in these three models can be abrogated by various point mutations within the HFRIGRCHSRIG domain indicating that Vpr possesses transdominant activity. Recently, Sawaya et al (Sawaya et al., 2000) demonstrated that two Vpr variants, at position R77S or R77A abrogate cell cycle arrest and activation of LTR mediated transcription induced by wildtype protein. Furthermore, using an inducible expression system, Zhou et al (Zhou and Ratner, 2001) show that single amino acid substitutions at position 79A, 79D and 79Q dramatically reduced G2 cell cycle arrest induced by wildtype Vpr.

Vpr induces apoptosis by binding to the ANT (Jacotot et al., 2000) component of the PTPC (Jacotot et al., 2000), causing $\Delta_{\psi Tm}$, release of cytochrome c and AIF, uncoupling of the electron transport chain and oxidative phosphorylation collapse (Jacotot et al., 2000; Macreadie et al., 1995; Macreadie et al., 1997). The interaction of

Vpr with ANT is specific, with an affinity in the nanomolar range, and is inhibited by Bcl-2 (Jacotot et al., 2000). Mutations in the $(H(F/S)RIG)_2$ domain of Vpr (i.e. R73A, R77A, R80A) abrogate the ability of Vpr to induce apoptosis in T lymphocytes ((Jacotot et al., 2001; Jacotot et al., 2000). Therefore it is possible that naturally occurring mutations in Vpr may also modulate T cell death and/or survival in HIV infected persons and thereby impact disease progression. We therefore assessed the frequency of Vpr mutations within the $(H(F/S)RIG)_2$ domain of HIV infected patients with progressive HIV disease, and in LTNP HIV infection. We also compared the *in vitro* and *in vivo* effects of the naturally occurring mutations of Vpr that are seen in our LTNP cohort to WT Vpr.

5.2 Materials and Methods.

VSV-G pseudotyped virus infections. To evaluate the effects of Vpr mutations on cell killing in the context of viral infection, a single cycle HIV-1 superinfection was used. VSV-G pseudotyped virus particles were prepared as previously described (Yao et al., 1998). Briefly, the envelope defective proviral plasmids HxBRUR+/Env-, HxBRUR-/Env- or HxBRUR+/Env- with an introduced R77Q (CCC to CAA) mutation were cotransfected into 293T cells stably expressing the VSV-G expressor (SVCMV-VSV-G) and 72 hours post infection culture supernatants was collected, preclarified and ultracentrifuged to pellet pseudotyped virus. The resultant virus preparations (VSV-G Vpr+, VSV-G Vpr-, or VSV-G Vpr R77Q respectively) were quantified in counts per minutes (cpm) by RT activity. One hundred cpm RT activity was used subsequently to infect 10^6 cells. Similarly, virus was titrated by Magi assay (Yao et al., 1998) and Jurkat cells were infected with 0.1 M.O.I. Jurkat T cells were infected in the presence of 10ug/ml polybrene (Sigma, St Louis MI) for 6 hours, followed by two washes with PBS. Cells were then maintained in complete media as described below.

Vpr Peptides. C-terminus (52-96) Vpr wild type (WT) and mutant (MT) R77Q peptides were synthesized (Genemed Synthesis Inc., San Francisco) by automated solid phase synthesis using FMOC and purification was performed using reverse-phase HPLC (Jacotot et al., 2001; Jacotot et al., 2000). The peptides were analyzed by electrospray mass spectrometry and purity of both peptides was ≥95%.

Cell culture and flow cytometry . Jurkat T cells (ATCC Rockville, MD) were cultured in RPMI 1640 (all cell culture products were purchased from Canadian Life Technology, Montreal Canada, unless otherwise stated) with 10% heat inactivated fetal bovine serum (Sigma St. Louis, MI), 100U/mL of each penicillin and streptomycin and 2mM L-glutamine. Cells were plated at 1 x 10^6 cells/well and incubated with varying concentrations of Vpr WT or Vpr R77Q peptides in isotonic buffer for 30 minutes followed by culture in complete media. Cells were collected at 8-20 hours and stained either with annexin-V FITC (BD Pharmingen) and 1 mg/mL PI (Sigma St. Louis, MI) or by TUNEL (Boehringer Mannheim). For TUNEL stains, cells were fixed in 4% paraformaldehyde (Sigma St Louis MI) for 30 minutes followed by two washes in PBS. Permeabilization was performed using ice cold 70% ethanol for 30 minutes at 4°C, followed by two washes with PBS. TUNEL staining was then performed according to the manufacturers instructions. Mitochondrial transmembrane potential was quantified using 40 μM $DiOC_6$ (Molecular Probes Eugene, OR) according to manufacturer's instructions and 4μM hydroethidine (Molecular Probes Eugene, OR) was used to determine superoxide anion generation (ROS). For inhibition studies, 100 μM of the caspase inhibitor zVAD-fmk (Bachem Bioscience Inc) was used. All flow cytometry analysis was performed using Coulter Epics Altra XL with 10000 acquired events. Cell cycle analysis was performed by resuspending cells in 80% ethanol on ice for 30 minutes. Following washing, cells were incubated with 180U/mL RNAse H (Sigma. St. Louis MI) and subsequently stained with 30μg PI/ml for 30 minutes at 37°C. DNA content was then analyzed using consort 30 software.

Immunoblotting. Cytosolic extracts from treated Jurkats were prepared by resuspending cell pellets for 15 minutes in ice cold caspase extraction buffer (100mM HEPES, pH 7.5, 10% sucrose (W/V), 0.5mM EDTA, 10mM DTT) followed by 40-60 strokes using a dounce homogenizer. Cells were spun at 2700 rpm (720g) for 5 minutes and the cytosolic supernatant fraction was frozen at -80 °C until use. Cytosolic proteins were separated by (4-15% gradient) SDS PAGE and transferred to Immobilon-P (Millipore) membrane. Membranes were blocked with 5% skim milk and incubated with: caspase 8 (a generous gift from Dr. P. Krammer), caspase 9 (MBL), caspase 3 (Transduction Laboratories - Lexington, KY), DFF (Santa Cruz) primary antibodies followed by the appropriate secondary antibody (anti-mouse IgG HRP (Amersham), anti-rabbit HRP (Amersham) and anti-goat IgG (Santa Cruz). Detection of proteins was performed by supersignal enhanced chemiluminence (Pierce). Equal loading of proteins was ensured using PCNA (Santa Cruz) specific antibodies followed by anti-mouse IgG HRP. All proteins were blotted using a 1:1000 dilution of primary and secondary antibodies in 5% skim milk containing 1 μg/mL BSA.

Isolation of mitochondria from mouse liver. Mouse livers were maintained on ice cold 0.9% NaCl and cut into small pieces, homogenized in 2 mL homogenizing medium (0.25 M sucrose, 3 mM HEPES buffer, 1 mM EDTA, pH 7.2). Homogenates were spun for 10 minutes at 500g (2600 rpm), and supernatants spun for 10 minutes at 500g (2600 rpm) and nuclear pellets discarded. Supernatants were then collected and spun at 11000 rpm for 10 minutes to pellet the mitochondria, which was resuspended in homogenizing medium. Mitochondrial fractions were treated with R77Q or WT Vpr at 37°C for 6 hours

and stained with $DiOC_6$. The remaining mitochondria were spun at 10000 rpm at 4°C for 10 minutes and separated into pellet or supernatant fractions for western blot analysis for cytochrome c release.

Vpr Sequencing. The clinical characteristics of LTNP used in this study have been previously published (Cohen et al., 1997b). Patients attending the immunodeficiency clinic at the Ottawa Hospital were used as controls. Eligible patients were naive to antiretroviral therapy (so as not to allow selection of mutations due to drug pressure) and had evidence of immunodeficiency (absolute CD4 count of <500 cells and viral load >1000 copies/mL). Following explanation of the study, patients who gave informed consent donated 10 mls of blood in heparinized tubes. This study was reviewed and approved by the Ottawa Hospital Research Ethics Board. Viral RNA was extracted from plasma samples (0.5 ml - 1.0 ml) using an automated nucleic acid extraction instrument (NucliSens; Organon Teknika, Toronto, Ontario). One fifth of the isolated RNA was reverse transcribed and amplified by polymerase chain reaction (RT-PCR using a single-tube system-Qiagen One-Step RT-PCR; Qiagen, Toronto, Ontario). The following primers were used Vpr1F GAGACTGGCATTTGGGTCA and Vpr1R TTTGTAAAGGTTGCATTACAT. Reaction conditions: 1 cycle 50 °C, 30 min, 1 cycle 95 °C, 15 min, 30 cycles 95 °C, 1 min; 50 °C, 1 min, 72 °C, 1.5min, 1 cycle 70 °C, 1.5 min. Three microlitres of the primary RT-PCR reaction were added to a secondary PCR mixture (Qiagen Hot-Start with primers Vpr2F GCAGGACATAACAAGGTAGGA and Vpr2R GTCGCTGTCTCCGCTTC) and amplified. Reaction conditions: 1 cycle 95 °C, 15 min, 30 cycles 95 °C, 1 min; 50 °C, 1 min, 72 °C, 1.5min, 1 cycle 70 °C, 10 min. The

secondary PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen; Carlsbad, California) as recommended by the manufacturer. Recombinant clones were identified by blue-white screening. Cloned DNA (4-8 clones/patient) was used in simultaneous bi-directional sequencing on a Li-Cor 4200L automated sequencer with dye-labeled primers complementary to plasmid vector sequences. Sequences were assembled, aligned and translated using Sequencher software (Gene Code; Ann Arbour, Michigan).

Database search for Vpr sequences. All sequences were found in the Los Alamos database (http://hiv-web.lanl.gov/seq-db.html) LTNP versus progressor status was determined by (a) specific notation within the Los Alamos database, or (b) by cross referencing the sequence with the original citation. If no mention was made either in the database, or in the original citation as to the patients clinical status (i.e. LTNP versus progressor) the sequence was excluded from the analysis. Sequences were aligned and compared using Sequencher software (Gene Code; Ann Arbour, Michigan).

Mouse Studies. 21 to 28 day old BALB/c mice received tail vein injections of 200µls of H_2O (control) alone or containing 10 mg/kg of R77Q or WT Vpr peptide (residues 52-96). Immediately prior to injection and again 24 hours later 200µls of blood was collected. 48 hours following injection, blood was removed by cardiac puncture, the mice were euthanised and necropsies were performed. Histology was performed on the injection site, liver, spleen and kidneys. Absolute CD3+, CD4+, CD8+ T cell numbers were determined from whole blood samples by single platform flow cytometry (Baudouin et al., 1999). Briefly, prior to mouse experiments the performance

characteristics of this assay were validated by dilution experiments using whole blood from mice (data not shown). Alignment and calibration of flow cytometer for absolute T cell counting was performed by using Coulter flow check and absolute lymphocyte count was calculated using Coulter Flow Count Fluorospheres. Peripheral blood from mice were stained with a monoclonal antibody panel CD3-FITC, CD4-PE and CD8-PC5 (BD Pharmingen). After staining, peripheral blood cells were lysed and fixed using the Multi-Q-Prep system (Coulter). Before analysis of whole blood samples, 100µl of Coulter Flow Count beads were added to the samples. Absolute count (cell/µl) was determined using the following formula:

Total # of cells counted / total # of Fluorospheres X flow count Fluorospheres assayed.

All studies involving mice were reviewed and approved by the Animal Care Committee of the University of Ottawa.

Statistics. For comparisons of the frequency of R77Q mutations between LTNP and progressor cohorts, 2 tailed Fishers exact test was used. For comparisons between treatment groups, a Wilcoxon rank test was used.

5.3 Results

Frequency of Vpr R77Q mutation in patients with progressive HIV infection and in LNTPs.

The clinical characteristics of LTNP used in this study have been previously published (Cohen et al., 1997b), and all patients included in our analysis had a viral load ≥ 1000 copies/mL, had never received antiretroviral therapy, had documented HIV infection for > 7 years and had CD4 counts > 600 cells/mm^3. Furthermore, no patients were homozygous for CCR5 mutations. Importantly, 2 out of 10 patients had viral loads of >30,000 copies/mL. Sequence analysis from our cohort of 10 LTNP found that 80% of patients had the mutation R77Q in Vpr while the remaining 20% had wild type Vpr (Fig 19). In parallel, plasma virus from 15 antiretroviral naive HIV infected patients with CD4 counts below 500 were sequenced for Vpr. In this second cohort of patients with progressive HIV disease only 5 out of 15 individuals (33%) had the R77Q mutation ($p = 0.041$, Fig 19). Therefore, viral isolates from patients with LTNP HIV infection have a significantly higher frequency of mutations at R77Q within the $(H(F/S)RIG)_2$ domain than patients with progressive disease.

We next assessed whether a similar association was present in viral sequences contained within the Los Alamos database. Sequences were only included in this analysis if the patients clinical status was mentioned (i.e. progressor versus LTNP). In this

Figure 19. *Vpr* sequence alignment of control patients and of LTNPs. DNA from 15 HIV infected patients with progressive disease and 10 LTNP patients were sequenced for *Vpr* and compared to *Vpr* consensus sequence.

Progressor Cohort

HIVNL4-3	TLQQLLFIHFRIGCRHSRIGITQ-QR-RTRNGASRS
1	TLQQLLFIHFRIGCRHSRIGITQ-QR-RTRNGASRS
2	ILQQLLFIHFRIGCRHSRIGITQ-RG-RTRNGAGRS
3	ILQQLLFTHFRIGCRHSRIGITR-QR-RARNGTSRS
4	ILQQLLFIHFRIGCRHSRIGIIR-QR-RTRNGASRS
5	ILQQLLFIHFRIGCQHSRIGLMRRTRNRTRNGASRS
6	ILQQLLFTHFRIGCRHSRIGITQ-R--RARNGASRS
7	ILQQLLFIHFRIGCRHSRIGITP-QR-RARNGSSRP
8	MLQQLLFIHFRIGCQHSRIGIIR-QR-RARNGSSRS
9	ILQQ-LFIHFRIGCRHSRIGIIP-QRRRR-DGASRS
10	ILQQLLFIHFRIGCPHSRIGITP-RR-SARNGASRS
11	ILQQLLFIHFRIGCRHSRIGIIQ-QRRARRNGASRS
12	ILQQLLFIHFRIGCRHSRIGIIQ-QR-RARNGASRS
13	ILQQLLFIHFRIGCQHSRIGIIR-QR-RTRNGASRS
14	TLQQLLFIHFRIGCQHSRIGITP-RR-RARNGASRS
15	VLQQLLFIHFRIGCQHSRIGILP-WR-RARNGASRS

Long Term Non Progressor Cohort

L1	ILQQLLFIHFRIGCQHSRIGITR-QR-RARNGASRP
L2	ILQQLLFIHFRIGCQHSRIGITR-QR-RARNGASRP
L3	ILQQLLFIHFRIGCRHSRIGIT?-R--RRARNGARS
L4	ILQQLLFIHFRIGCRHSRIGISL-RG-RARNGAGRS
L5	ILQQLLFIHFRIGCQHSRIGLMRRTRNRTRNGASRS
L6	ILQQLLFIHYRIGCQHSRIGIQQ-RRARRARNGARS
L7	ILQQLLFIHFRIGCQHSRIGLMRRTRNRTRNGASRS
L8	ILQQLLFIHFRIGCQHSRIGIIRQRRTRNGASRS
L9	ILQQLLFIHFRIGCQHSRIGLMRRTRNRTRNGASRS
L10	ILQQLLFIHFRIGCQHSRIGITPRRRARNGASRS

analysis (Appendix I and II) R77Q was present in 20/55 (36%) of progressors, as compared to 109/146 (75%) of LTNP ($p<0.001$).

HIV Infection R77Q Vpr results in less death than infections with wild type virus.

To assess whether R77Q causes less cell death than WT, we analyzed whether Jurkat T cell death was different in parallel viral infections that differ only by virtue of a mutation at position 77 of Vpr (CCC to CAA). VSV-G pseudotyped virus was prepared by cotransfecting an envelope deficient HIV-1 provirus with VSV-G envelope expressing plasmid in 293T cells. Progeny virions were then used to infect Jurkat T cells. On day 4, cells infected with WT Vpr began to display characteristic light scatter profiles of dying cells when compared to mock or MT Vpr R77Q (Fig 20 and Fig 21A). Detection of phosphatidylserine exposure on the outer plasma membrane by annexin V was used to quantify the percentage of apoptotic cells. In comparison to cells infected with WT virus, cells infected with R77Q vpr showed less annexin positive staining ($p=0.01$, Fig 20). The loss in cellular morphology was more apparent with the majority of WT Vpr infected cells in the dead gate. In contrast, Jurkats infected with MT Vpr R77Q or mock infected remained in the live gate (Fig 21A). In parallel, cell cycle analysis was performed on infected cells, demonstrating no differences in cell cycle arrest between infections (data not shown). When we analyzed the effects of VSV-G infections on $\Delta\Psi Tm$, we found that MT Vpr induced significantly less $\Delta\Psi Tm$ than infections using WT Vpr (Fig 21B). Whereas WT vpr infection resulted in the complete cleavage of procaspase 8 into an active caspase 8 fragment, there was no evidence of caspase 8 activation in MT Vpr

Figure 20. R77Q *Vpr* induces less apoptosis in single cycle infections using VSV-G pseudotyped HIV-1 virus. Jurkat T cells were infected with 100 cpm/cell RT activity. Cells were analyzed by annexin V FITC / PI staining for apoptotic cells, at the indicated time points post infection. Values are representative of three independent experiments.

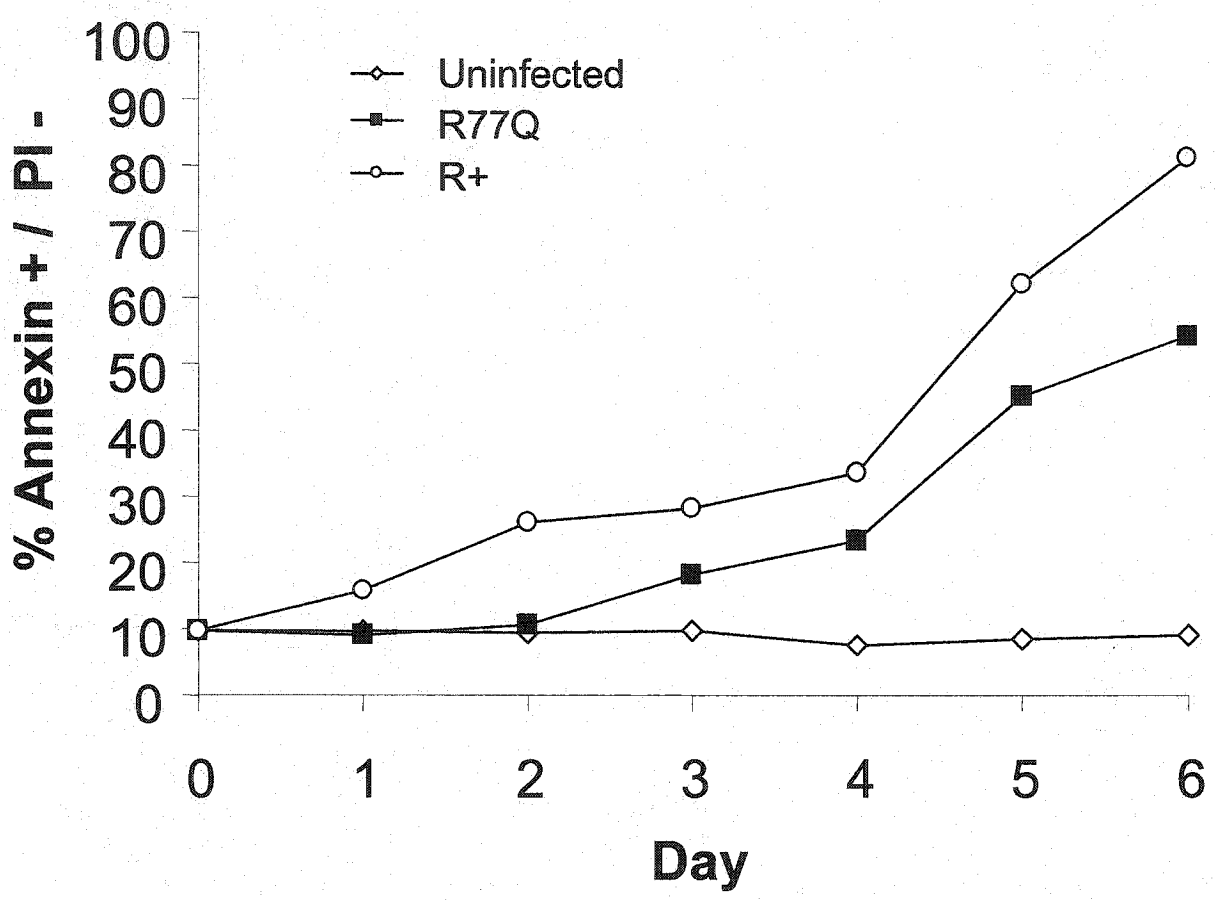
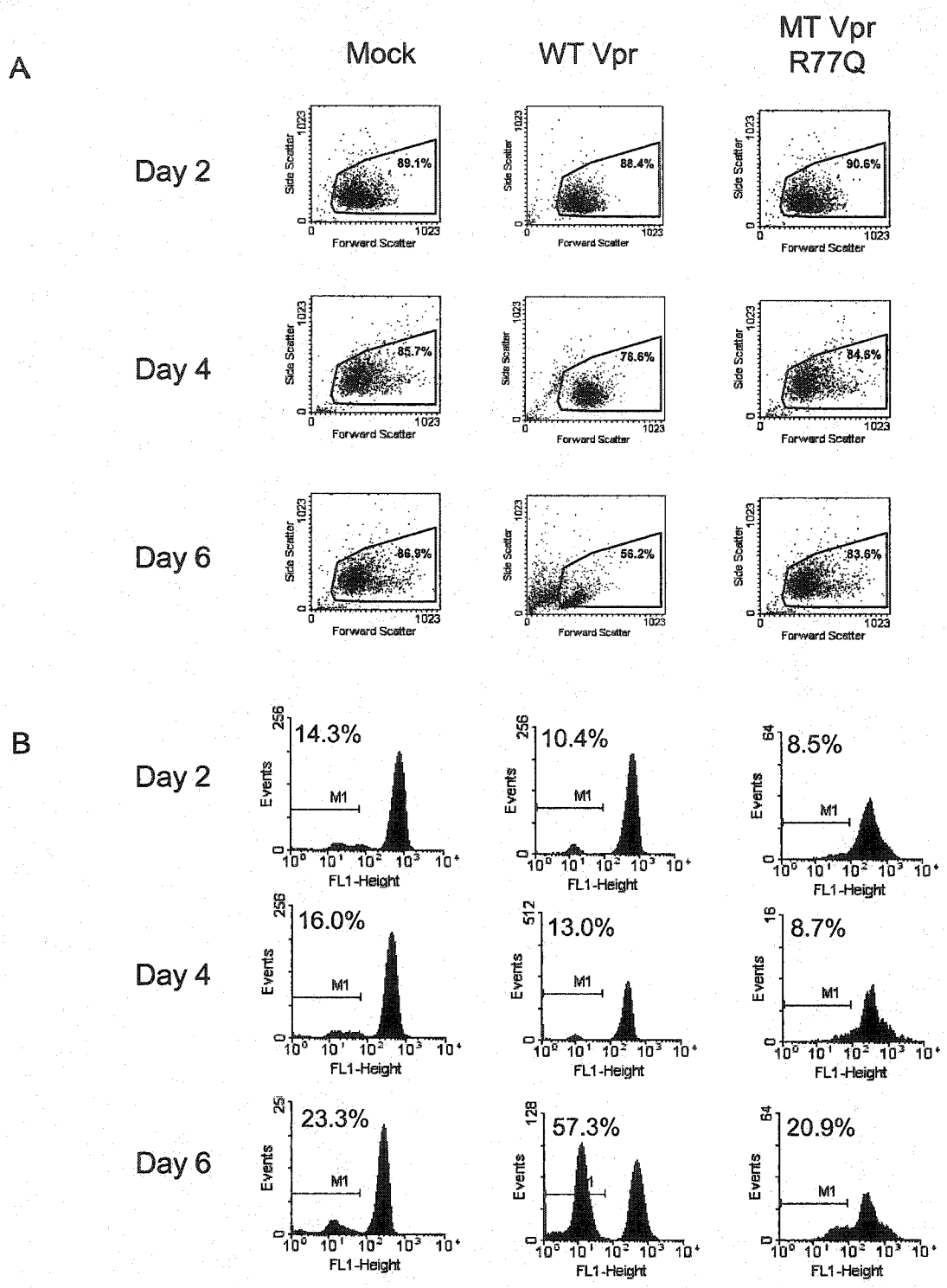

100
90
80
70
60
50
40
30
20
10
0
% Annexin + / PI -
Uninfected
R77Q
R+
0
1
2
3
4
5
6
Day

Figure 21. Decreased Levels of Apoptosis in Jurkat cells infected with VSV-G pseudotype virus containing Vpr R77Q. Jurkat cells were mock or infected with (0.01 MOI) WT Vpr or MT Vpr R77Q VSVG. Cells were collected on day 2, 4 and 6 for analyzed for apoptosis by (A) light scatter and (B) $\Delta\psi$Tm. Cells infected with MT Vpr R77Q VSVG had less evidence of morphology changes typical of dying cells and less $\Delta\psi$Tm compared to WT Vpr VSVG infections. (C) MT Vpr R77Q induces less caspase processing than WT Vpr. Cytosolic extracts from day 6 infections were immunoblotted for procaspase 8 and DFF cleavage. Protein was normalized by cell numbers. Lane 1 – control, 2 – WT Vpr, 3 – Vpr (-), 4 – R77Q.

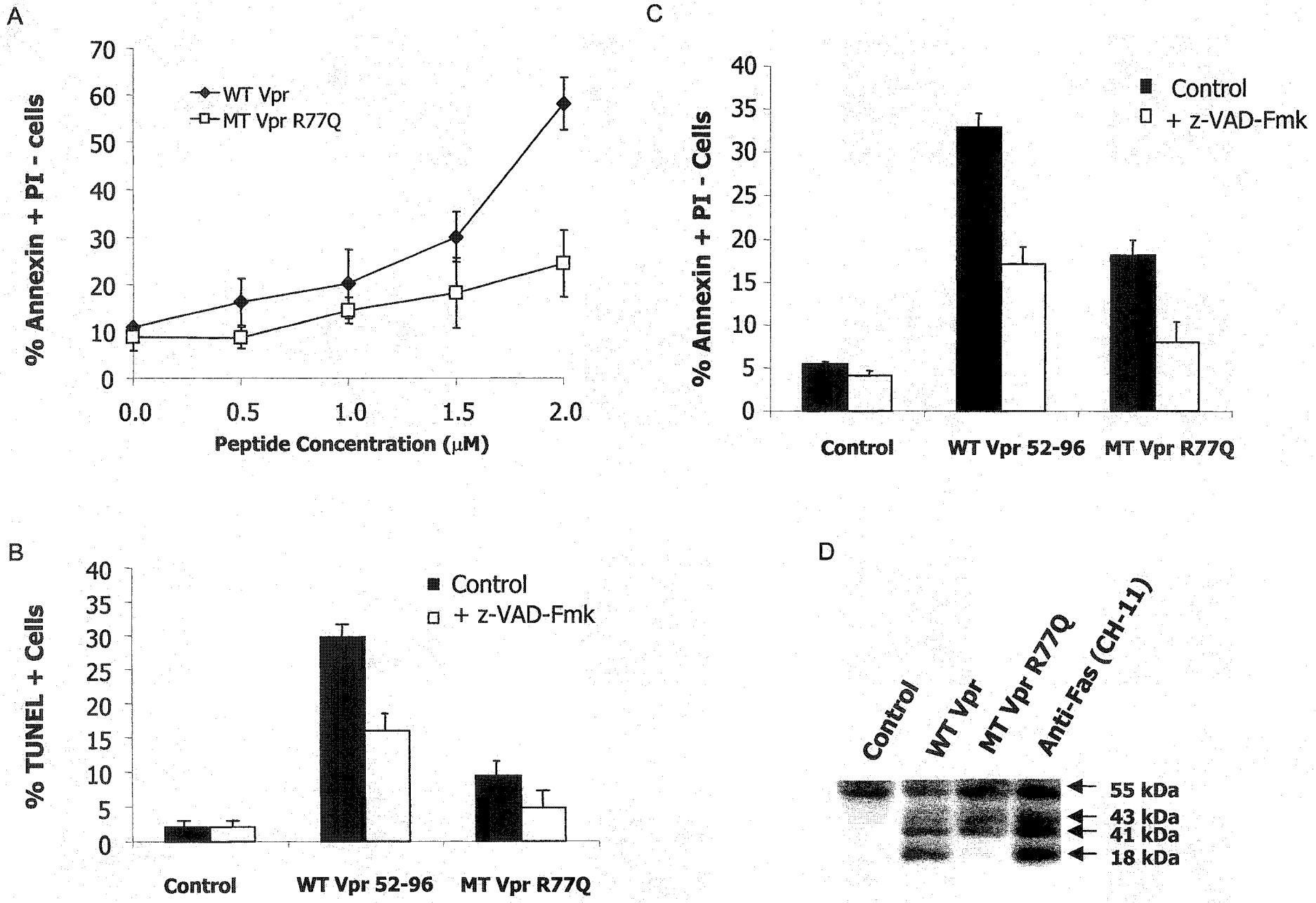

A
Mock
WT Vpr
MT Vpr R77Q
Day 2
Day 4
Day 6
Side Scatter
Forward Scatter
89.1%
88.4%
90.6%
85.7%
78.6%
84.8%
86.9%
56.2%
83.6%
B
Day 2
Day 4
Day 6
Events
FL1-Height
M1
14.3%
10.4%
8.5%
16.0%
13.0%
8.7%
23.3%
57.3%
20.9%
C
1 2 3 4
Procaspase 8
Active Caspase 8
55kDa
18kDa
Procaspase 9
DFF
46kDa
45kDa

R77Q infected cells (Fig 21C). Similar results were seen when procaspase 9 and DFF were monitored indicating that MT Vpr R77Q infections of Jurkat cells results in less apoptosis compared to WT Vpr.

Decreased Apoptosis induced by Vpr (52-96) R77Q peptide.

Previous investigators have established that the c-terminal peptide 52-96 of Vpr which contain the $(H(F/S)RIG)_2$ domain are responsible for Vpr mediated initiation of apoptosis (Azad, 2000; Bartz et al., 1996; Chen et al., 1999; Conti et al., 2000; Conti et al., 1998; Goh et al., 1998; He et al., 1995; Jacotot et al., 2000; Jowett et al., 1995; Macreadie et al., 1996; Macreadie et al., 1995; Macreadie et al., 1997; Nishizawa et al., 2000; Patel et al., 2000; Piller et al., 1998; Shostak et al., 1999; Stewart et al., 1997; Stewart et al., 2000). We studied the effect of c-terminal Vpr peptides (52-96) with or without the R77Q mutation to evaluate the effect of this mutation on Vpr induced apoptosis. Jurkat cells were incubated with increasing doses of Vpr WT peptide or R77Q peptide (Fig 22A) and analyzed for apoptosis. Similar results were seen using either annexin V and propidium iodide staining (Fig 22A; WT Vpr = 56.0% vs. R77Q Vpr = 27.3%, $n = 3$, $p = 0.02$) or TUNEL staining (Fig. 22B; WT Vpr = 30.0% vs. R77Q Vpr = 9.2%, $n = 3$, $p < 0.01$). Consistent with other published reports suggesting that Vpr induced apoptosis is caspase dependent (Shostak et al., 1999; Stewart et al., 2000), the induction of apoptosis by both WT and R77Q Vpr was blocked by the pancaspase inhibitor zVAD-fmk (Fig 22B,C). To confirm differences in apoptosis activation, cleavage of pro-caspase 8 was assessed and was greater in cells treated with WT Vp

Figure 22. MT R77Q Vpr peptide induces less death of cells and caspase 8 processing compared to WT Vpr. (A) Jurkats were treated with varying doses of synthetic C terminal WT or R77Q *Vpr* peptides for 16 hours followed by annexin V FITC / PI staining or (B) TUNEL staining. Data represents average of three independent experiments. (C) Jurkats were pretreated in the presence or absence of the pancaspase inhibitor zVAD-fmk. (D) WT *Vpr* causes complete cleavage of caspase 8 into the intermediate and active p18 fragments in a similar manner to that seen with Fas ligation. Treatment with R77Q peptide has decreased caspase 8 activation and undetectable p18 fragments.

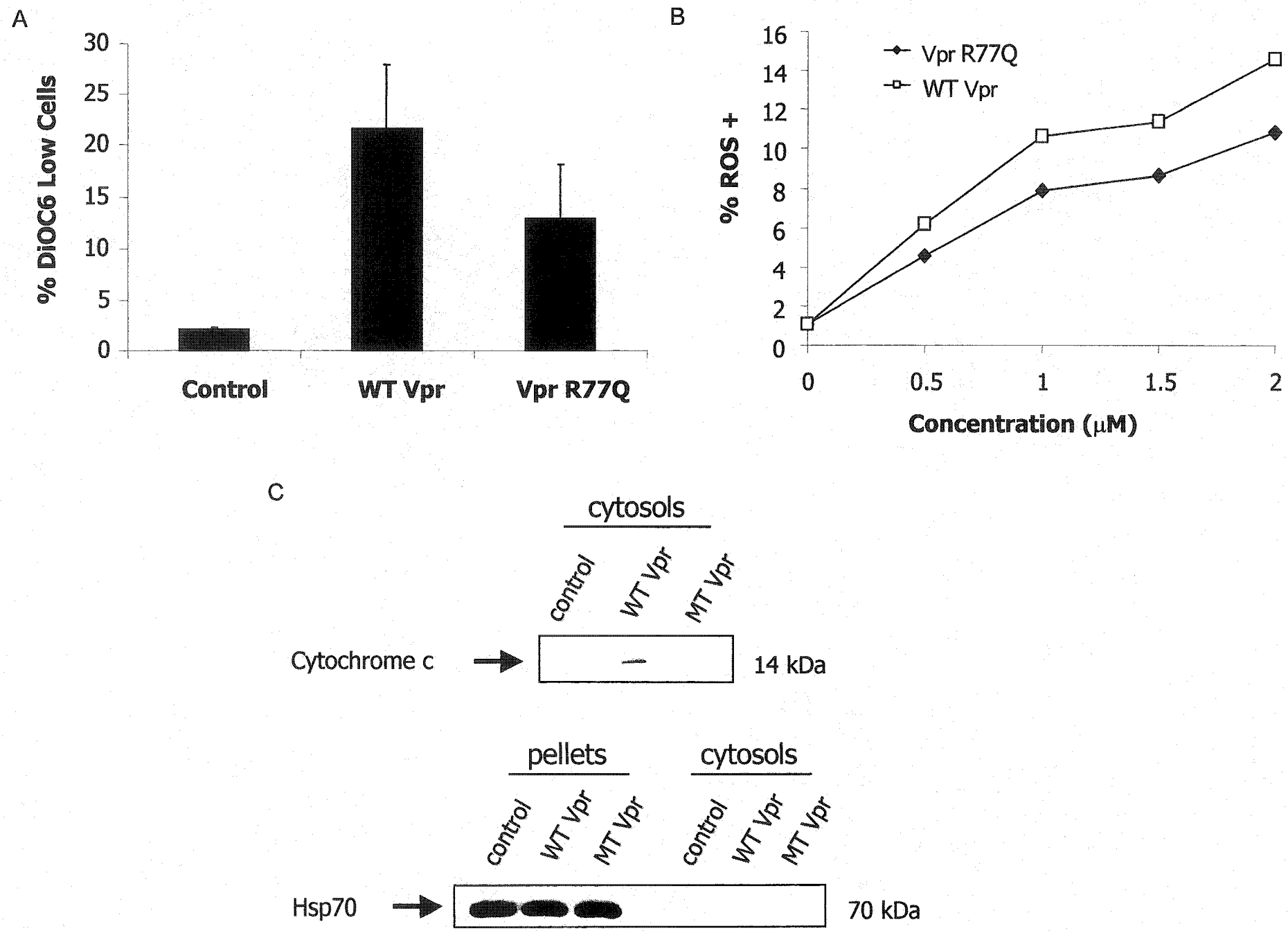

A
% Annexin + PI - cells
WT Vpr
MT Vpr R77Q
Peptide Concentration (μM)
C
% Annexin + PI - Cells
Control
+ z-VAD-Fmk
Control
WT Vpr 52-96
MT Vpr R77Q
B
% TUNEL + Cells
Control
+ z-VAD-Fmk
Control
WT Vpr 52-96
MT Vpr R77Q
D
Control
WT Vpr
MT Vpr R77Q
Anti-Fas (CH-11)
55 kDa
43 kDa
41 kDa
18 kDa

peptides where a p18 fragment is clearly visible, yet in Vpr R77Q peptide treated samples, caspase 8 is partially only cleaved (Fig 22D).

Vpr R77Q induces less mitochondrial release of cytochrome c than WT.

Vpr directly binds to ANT (Jacotot et al., 2000) and opens mitochondrial PTPC (Jacotot et al., 2001; Jacotot et al., 2000) resulting in loss of $\Delta_{\psi Tm}$, and the release of apoptogenic factors into the cytoplasm. While 16 hour treatment with WT Vpr caused 21.6% of Jurkat cells to lose $\Delta_{\psi Tm}$, treatment with R77Q resulted in $\Delta_{\psi Tm}$ in only 12.6% of Jurkats ($p = 0.02$, $n = 2$) (Fig 23A) consistent with differences in $\Delta_{\psi Tm}$ induced by WT versus R77Q VSV-G infections. The production of reactive oxygen species (ROS) was attenuated with R77Q peptide when compared to WT peptide (Fig 23B; R77Q Vpr = 10.8% vs WT Vpr = 14.4, $n = 3$, $p = 0.05$). Furthermore, cytochrome c release as a consequence of PTPC opening was monitored in the cytosolic fractions of Jurkats treated with either R77Q *Vpr* peptide or WT (Fig 23C - upper panel). Whereas cytochrome c release was abundantly detected in the cytosols of cells treated with WT Vpr, R77Q Vpr did not have the same effect. To ensure that the cytosolic fractions were not contaminated with mitochondria, immunoblots for a mitochondria specific protein, heat shock protein 70 (hsp70) were performed. As hsp70 is present only inside the mitochondrial matrix, it is a marker for the presence of mitochondria (Fig 23C - lower panel) (Marchenko et al., 2000).

Figure 23. Effect of WT or R77Q *Vpr* on mitochondria in Jurkat cells. Jurkats were treated with WT or R77Q peptide and assayed for (A) transmembrane potential loss using $DiOC_6$ (B) production of reactive oxygen species. (C) Jurkat T cells were treated with WT or R77Q (MT) *Vpr*, lysed and fractionated into mitochondrial and cytosolic fractions. The cytosolic fraction was blotted for cytochrome c (top) and each fraction was probed for the mitochondrial specific protein Hsp 70 (bottom) to confirm no mitochondrial contamination within the cytosols. Data represents average of 3 independent experiments.


A
% DiOC6 Low Cells
30
25
20
15
10
5
0
Control
WT Vpr
Vpr R77Q
B
% ROS +
16
14
12
10
8
6
4
2
0
Vpr R77Q
WT Vpr
0
0.5
1
1.5
2
Concentration (μM)
C
cytosols
control
WT Vpr
MT Vpr
Cytochrome c
14 kDa
pellets
cytosols
control
WT Vpr
MT Vpr
control
WT Vpr
MT Vpr
Hsp70
70 kDa

Vpr R77Q induces less caspase activation than WT Vpr

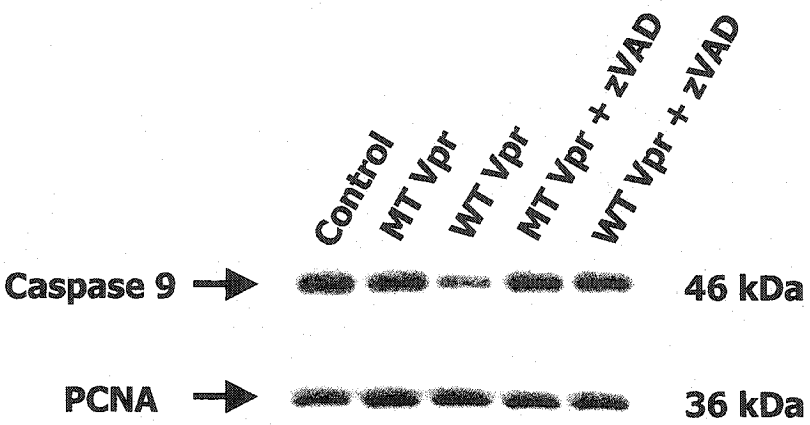
Following release of cytochrome c from mitochondria, caspase 9 is activated, which subsequently activates caspase 3 which in turn cleaves and activates DNA fragmentation factor (DFF) which is the DNase responsible for apoptotic DNA fragmentation. We therefore evaluated caspase 9, caspase 3 and DFF cleavage in cells treated with R77Q or WT Vpr peptides. Procaspase 9 and procaspase 3 were both reduced only in the cells treated with WT Vpr peptide (Fig 24A, B), indicating cleavage, yet in cells treated with R77Q, the reduction of procaspases 9 and 3 was less apparent. In parallel, caspase 3 activity was assayed in cells treated with either WT or R77Q Vpr peptide, demonstrating that caspase 3 activation was less in cells treated with R77Q than in cells treated with WT peptide (data not shown). Similarly, DFF cleavage was detected only in cells treated with the WT peptide (Fig 24C), again demonstrating an impaired ability of R77Q peptide to induce apoptotic changes when compared to WT. All immunoblots were normalized for equal loading using PCNA.

Effects of R77Q or WT Vpr on isolated mitochondria

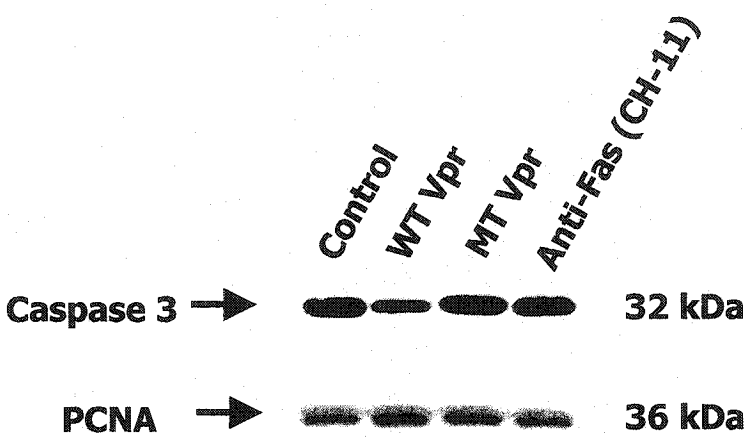
Conceivably, the impaired ability of R77Q to induce apoptosis in intact cells might be due to differences in uptake of Vpr into cells. To assess this possibility, we incubated freshly isolated mitochondria from mouse liver and measured $\Delta_{\psi Tm}$ using $DiOC_6$. Within 6 hours of incubation at the lowest concentration of peptides, WT Vpr caused 62.7% of mitochondria to lose $\Delta_{\psi Tm}$. Whereas only 26.6% of isolated

Figure 24. Effect of WT or R77Q *Vpr* on caspase 9, 3 and DFF. Jurkat T cells were treated overnight with WT or R77Q (MT) *Vpr* and analyzed for (A) caspase 9 activation (B) caspase 3 activation and (C) DFF cleavage. Blots were stripped and reprobed for PCNA to ensure equal loading.

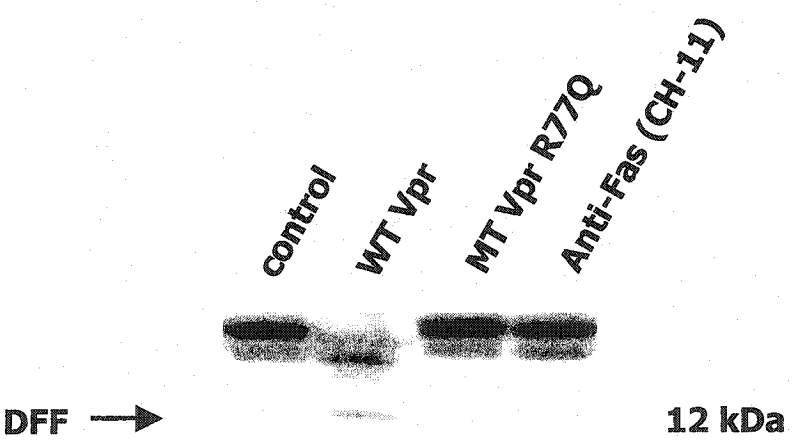
A


Control
MT Vpr
WT Vpr
MT Vpr + zVAD
WT Vpr + zVAD
Caspase 9
46 kDa
PCNA
36 kDa

B


Control
WT Vpr
MT Vpr
Anti-Fas (CH-11)
Caspase 3
32 kDa
PCNA
36 kDa

C


control
WT Vpr
MT Vpr R77Q
Anti-Fas (CH-11)
DFF
12 kDa

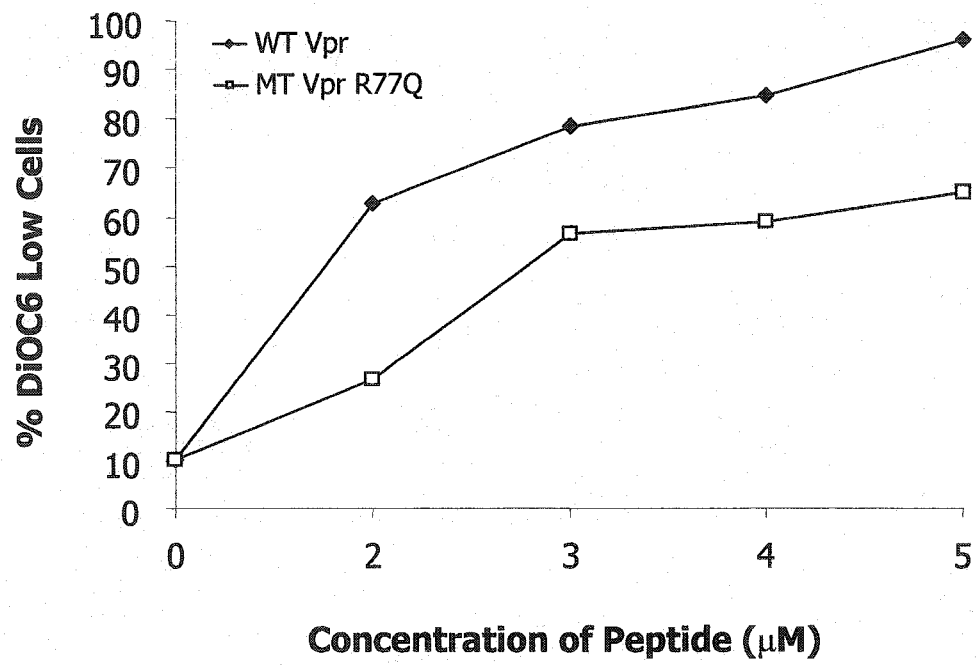
mitochondria treated with R77Q Vpr lost $\Delta_{\Psi Tm}$ ($p = 0.02$; Fig 25A). We also assessed the release of cytochrome c from isolated mitochondria after treatment with Vpr WT or R77Q peptides. Cytochrome c release was detected at all concentrations of WT Vpr, but only at the highest concentration of R77Q Vpr (Fig 25B).

R77Q Vpr is less toxic than WT in vivo

To assess the physiologic relevance of impaired apoptosis induction of R77Q Vpr *in vivo*, 21 - 28 day old BALB/c mice received intravenous tail vein injections of 200 µl of diluent alone or with either WT or R77Q Vpr peptide (10 mg/kg). CD4, CD8 and CD3 T cell numbers were assessed at baseline as well as 24 and 48 hours post injection. Control mice had stable T cell counts over the 48 hour period of observation (data not shown), whereas mice treated with WT Vpr experienced a dramatic reduction of both CD4 and CD8 T cell numbers by 48 hours. Consistent with our *in vitro* observations, when compared to control mice, we found a decrease in CD4 T cell numbers in mice injected with WT Vpr ($n = 5$) yet in comparison to WT vpr R77Q caused less T cell depletion (Fig 26A, $n = 5$, $p < 0.001$). Similar results were found when we analyzed CD8 T cell numbers. Injection of WT Vpr resulted in a greater decrease in CD8 T cells compared to R77Q injected mice (Fig 26B, $n = 5$, $p = 0.002$). Furthermore the magnitude of CD4 T cell depletion with WT peptide was greater than that of CD8 T cells (CD4% of baseline at 48 hrs = 15.6%, CD8% of baseline at 48 hrs = 26.3%, p=0.0035). Of interest, local injection site toxicity was apparent in treated mice; severe tail vein vasculitis resulting in tail discoloration and petechia was seen in all mice who received WT Vpr

Figure 25. Effect of WT or R77Q *Vpr* on isolated mitochondria. Mitochondria were isolated from mouse livers and treated with the indicated concentration of WT *Vpr* or R77Q (MT) *Vpr* and supernants analyzed for (A) $\Delta_{\psi Tm}$ or (B) cytochrome c release. Equal loading of protein was confirmed using PCNA (data not shown). Data represents 1 of 3 independent experiments.

A


100
90
80
70
60
50
40
30
20
10
0
% DiOC6 Low Cells
WT Vpr
MT Vpr R77Q
0
2
3
4
5
Concentration of Peptide (μM)

B

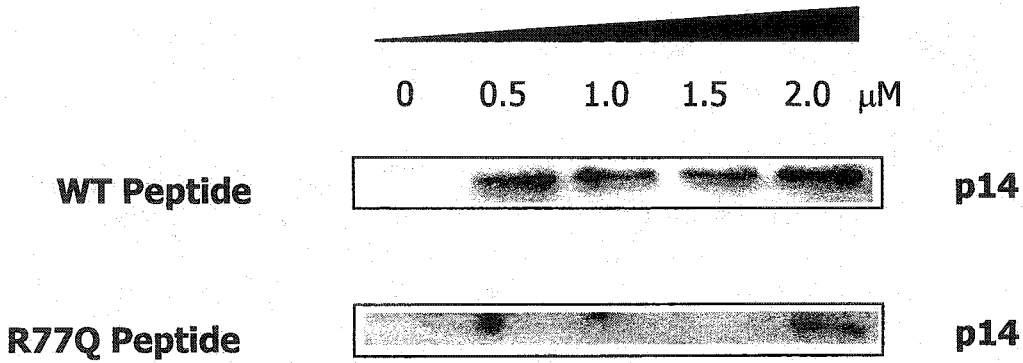
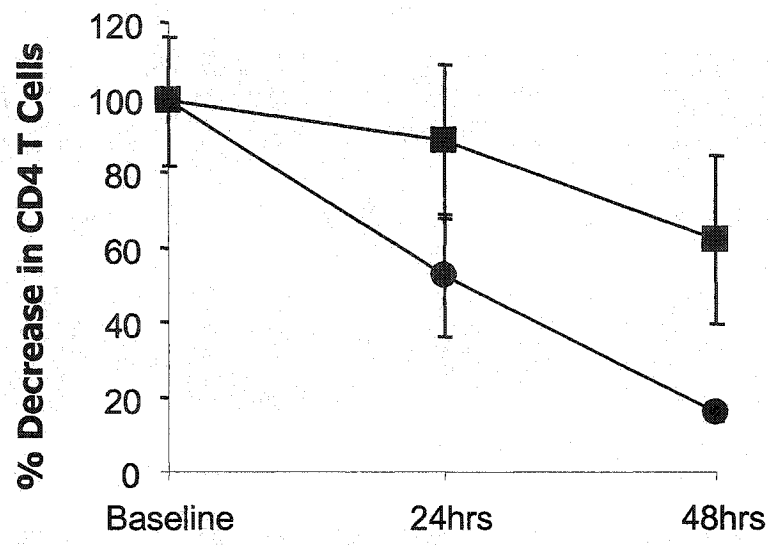
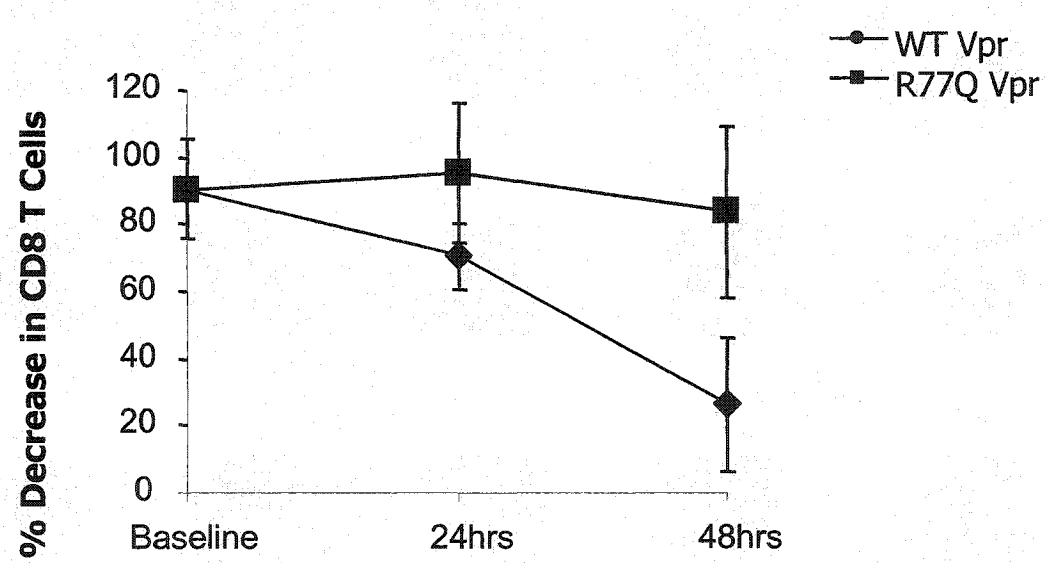

0 0.5 1.0 1.5 2.0 μM
WT Peptide
p14
R77Q Peptide
p14

Figure 26. Effect of WT or R77Q *Vpr* on T cell depletion *in vivo*. 21-28 day old Balb/c mice were IV infected with vehicle control (data not shown), or containing WT *Vpr* or MT *Vpr*. The absolute CD4 or CD8 T cell number at baseline, 24 and 48 hours after injection was determined by FACS analysis. Data is presented as percent decrease from baseline normalized to control mice for (A) CD4 T cells or (B) CD8 T cells.

A


% Decrease in CD4 T Cells
120
100
80
60
40
20
0
Baseline
24hrs
48hrs

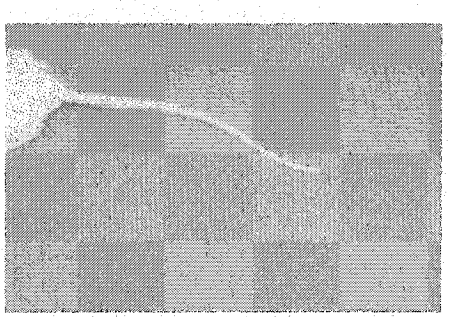
B

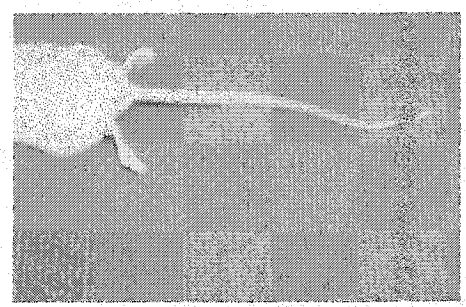

WT Vpr
R77Q Vpr
% Decrease in CD8 T Cells
120
100
80
60
40
20
0
Baseline
24hrs
48hrs

(Fig 27), while no toxicities were apparent in vehicle control treated mice. A mild form of tail vein vasculitis that was associated with slight tail vein discoloration was present in the R77Q treated mice (Fig 27). Necropsy samples of liver, spleen and kidney showed no evidence of systemic vasculitis in any mice (data not shown). Thus, the phenotypic consequences of Vpr injection confirm an impaired ability of R77Q to induce CD4 and CD8 T cell depletion and to induce local tissue injury.

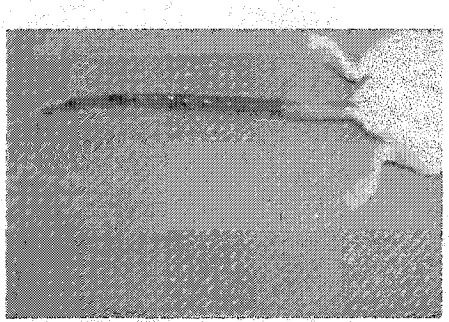
Figure 27. Local injection site and effect of WT or R77Q *Vpr*. 21-28 day old Balb/c mice were injected with vehicle control alone or containing WT *Vpr* or MT (R77Q) *Vpr*. Tail discoloration appeared in WT treated mice as early as 8 hours post injection and persisted up to 48 hours. Minimal tail discolouration occurred in MT treated mice and no discolouration observed in control.

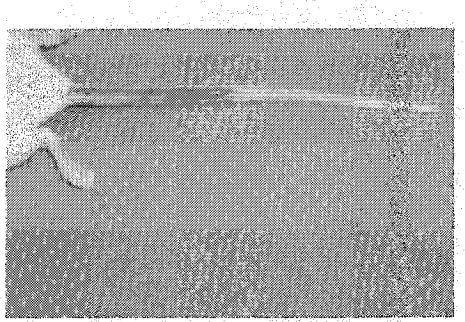
Control



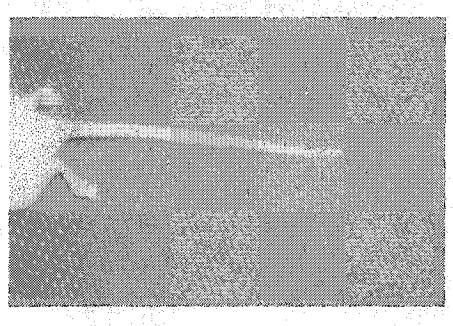


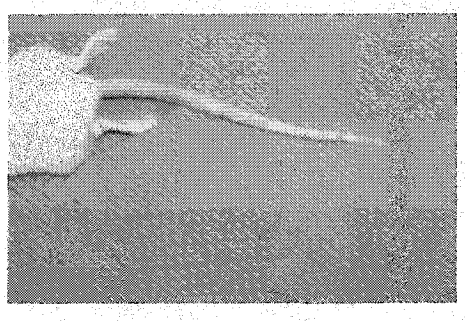
WT Vpr





MT Vpr





5.4 Discussion

Only recently has the link between Vpr and apoptosis been established, and more importantly the pivotal role of the $(H(F/S)RIG)_2$ domain. Thus, while the Vpr sequence from LTNP has been previously analyzed, the importance of point mutations within this domain has not been fully investigated. In our analysis of Vpr mutations, we examined both a cohort of 10 LTNPs all of whom had detectable levels of viral replication, and 15 HIV infected progressors who were antiretroviral naive with detectable viral replication, and who had CD4 T cells counts below 500. According to this analysis, 80% of LTNPs but only 33% of progressors have the Vpr mutation R77Q. These data are consistent with other studies demonstrating high frequency (approximately 80%) of R77Q mutations in LTNP (Wang et al., 1996; Zhang et al., 1997). Conversely the low frequency of R77Q in patients with progressive disease has not been previously reported, but is consistent with a search of the *Los Alamos* database in which approximately 17% of progressors had this mutation. In addition, in a study of an HIV infected mother-child (Wang et al., 1996), both infected mother and child had long term non-progressive disease which was associated with an R77Q mutation of Vpr, yet reversion to wild-type genotype at position 77 was temporally associated with progressive disease. Thus, our data are consistent with all available data which suggest an association of Vpr R77Q with delayed disease progression. A recent study by Stevenson's group have identified a new mutation in one long term non progressive patient. This point mutation in the n-terminal of Vpr, Q3R demonstrates reduced cytopathogenicity, thus providing a further piece of evidence that

loss of apoptotic activity in Vpr is correlated to asymptomatic HIV disease progression (Somasundaran M et al., 2003).

Since early in the HIV epidemic, elevated levels of T cell depletion have been observed in the peripheral blood of infected patients. Furthermore, both HIV infected and uninfected cells from patients display enhanced susceptibility to apoptosis induced by activation, mitogen, antigen and death receptor initiated signalling (reviewed in (Badley et al., 2000)). While considerable debate exists concerning the signals which predominate *in vivo*, HIV infection leads to apoptosis induced by activation signals, enhanced expression of apoptosis inducing ligands, and apoptotic signals produced by viral proteins including each of gp120, Nef, Tat, protease and Vpr (reviewed in (Badley et al., 2000)). A causal role for apoptosis as the mediator of CD4 T cell depletion in HIV infection is suggested by the following: (1) animal models of lentivirus infection in which enhanced apoptosis is observed are associated with immunodeficiency, whereas those without apoptosis are not (Estaquier et al., 1994). (2) The disease course of human infection with HIV correlated inversely with apoptosis both in cross sectional and longitudinal studies (Patki et al., 1997; Samuelsson et al., 1997). (3) Initiation of antiretroviral therapy which results in immune reconstitution causes a dramatic reduction in T cell apoptosis both within the peripheral blood and in lymphoid tissues (Badley et al., 1998; Badley et al., 1999). Finally (4), whereas patients with rapidly progressive HIV disease have high levels of apoptosis, patients with LTNP HIV infection have levels of apoptosis similar to those of HIV uninfected patients (Liegler et al., 1998; Wasmuth et al., 2000). Intriguingly mechanisms of LTNP defined thus far have identified host and

viral means of impaired viral infectivity or replication, yet no mechanism has been defined which impairs HIV's ability to induce T cell death.

Since the $(H(F/S)RIG)_2$ domain of Vpr is responsible for inducing apoptosis and mutations within this domain are associated with LTNP, we compared the apoptotic effects of R77Q Vpr and WT Vpr *in vitro*. Jurkats cells infected with VSV-G or treated with R77Q peptides underwent less apoptosis, $\Delta_{\psi Tm}$, caspase activation, and DFF activation. Further, these differential effects were also seen when isolated mitochondria were treated with WT or R77Q peptide. Thus, R77Q mutations of Vpr are both associated with a LTNP phenotype and have a decreased ability to induce apoptosis *in vitro*.

Transgenic mice which overexpress Vpr display CD4 and CD8 T cell depletion and thymic atrophy (Iwakura, 1998), thereby suggesting that Vpr influences T cell survival *in vivo*. Therefore, we assessed the *in vivo* effects of mutant versus wild type Vpr in mice following tail vein injection. Strikingly while both CD4 and CD8 T cell depletion are seen, the magnitude of CD4 T cell depletion is greater than that seen for CD8 T cells. These changes very closely mimic changes in CD4 and CD8 T cell number seen in patients during the course of HIV disease, suggesting that Vpr may impact the CD4 and CD8 T cell decline *in vivo*. Whereas significant CD4 and CD8 T cell depletion and local tail vein vasculitis occurs following injection of WT Vpr, all of these effects are attenuated following injection of R77Q Vpr. Thus, both *in vitro* and *in vivo*, in

experimental systems and in HIV infected patients, mutation within the $(H(S/F)RIG)_2$ apoptosis inducing domain of Vpr is associated with attenuated T cell depletion.

Our cumulative data thus demonstrate an impaired ability of R77Q mutations of Vpr to induce apoptosis *in vitro* and *in vivo*, and this mutation is seen in a high frequency of LTNP as opposed to patients with progressive HIV disease. These observations suggest that Vpr plays a significant role in CD4 T cell depletion in individuals infected with HIV, perhaps to a greater degree than other proposed mechanisms of CD4 T cell depletion. Moreover, it suggests a therapeutic opportunity for the development of Vpr inhibitors to reduce T cell death during HIV infection.

Section 6:
Vpr $(HF/SRIG)_2$ Domain Mutations are Transdominant Inhibitors of Apoptosis

6.1 Introduction

Based on the results in section 5.3, we postulated that some c-terminal vpr peptides possess antiapoptotic activity. The R77Q mutant found in long term non progressors is associated with reduced ability to induce apoptosis. This substitution had modest dominant negative activity (data not shown) and prompted us to investigate other mutations in vpr that possess more potent antiapoptotic transdominant activity. Several other groups have identified vpr peptides that have decreased ability to induce cell cycle arrest, HIV transactivation and nuclear localization compared to wildtype vpr (Sawaya et al., 2000; Zhou and Ratner, 2001). However, no group to date has examined whether dominant negative vpr petides can block other forms of apoptotic stimulus or what effect these mutations have on cell death. We have identified a short c-terminal double mutant (R73A, R77A), termed DN2, which protects cells from apoptosis induced by wildtype vpr *in vitro*. Interestingly, this protection was independent of HIV as DN2 inhibited apoptosis by Fas agonists and DNA damaging agents. These results provide the first evidence for protection against cell death using a transdominant peptide and suggests a novel target to inhibit death through the mitochondria.

6.2 Materials and Methods

Cell culture. Jurkat T cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Sigma, St. Louis), 100U/mL of each penicillin and streptomycin (Canadian Life Technologies Products. Montreal, Canada unless otherwise stated). Blood was isolated from healthy donors following informed consent outlined by the Ottawa Hospital Research Ethics Board. PBMCs were maintained as described above. Vpr peptide (all N-terminally conjugated with biotin) sequences were as follows: WT: N' TWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS 'C, DN1: N' LFIHFAIGCRHSRIGVT 'C, DN2: N' LFIHFAIGCAHSRIGVT C' DN3: N' LFIHFAIGCRHSAIGVT 'C, DN4: N' LFIHFAIGCAHSAIGVT C'. Purity for all peptides was confirmed by mass spectrometery (Genemed Synthesis Inc., San Francisco, CA). All peptides were reconstituted at 1mM concentration in ddH_2O and stored at -80°C until use.

Detection of Cell Death. Cells were pretreated in isotonic buffer for 30 minutes with DN Vpr peptides, followed by resuspension in complete media. Cell death was induced using: 0.05ug/mL agonist Fas antibodies (Immunotech, CH-11) for 16hrs, 500ng/mL camptothecin (Sigma, St. Louis) for 16 hrs or 1μM WT Vpr for 16 hr. Cells were collected at the indicated time points and stained with Annexin V-FITC (BD Pharmingen) for 30 minutes at 37°C followed by addition of 1μg/mL propidium iodide (Sigma, St. Louis). For mitochondria transmembrane potential analysis, cells were stained for 20 minutes with 40μM 3'3'-dihexyloxacarbocyanine iodide, $DiOC_6$

(Molecular Probes, Eugene. OR). All flow cytometry acquisitions were performed using Coulter Epics Altra (Coulter) with no less than 10000 collected events.

Immunoblotting analysis. Cells lysates were prepared by resuspending cells in lysis buffer for 15 minutes on ice. Protein concentration was normalized to cell numbers. For detection of cytochrome c, Jurkat cytosols were prepared by 15 minute incubation in ice cold isotonic buffer, followed by 40-80 strokes using a dounce homogenizer type B 2mL pestle. Complete membrane dissociation was verified by > 90% trypan blue inclusion. Cleared lysates were resuspended in SDS loading buffer and heated to 100°C for 5 minutes followed by separation on 12% SDS PAGE. Proteins were transferred to PVDF membranes (Millipore) and blocked with 5% skim milk for 4 hours at room temperature. Caspase 8 (Biosource), caspase 3 (generous gift Dr. R.P Sekaly) and caspase 9 (MBL) primary antibodies were used at 1:1000 blotted overnight at 4°C. Western blots were visualized with SuperSignal (Pierce).

In vivo induction of Apoptosis. Eight week old female BALB/c mice (Charles River) were maintained in germ free housing, acclimatized for 7 days prior to initiation of experimental protocols and handled according to regulations approved by the Animal Care Committee at the University of Ottawa. Fifty micrograms of the Fas agonist antibody, Jo2 (BD Pharmingen) was diluted in sterile PBS and stored at 4°C prior to use. 100mg/kg of DN2 was diluted in sterile water and stored at -20°C prior to use. Mice were injected by intravenous tail vein in a total volume of 200μL with PBS, DN2 or Jo2. For challenges with Jo2, mice were simultaneously injected with DN2 and Jo2. Mice

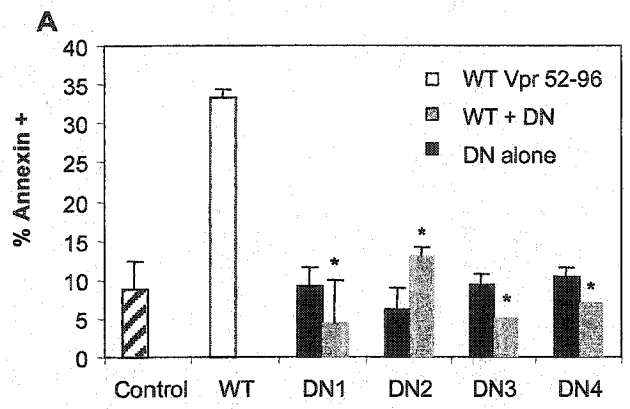
were euthanized at 24 hours and tissues were collected in paraformaldehyde for histopathological analysis. Samples were sent to the Guelph animal care and histopathology centre, School of Veterniary Medicine for necropsy (Guelph, Ontario). TUNEL analysis were performed by Molecular Histology Inc., (Montegomery Village, MD).

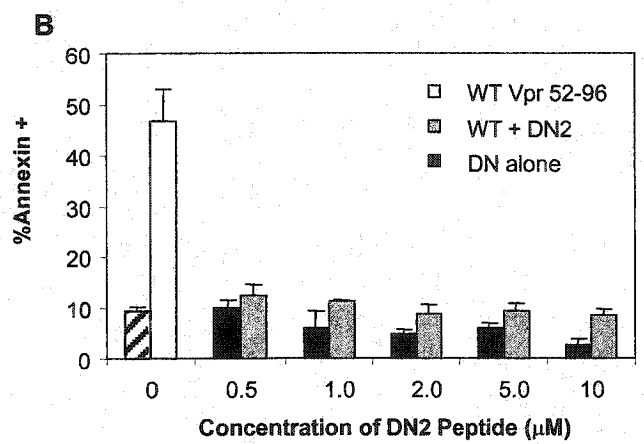
6.3: Results

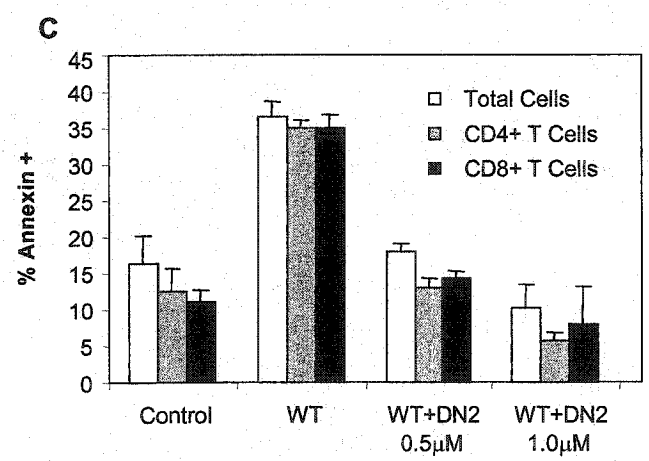
Dominant Negative Vpr Peptides Lack Cytotoxicity and Blocks wildtype Vpr Induced Apoptosis in vitro.

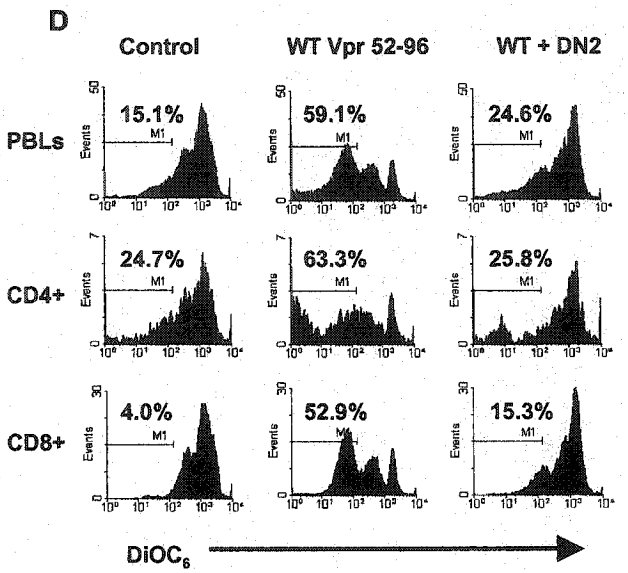
We generated a c-terminal library of synthetic Vpr peptides with specific mutations spanning positions 73-79. Four potential peptides were chosen for further study based on their ability to inhibit WT Vpr induced apoptosis (Fig 28A). Jurkat cells treated with DN2 Vpr peptides had a dose dependent inhibition of spontaneous apoptosis (Fig 28A) that could be seen within 1 hour of treatment (data not shown). No observable toxicity was observed using the remaining three DN peptides with concentrations up to 10μM (Fig 28B). As expected, incubation of Jurkat cells with WT Vpr peptide resulted in a significant number of annexin + cells (33.2%) (Jacotot et al., 2001; Jacotot et al., 2000). However, pretreatment of Jurkats with DN peptides blocked WT Vpr induced apoptosis to levels similar or below control treatments (Fig 28A). The inhibitory effect of DN2 against WT Vpr was dose dependent and the blocking effect could be observed at concentrations as low as 0.5μM (Fig 28B). When these experiments were repeated using primary human PBLs, we obtained similar results. In bulk PBLs, all four peptides dramatically reduced the amount of apoptosis induced by WT Vpr (Fig 28C). As expected, three colour FACs analysis of annexin +, CD4+ or CD8+ revealed a substantial decrease in DN2 pretreated cells undergoing apoptosis (Fig 28D). In all cases, DN2 blocked cell death back to baseline levels. The inhibition in total PBLs, CD4 and CD8 cells was observed at 0.5μM and maximal at 1.0μM (Fig 28C). The $\Delta_{\Psi Tm}$ is an early

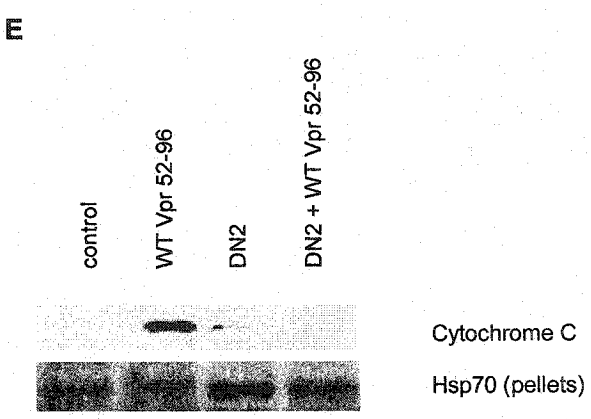
Figure 28. Antiapoptotic Effect of *Vpr*-Derived c-terminal peptides. (A) Four vpr derived peptides were generated as: DN1 - R73A, DN2 - R73,77A, DN3 - R73,80A and DN4 – R73,77,80A. DN peptides treatment alone (black bars) in Jurkat cells did not induce apoptosis. Jurkats were treated with WT Vpr peptide (white bars) alone or DN followed by WT Vpr (grey bars) and assayed for apoptosis by annexin V staining. Data are averages from 4 independent experiment, * $p < 0.03$ (versus WT vpr alone). (B) Dose response to DN2 alone or WT Vpr + DN2 in Jurkat Cells. (C) DN2 protects primary T cells from WT Vpr induced cell death. Three colour FACS analysis on CD4+ and CD8+ T cell apoptosis was performed after treatment with DN2 alone or WT Vpr + DN2. Each data set was performed three times. (D) Loss of TMP in PBLs was assessed using DiOC6 staining following treatment with DN2 alone or WT Vpr + DN2. (E) Undetectable release of cytochrome c from mitchondria of Jurkats pretreated with DN2 and challenged with WT Vpr. Experiments were performed at least 2 times and cytosolic release of cytochrome c was confirmed by probing with hsp70 in mitochondria pellets.


A
% Annexin +
WT Vpr 52-96
WT + DN
DN alone
Control
WT
DN1
DN2
DN3
DN4


B
%Annexin +
WT Vpr 52-96
WT + DN2
DN alone
Concentration of DN2 Peptide (μM)


C
% Annexin +
Total Cells
CD4+ T Cells
CD8+ T Cells
Control
WT
WT+DN2 0.5μM
WT+DN2 1.0μM


D
Control
WT Vpr 52-96
WT + DN2
PBLs
CD4+
CD8+
15.1%
59.1%
24.6%
24.7%
63.3%
25.8%
4.0%
52.9%
15.3%
DiOC6


E
control
WT Vpr 52-96
DN2
DN2 + WT Vpr 52-96
Cytochrome C
Hsp70 (pellets)

marker for apoptosis and can be measured using the cationic lipid dye, $DiOC_6$, which readily stains mitochondria with intact potential (Jacotot et al., 2001; Jacotot et al., 2000). PBLs treated with WT Vpr had 59.1% $DiOC_6$ low cells compared to only 24.6% in cells that were pretreated with 0.5μM DN2 (Fig 28D). The number of cells which lost $\Delta\psi_{Tm}$ as a result of WT Vpr stimulation decreased from 63.3% to 24.7% in CD4+ gated cells and from 52.9% to 15.3% in CD8+ gated DiOC6 low cells (Fig 28D). This implies that DN2 reduces $\Delta\psi_{Tm}$ loss in cells stimulated to undergo apoptosis by WT Vpr. Moreover, the release of cytochrome c in Jurkat cytosols by WT Vpr was completely prevented by DN2 (Fig 28E). This indicated that DN2 is capable of blocking early mitochondrial apoptotic events.

DN2 Peptide inhibits non viral forms of apoptosis

We used Fas agonists and a DNA damaging agent camptothecin (CPT), to study the effects of DN2 on other forms of Jurkat T cell death irrespective of HIV. All 4 candidate peptides significantly reduced the amount of cell death induced by Fas by greater than 69% (Fig 29A) while 2 of 4 peptides blocked CPT induced apoptosis (Fig 29B). The blocking effect by Fas was dose dependent (Fig 29C) and observed within 1 hour (data not shown). DN2 also inhibited the loss of $\Delta\psi_{Tm}$ induced by anti-Fas from 26.0% DiOC6 low to 4.3% (Fig 29D). Furthermore, the release of cytochrome c induced by Fas (Fig 29E) was completely blocked by DN2, consistent with the ability of DN2 to suppress opening of PTPC. When downstream caspase activation was analyzed, cells treated with all four DN peptides alone did not activate procaspase 8 (Fig 30 – top panel),

Figure 29. Inhibition of Cell Death is Independent of HIV vpr. Jurkats were stimulated with Fas (A) or CPT (B) in the presence or absence of DN peptides. Apoptosis was assayed using annexin V staining. (C) Dose responsive inhibition of Fas by DN2 in Jurkat cells. (D) Jurkats pretreated with DN2 followed by stimulation with Fas have reduced TMP loss and (E) release of cytochrome c from the mitochondria.

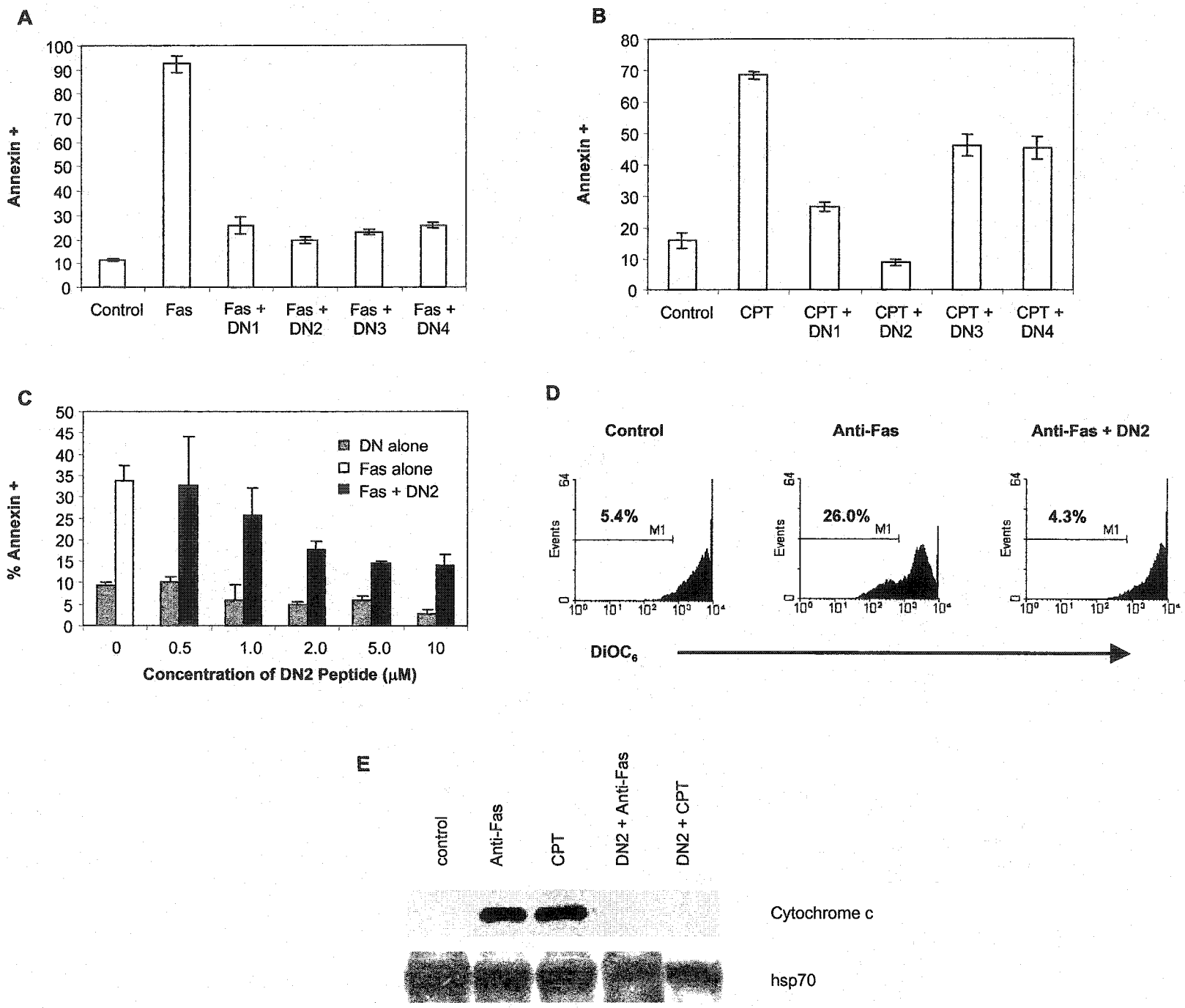
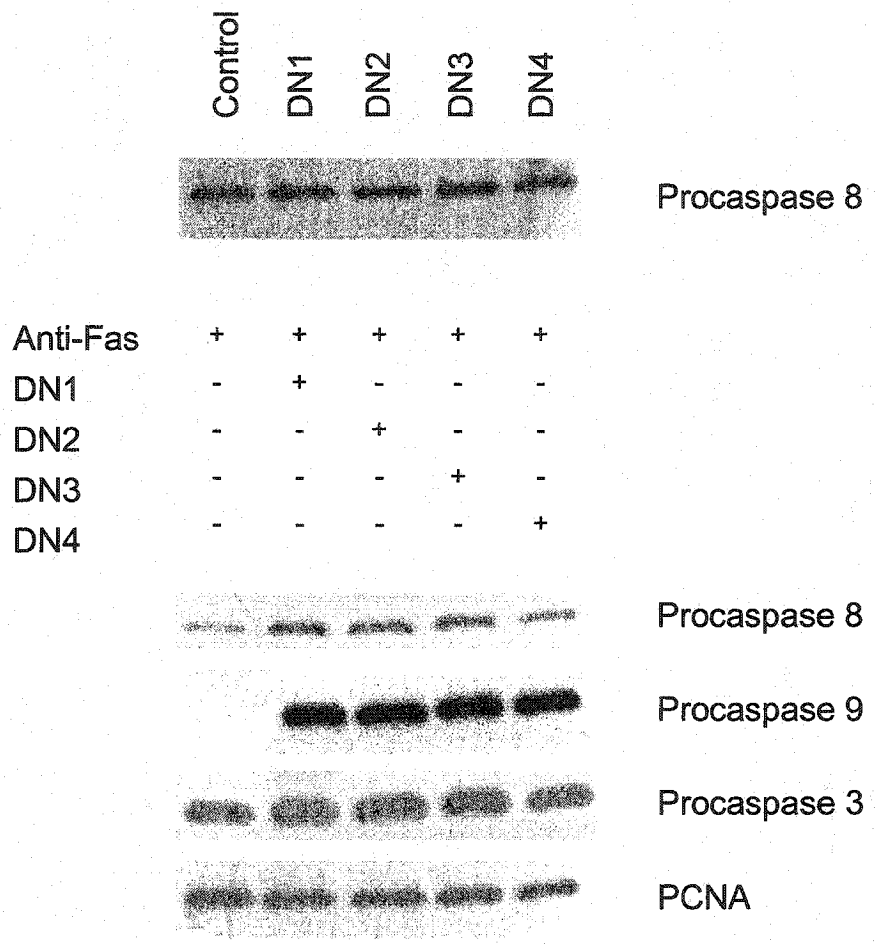

A
Annexin +
100
90
80
70
60
50
40
30
20
10
0
Control
Fas
Fas + DN1
Fas + DN2
Fas + DN3
Fas + DN4
B
Annexin +
80
70
60
50
40
30
20
10
0
Control
CPT
CPT + DN1
CPT + DN2
CPT + DN3
CPT + DN4
C
% Annexin +
50
45
40
35
30
25
20
15
10
5
0
DN alone
Fas alone
Fas + DN2
0
0.5
1.0
2.0
5.0
10
Concentration of DN2 Peptide (μM)
D
Control
Anti-Fas
Anti-Fas + DN2
Events
64
0
5.4%
M1
26.0%
M1
4.3%
M1
DiOC6
E
control
Anti-Fas
CPT
DN2 + Anti-Fas
DN2 + CPT
Cytochrome c
hsp70

Figure 30. Downstream mitochondria activation of procaspases 8, 3, 9 by WT Vpr is inhibited by DN2. Cells were treated as indicated and cytosols or whole cell lysates were analyzed for caspase activation by immunoblotting.


Control
DN1
DN2
DN3
DN4
Procaspase 8
Anti-Fas + + + + +
DN1 - + - - -
DN2 - - + - -
DN3 - - - + -
DN4 - - - - +
Procaspase 8
Procaspase 9
Procaspase 3
PCNA

consistent with previous experiments demonstrating a lack of cytotoxicity in Jurkats treated with DN peptides. Whereas anti-Fas treatments resulted in the loss of procaspase 8 (Fig 30 – bottom panel), Jurkats pretreated with DN2 blocked activation of procaspase 8. In contrast, treatment of cells with Fas resulted in complete disappearance of procaspase 9 which was inhibited by DN2. Downstream of caspase 9, we detected partial cleavage of procaspase 3 (Fig 30 - bottom) which was blocked by DN2. These data indicate that pretreatment of cells with DN2 resulted in blockage of apoptosis at all the levels tested, which was independent of HIV proteins.

In vivo protection against anti-Fas

As previously described, intraperitoneal or intravenous (IV) injection of the anti-mouse Fas agonist antibody causes fulminant heptatic failure and death of BALB/c mice within 3-5 hours. In order to study whether the *in vitro* effects of DN2 could also be seen *in vivo*, mice were IV injected simultaneously with 50μg Jo2 and 100mg/kg DN2. There was a 0% survival in mice injected with anti-fas only. Strikingly, at 12 hours, 60% of mice injected with DN2 and Jo2 survived (Fig 31). By 24 hours, 40% of mice injected with both Jo2 and DN2 remained alive. In liver sections, DN2 significantly reduced the number of TUNEL positive cells compared to mice treated with Jo2 alone. Mice injected with Jo2 had greater than 25 TUNEL positive cells per high power field compared to only 5 TUNEL positive cells per high power field when DN2 was coinjected (Fig 32). In addition, severe liver hemorrhage was observed in Jo2 treated mice (Fig 32). We also performed toxicity assays of DN2 and with concentrations up to 100mg/kg, no

Figure 31. Protection from lethal anti-Fas treatment by DN2 in BALB/c mice. 8 week old mice were simultaneously coinjected by tail I.V. with DN2 and Jo2. Surivival of mice was monitored for 24 hours followed by euthanasia of all remaining mice.

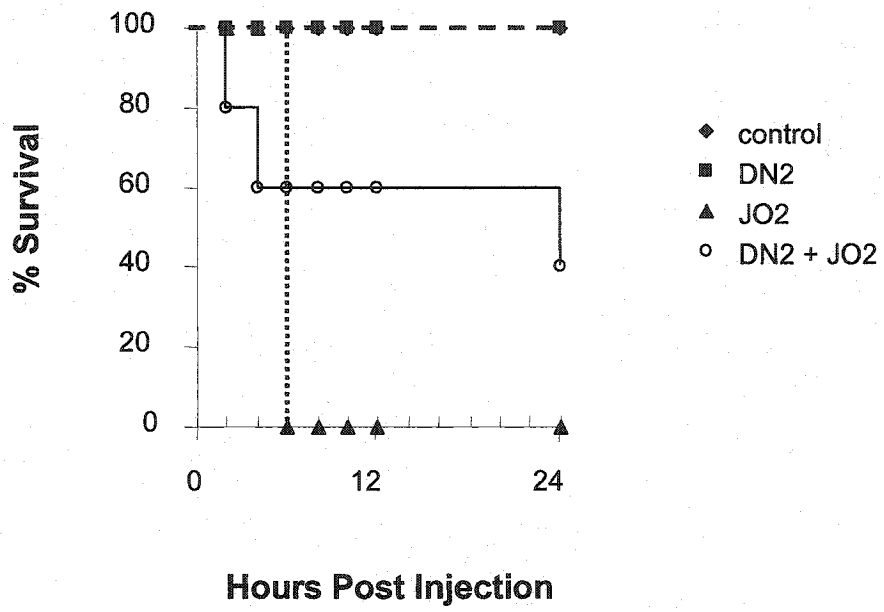
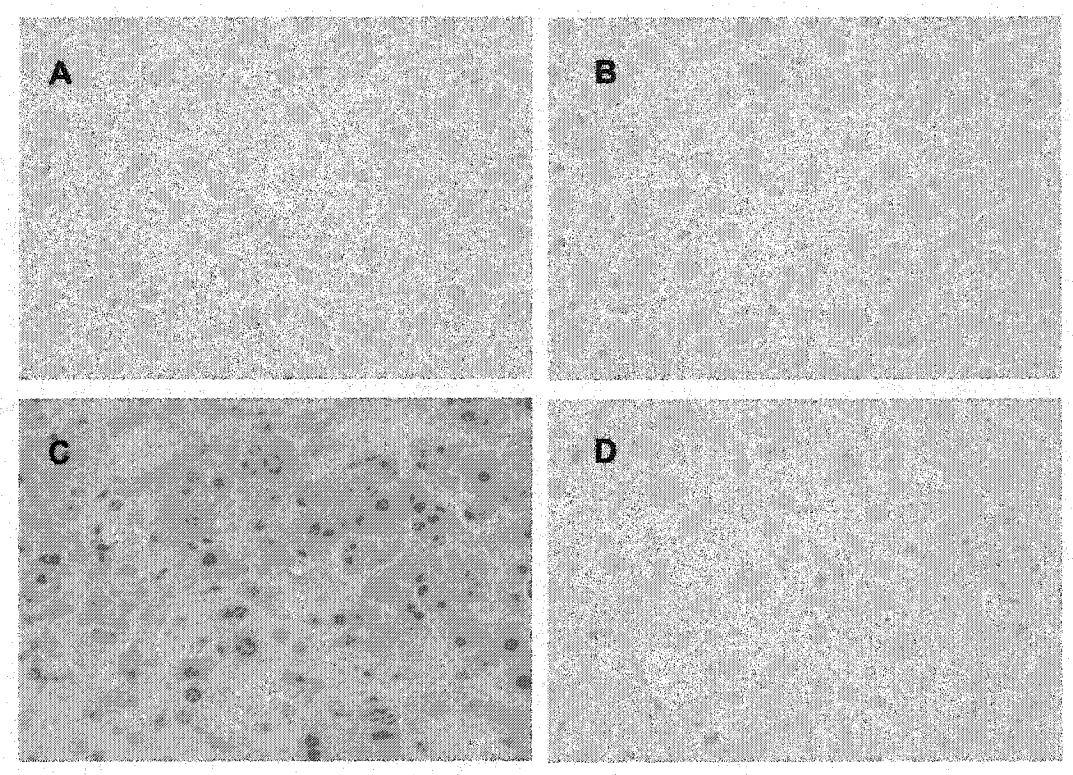

100
80
60
40
20
0
% Survival
0
12
24
Hours Post Injection
control
DN2
JO2
DN2 + JO2

Figure 32. Decreased number of TUNEL positive cells in livers of mice coinjected with DN2. Mice were injected simultaneously with DN2 and J02 followed by tissue collection at 24 hours. A – Control, B – DN2 alone, C – JO2 alone, D – DN2 + JO2.


A
B
C
D

detrimental effect was observed in the kidney, brain, tail, liver, spleen or thymus of these mice (data not shown).

6.4 Discussion

Apoptosis is the underlying cause of many viral and nonviral pathologies of disease including influenza (Hinshaw et al., 1994) HIV (reviewed in (Badley et al., 2000)), cerebral ischemia (Silverstein, 1998) and idiopathic CD4 T lymphocytopenia syndrome (Laurence et al., 1996). Mitochondrial deregulation and loss of function are pivotal steps in these processes. This observation has led to the intense search for novel mitochondria inhibitors of apoptosis. Although the mechanisms of CD4+ T cells loss during HIV infection are not completely understood, recent data implicate Vpr as a cofactor in initiating death through direct interactions with the mitochondria (Jacotot et al., 2001; Jacotot et al., 2000). Commitment to death by Vpr involves direct mitochondrial damage with the breakdown of membrane potential and liberation of cytochrome c into the cytoplasmic compartment. This translocation of cytochrome c triggers the formation of a complex with an oligomerized Apaf-1 which recruits caspase 9 (Jacotot et al., 2001; Jacotot et al., 2000). This results in autocatalytic activation of caspase 9 and cleavage of the initiator caspase 3. In this way, activated caspase 3 can auto feedback and cause the cleavage of procaspase 8, further amplifying the apoptotic cascade (Jacotot et al., 2001; Jacotot et al., 2000), an event exemplified by type II cells (Scaffidi et al., 1998).

Vpr has also been shown to induce cell cycle arrest and provide additional signals for efficient replication in macrophages. Although transdominant activity of Vpr has been recently described by Sawaya *et a,l* (Sawaya et al., 2000) on abrogating cell cycle

arrest, no study has characterized the antiapoptotic effect of these mutants. We recently show that the point mutation R77Q is associated with decreased ability to induce cell death compared to WT Vpr (submitted). This indicated that specific mutations in the apoptotic domain of Vpr may possess antiapoptotic function, consistent with the dual role of Vpr in apoptotic responses of T cells (Conti et al., 2000). Thus, we generated a Vpr C-terminal peptide library and isolated 4 candidate peptides which possess potent antiapoptotic activity. We isolated a double mutant termed DN2 which decreased spontaneous death in cell culture and within 1 hour completely prevented WT Vpr induced cell death of Jurkats and human primary PBLs. We chose to study DN2 in detail since it had the most potent antiapoptotic activity against a wide range of apoptotic stimuli. When Jurkats were pretreated with DN2, we were unable to detect typical changes associated with mitochondria dysfunction including loss of $\Delta_{\Psi Tm}$ and cytochrome c release.

Although the precise pathway by which DN2 acts is not evident from these experiments, coimmunoprecipitation studies did not reveal preferential binding of DN2 over WT Vpr to ANT (data not shown), in contrast to the single R73A or R80A mutations which have lower affinity for ANT (Jacotot et al., 2001; Jacotot et al., 2000). The disparity between the results maybe explained by single mutations versus double mutations within the same peptide and the sensitivity of the assay used. However, these data are consistent with reports of other Vpr mutants, namely R73A having transdominant activity both *in vitro* and in *vivo* (Sawaya et al., 2000; Zhou and Ratner, 2001). In addition, immunofluorescence experiments demonstrated that DN2 and WT

Vpr colocalized to the mitochondrial compartment (Jacotot et al., 2001 and data not shown; Jacotot et al., 2000). Therefore, the inhibitory effect of DN2 on WT Vpr may not be due to competitive inhibition but associated with structural changes that preclude oligomerization of Vpr (Piller et al., 1998), thus preventing signals for ANT opening. It is also unclear whether DN2 is capable of having inhibitory effects on the Bcl-2 family members including Bcl-xL, Bax and Bid and is indeed a likely possibility as both WT Vpr and Bcl-2 modulate $\Delta\Psi_{Tm}$ through a direct interaction with ANT (Jacotot et al., 2001; Jacotot et al., 2000). The ability of mutant forms of vpr such as R77Q to induce less apoptosis may result from a dominant negative effect such as the effect described with DN2. In this model, mutant forms of vpr would still bind to ANT but with stronger affinity or binding of dominant negative peptides would prevent binding of the wildtype protein. In addition, the general affect of DN2 bears resemblances to Bax/Bak double deficient cells in that these cells are completely resistant to many forms of apoptosis including death receptor ligation, serum withdrawal, UV irradiation and mitochondrial apoptosis inducers. This suggests that DN2 may affect the ability of Bax/Bak to induce the release of cytochrome c from the mitochondria possibily by binding to these two proteins and preventing their channel forming activity. These effects have not been formally tested but are currently underway to evaluate these possibilities. Interestingly, DN2 significantly blocked death induced by Fas agonists and CPT suggesting that DN2 has HIV independent antiapoptotic activity. With both Fas and CPT, DN2 prevented the loss of $\Delta\Psi_{Tm}$ and release of cytochrome c. DN2 further inhibited the activation of caspase 8, 3, and 9 indicating that the initial events of mitochondrial induced apoptosis were blocked.

In contrast to mice injected with Jo2 alone, simultaneous coinjection of DN2 and Jo2 in BALB/c significantly retarded death by 40%. The inhibition of death correlated with decreased TUNEL staining in mice coinjected with DN2. Thus, the effects of DN2 on Fas induced apoptosis in human PBLs *in vitro* correspond to experimentally induced suppression of cell death *in vivo*.

The range of distinct apoptosis pathways inhibited by DN2 *in vitro* indicates that DN2 exerts its activity by modulating a fundamental and common regulatory component of the apoptotic program. The present study provides evidence that DN2 is a potent general suppressor of apoptosis *in vivo* and *in vitro*, by inhibiting key initiator events arising through mitochondrial dysfunction. These results provide insight into the development and evaluation of strategies targeting mitochondrial induced apoptosis and is not limited to HIV infection, but also for intervention of organ trauma and neurodegenerative diseases due to excessive apoptosis.

7.0 General Discussion

In addition to the documented toxicities of HIV therapy, the discovery that HIV remains in quiescent reservoirs and is not affected by antiretroviral drugs has strengthened the emphasis on developing new microbicides, vaccines and strategies to treat HIV. The first section of this thesis examines the role of TRAIL during HIV infection and evaluates whether TRAIL could be used as a possible therapy to target HIV reservoirs. Both cells infected *in vitro* and cells from patients infected with HIV have gross alterations in TRAIL receptor and TRAIL expression. These cells undergo apoptosis following exogenous addition of recombinant TRAIL or TRAIL agonists indicating that HIV infection results in a TRAIL sensitive phenotype. To test whether TRAIL could promote the containment or elimination of the major reservoirs for HIV, an enhanced virus culture technique was used to assay (Chun et al., 1998; Chun et al., 1997b; Finzi et al., 1997) the size of the latent pool following *in vitro* TRAIL or TRAIL agonist treatments. For the first time, we show that TRAIL reduces the number of latently infected cells, in some cases to undetectable levels, opening the possibility of complete clearance of HIV. In cases where replication competent virus was still detectable, it was demonstrated that two and three rounds of TRAIL treatment further reduced the size of the latent reservoirs (data not shown). This indicates that multiple treatments may be necessary to achieve sterile cure. The reasons for multiple rounds of treatment are not known at this time. However, further examination of these data in animal models will be required to fully address these conclusions.

The use of recombinant TRAIL preparations may be many years away from clinical trials. Moreover, recombinant TRAIL suffers from a poor pharmacological half life, clearance and stability. An alternative approach is to attempt to use cytokines to enhance TRAIL expression via the intrinsic function of NK cells. The goal is to use IL-15 to stimulate NK cells as 'natural viral killers' against any infected cell. In section 3.0, we demonstrate that *in vitro* treatment of human NK cells with IL-15 dramatically increases the functional expression of TRAIL. More importantly, IL-15 stimulated NK cells caused specific TRAIL lysis of cells harboring persistent HIV providing evidence that IL-15 could be used as a therapy for HIV. Surprisingly, IL-7 upregulated FasL expression which led to an increase in FasL mediated NK killing. These results show that TRAIL and FasL are differentially controlled and provides further evidence for the use of IL-15 as a possible therapy to treat HIV disease.

The intense effort in developing TRAIL as a treatment for human cancers has also shed important information on the cellular death receptor pathways and their implication in HIV disease. However, despite the encouraging data in section 2.0 and 3.0 on TRAIL, the molecular regulation of TRAIL and TRAIL receptors during HIV infection remains unclear. To examine factors controlling TRAIL receptor and TRAIL sensitivity, cells were treated with various sources of gp120. In all cases, gp120 increased TRAIL receptor expression leading to susceptibility to TRAIL mediated cell death. These experiments suggest a possible molecular control by which TRAIL sensitivity is regulated. Interestingly, ligation of the chemokine coreceptor for HIV, CXCR4 with its natural ligand SDF-1 or antibodies directed at CXCR4 also resulted in TRAIL receptor

upregulation and TRAIL sensitivity. This indicated that TRAIL is controlled independently of HIV. Given studies showing the involvement of TRAIL in autoimmunity, we postulated that TRAIL could be a regulator of inflammatory responses. Using neutrophils as a model system, we determined that neutrophils are susceptible to TRAIL dependent activated induced cell death. Collectively, these results demonstrate TRAIL as a key factor in controlling inflammatory responses. However, further studies using gene deleted or knock down models of TRAIL will be required to fully address these conclusions.

As apoptosis plays an important role in HIV disease, we examined factors which contribute to the destruction of CD4 T cells in attempts to develop potential inhibitors of cell death induced by HIV. Accumulating evidence demonstrates that expression and soluble forms of Vpr mediated apoptosis of lymphocytes (Ayyavoo et al., 1997; Ferri et al., 2000; Jacotot et al., 2001; Levy et al., 1994; Levy et al., 1995; Macreadie et al., 1996; Macreadie et al., 1997; Nishizawa et al., 2000; Piller et al., 1998). Therefore, we hypothesized that naturally occurring mutations within Vpr would correlate with the non-symptomatic disease progression seen in long term non progressors. Genetic analysis revealed an 80% glutamine to arginine mutation at position 77 within the C-terminus of Vpr. *In vitro* studies found a significant decrease in the ability of R77Q mutant to kill cells when compared to wildtype using several molecular approaches. However, these experiments were unable to determine the molecular basis for the decreased apoptotic ability of R77Q. Interestingly, R77Q mutants possessed mild dominant negative activity suggesting that other transdominant mutants may have more potent antiapoptotic activity.

Therefore, a small peptide library was prepared and individually tested for antiapoptotic properties. One novel peptide was identified, DN2, that dramatically blocked wildtype vpr induced apoptosis. DN2 was also found to inhibit Fas and DNA damaging forms of apoptosis. Moreover, experiments using a liver hepatic necrosis model showed that DN2 blocked apoptosis induced by anti-Fas, thus providing the first known *in vivo* mitochondria inhibitor. The range of pathways inhibited by DN2 suggests widespread application as a blocker of apoptosis induced by neurogenerative diseases, viral infections and organ trauma.

The scale of the HIV epidemic has far exceeded the expectations of health care workers, politicians and those who are infected. The spread has been greatly underestimated with serious consequences for sub-Saharan Africa. AIDS threatens to strike the advancement of human development and represents one of the most contentious calamity we currently face. The potential for further spread is imminent if the current rate of infections continues and effective measures commensurate with the epidemic are not implemented. The political, economic and social capital structure has been eroded by stalled prevention and treatment efforts among industrialized nations. Intervention with antiretroviral therapy has been a major stride forward in reducing morbidity and mortality associated with HIV in settings where populations can afford access. The urgent need to provide universal availability to affected communities will require restructuring of global economics and more importantly, intense research efforts among the entire scientific community.

STATEMENT OF CONTRIBUTION OF COLLABORATORS

A number of collaborators have assisted throughout the thesis work. Their percent contribution to the overall section is denoted in brackets.

Section 2: Dr. John Kim's group/staff (20.0%) conducted all viral load assays in accordance with strict Health Canada Guidelines and the PCR based viral DNA detection. All TRAIL reagents were kindly provided by Dr. David Lynch. Dr. Erling Rud's group/staff (5.0%) provided samples from SHIV infected macaques.

Section 5: Dr. Oren J. Cohen (2.0%) and Dr. Anthony S. Fauci provided DNA from their LTNP cohort for sequencing of vpr. Dr's. Kim and Montpetit's group/staff (5.0%) performed the vpr sequencing and analysis. Dr's Keets, Kilborn and staff handled (5.0%) all mice injection studies (5.0%). Dr. Eric Cohen (5.0%) and staff generated the initial VSV-G viral stocks and constructs for subsequently experiments.

Section 6. Histopathology on mice were performed by pathology lab at the University of Guelph (5.0%). TUNEL staining was performed by Molecular Histology Inc. (5.0%).

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Normal Progressors

Patient	Accession	Vpr Sequence	Clade
1	AF000314	Q	B
2	AF004394	Q	B
3	M17451	Q	B
4	K02007	Q	B
5	M38431	Q	B
6	M93259	Q	B
7	U51188	Q	CRF01_AE
8	AB052995	Q	CRF01_AE
9	U51189	Q	CRF01_AE
10	AF061642	Q	G
11	AJ271370	Q	N
12	AJ006022	Q	N
13	L20587	Q	O
14	AF325763	Q	NA
15	AJ006287	Q	B
16	U46016	Q	C
17	AF377954	Q	CRF02_AG
18	U888222	Q	NA
19	M17451	Q	B
20	K02007	Q	B
1	AJ237565	H	H
2	AF377959	H	AG
3	AF000323	R	B
4	AF133389	R	B
5	AF133392	R	B
6	AF133393	H	B
7	AF133394	R	B
8	AF133395	R	B
9	AF133397	R	B
10	U63141	R	NA
11	AJ271445	R	B
12	AB034516	R	B
13	AB034517	R	B
14	AB034518	R	B
15	U39362	R	B
16	M17449	R	B
17	U69584	R	B
18	L02317	R	B
19	U26546	R	B
20	U21135	R	B
21	AF110973	L	C
22	AF110979	H	C
23	AF286233	H	C
24	AF286235	L	C
25	AF193253	H	CRF05_DF
26	AF064699	H	CRF05_DF
27	AF064699	H	CRF06_cpx
28	AJ288982	H	CRF06_cpx
29	AJ288981	H	CRF06_cpx
30	AF077336	R	F1
31	AF075703	H	F1
32	AJ249236	H	F2
33	AF190128	H	H
34	AJ249235	H	K
35	AJ249239	H	K

PROGRESSORS R77Q	20 (36%)
PROGRESSORS R77?	35 (64%)
TOTAL PROGRESSORS	55

Long Term Non Progressors

Patient	Accession	Vpr Sequence	Clade
1	U61882	Q	NA
2	AF316105	Q	B
3	AB043906	Q	NA
4	AF316862	Q	O
5	AF316861	Q	O
6	AF316860	Q	O
7	AF316856	Q	O
8	AF316857	Q	O
9	AF316859	Q	O
10	AF316858	Q	O
11	AF193275	Q	A
12	AF004885	Q	A
13	AF069671	Q	A
14	AF069673	Q	A
15	M62320	Q	A
16	AF004885	Q	A
17	U51190	Q	A
18	AF067156	Q	C
19	U41704	Q	B
20	U41707	Q	B
21	U37270	Q	B
22	U37271	Q	B
23	U37268	Q	B
24	AF042100	Q	B
25	AF042101	Q	B
26	AF042102	Q	B
27	AF042106	Q	B
28	U71182	Q	B
29	Z68566	Q	B
30	AB034519	Q	B
31	AB034520	Q	B
32	AB034536	Q	B
33	AB034537	Q	B
34	AB034543	Q	B
35	AB034544	Q	B
36	AF224507	Q	B
37	Z68540	Q	B
38	Z68541	Q	B
39	Z68549	Q	B
40	Z68551	Q	B
41	Z68568	Q	B
42	Z68569	Q	B
43	Z68570	Q	B
44	Z68571	Q	B
45	Z68572	Q	B
46	Z68573	Q	B
47	Z68574	Q	B
48	Z68575	Q	B
49	Z68576	Q	B
50	Z68577	Q	B
51	Z68578	Q	B
52	Z68579	Q	B
53	Z68580	Q	B
54	Z68581	Q	B
55	Z68582	Q	B
56	Z68583	Q	B
57	AF005495	Q	C
58	U52953	Q	C
59	AF290027	Q	C
60	AF110959	Q	C
61	AF067157	Q	C
62	AF067158	Q	C
63	AF286234	Q	C
64	AF286235	Q	C
65	AF286227	Q	C
66	AF286224	Q	C
67	AF286225	Q	C

68	AB032740	Q	CRF01_AE
69	AB032741	Q	CRF01_AE
70	AF063223	Q	CRF02_AG
71	AF063224	Q	CRF02_AG
72	AJ245481	Q	CRF06_CPX
73	AJ288981	Q	CRF06_CPX
74	AF286226	Q	CRF07_BC
75	AF286230	Q	CRF08_BC
76	AY008717	Q	CRF08_BC
77	AY008716	Q	CRF08_BC
78	AY008715	Q	CRF08_BC
79	AF286229	Q	CRF08_BC
80	AF286550	Q	CRF10_CD
81	AF289549	Q	CRF10_CD
82	AF179368	Q	CRF11_CPX
83	AJ239083	Q	MO
84	Y16018	Q	O
85	Y16019	Q	O
86	Y16020	Q	O
87	Y16021	Q	O
88	Y16022	Q	O
89	Y16023	Q	O
90	Y16024	Q	O
91	Y16025	Q	O
92	Y16026	Q	O
93	Y16027	Q	O
94	Y16028	Q	O
95	Y16029	Q	O
96	Y16030	Q	O
97	Y16031	Q	O
98	see paper	Q	NA
99	see paper	Q	NA
100	see paper	Q	NA
101	see paper	Q	NA
102	U09126	Q	C
103	U08797	Q	B
104	94IN11246	Q	C
105	U4056	Q	U
106	93BR020	Q	F
107	see paper	Q	NA
108	see paper	Q	NA
109	see paper	Q	NA
1	AF316107	H	B
2	AF325749	S	O
3	AY006053	R	NA
4	U61885	R	NA
5	AF069670	R	A1
6	AF377959	H	AG
7	U73371	R	B
8	AF133382	R	B
9	AF133383	H	B
10	AF133384	R	B
12	AF133385	H	B
13	AF133387	H	B
14	AF042103	R	B
15	AF042104	R	B
16	AF042105	R	B
17	Z68555	R	B
18	Z68556	R	B
19	Z68562	R	B
20	AF224507	R	B
21	Z68542	R	B
22	Z68543	R	B
23	Z68544	R	B
24	Z68545	R	B
25	Z68546	R	B
26	Z68547	R	B
27	Z68548	R	B
28	Z68550	R	B
29	Z68552	R	B
30	Z68557	R	B
31	Z68579	H	B
32	Z68584	R	B
33	Z68585	R	B
34	Z68539	R	B
35	U24447	H	NA
36	U24444	R	B

LTNP R77Q	109	(75%)
LTNP R77?	37	(25%)
TOTAL LTNPS	146	