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Frequency and Significance of HIV-1 Infection of CD8+ T-Cells: Implications for Viral Pathogenesis

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FREQUENCY AND SIGNIFICANCE OF HIV-1 INFECTION OF CD8+ T-CELLS: IMPLICATIONS FOR VIRAL PATHOGENESIS

BY

NAVEED GULZAR

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ABSTRACT

To date, the effect of HIV-1 infection on CD8+ T-cells remains poorly studied. Previous studies have shown that the effector functions of CD8+ T-cells diminish during AIDS. We postulated that CD8+ T-cell functions decrease due to the tropism of HIV-1 for CD8+ T-cells. Therefore we examined whether CD8+ T-cells provide suitable targets for HIV-1 infection and the mechanism(s) by which the virus enters the cells. We hypothesized that HIV-1 entry into these cells may be facilitated through access to extracellular receptors.

Ex vivo experiments were performed using blood samples from a cohort of HIV-1 infected patients attending the Ottawa Hospital Immunodeficiency Clinic. Frequency of HIV-1 infection was monitored by flow cytometric detection of HIV-1 p24 antigen and viral production from separated CD8+ and CD4+ T-cell lineages was assayed by the quantitation of viral RNA transcripts. Primary CD8+ T-cells and CD8+ T-cell clones used in the *in vitro* studies were isolated from the peripheral blood of healthy volunteers. HIV-1 infection was monitored by both ELISA and flow cytometric analyses. Similarly, receptor analysis was performed by flow cytometry and confirmed by RT-PCR analysis.

There was a significantly higher frequency of CD8+HIV+ cells than CD4+HIV+ cells found in the *ex vivo* studies of patient samples, however, viral production from the CD8+HIV+ subset was 2-3 logs lower than that found in CD4+HIV+ T-cells. In addition, CD8+ T-cells served as suitable targets for productive HIV-1 infection *in vitro* and preferential HIV-1 replication occurred in the memory T-cell subset. Interestingly, the HTLV-I transformed CD8+ T-cell clones exhibited HIV-1 production 20-fold greater than CD4+ T-cells. Our research also demonstrated that during the course of infection, there was a decrease in mean expression of the CD8 and CXCR4 cell-surface molecules in the HIV-1 infected CD8+ T-cell clones. Accordingly, the use of antibodies to the CD8 or CXCR4 molecules eradicated viral adsorption and replication in the CD8+ T-cell clones.

Our research was the first to demonstrate the significance and susceptibility of CD8+ T-cells to HIV-1 infection through both *ex vivo* and *in vitro* analyses. We conclude, with multiple lines of evidence detecting and measuring HIV-1 infection of CD8+ T-lymphocytes, that this cellular target and reservoir may be central to HIV-1 pathogenesis. By identifying a novel target of HIV-1 and potential cellular receptors used by the virus, future therapeutic strategies may be designed to help prevent and treat HIV-1 infection.

ACKNOWLEDGEMENTS

My life as a graduate student started ominously on September 11th, 2001. Fortunately, things turned around after that fateful morning as I had the great pleasure of pursuing my studies under the supervision of Dr. Karen Copeland. Not only was she an excellent supervisor (no one can ask for one better) as she guided me with a steady hand the last 5+ years in my academic research, but was also a friend whom I felt as comfortable talking about current events and things going on in my life as I was about science. She would always make sure that I was comfortable with what I was doing in the lab and would constantly remind me that she was always there to help me if I needed it. Even when times were tough, she still kept up the optimism. I only hope that I lived up to the expectations that she placed upon me.

I would also like to thank the numerous technicians and students that passed through the Copeland lab (too many to name) for not only helping me with my experiments, but also in keeping me entertained. My gratitude is extended to all the students at the OHRI, the University of Ottawa and especially the Angel lab who put up with me and my pranks (especially Charlene Young who took the brunt of all these with a good smile). Special thanks go out to Bilge Diker for always being supportive and always volunteering to help me out with anything (and for being the only one who got me back with a good prank). Also thanks go out to Greg (nee Beaudoin) Harris for being my fellow graduate student in the lab and taking the time to look over my manuscripts/abstracts/applications.

Of course I couldn't be here today without the guidance of my thesis advisory committee, Dr. Kathryn Wright and Dr. Steffany Bennett. In addition, I would like to acknowledge the Canadian Institutes of Health Research and the Ontario Graduate Scholarship programs for providing me with funding to pursue my studies.

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I would like to dedicate this thesis to Dina Shafey, my partner in not-so-much crime for always being supportive of me. You put up with my complaints and frustrations and were able to put up with me and my jokes, no matter how bad you thought they were. You made waking up in the morning and coming to the lab a whole lot easier and I look forward to spending the rest of our lives together.

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LIST OF ABBREVIATIONS

AIDS: acquired immune deficiency syndrome
APC: antigen presenting cell
APOBEC: apolipoprotein B mRNA-editing catalytic
ART: antiretroviral therapy
ARV: aids-associated retrovirus
ATCC: american type culture collection
AZT: Azidothymidine
Bcl: B-cell lymphoma
BSA: bovine serum albumin
CAF: CD8+ antiviral factor
CCR: C-C chemokine receptor
CD: cluster differentiation
CMV: cytomegalovirus
CNAR: CD8+ T-cell non-cytotoxic antiviral response
CPT: camptothecin
CTL: cytotoxic T-lymphocyte
CXCR: C-X-C chemokine receptor
DC: dendritic cell
$DiOC_6(3)$: 3,3'-dihexyloxacarbocyanine iodide
DNA: deoxyribonucleic acid
DP: double positive
EBV: epstein-barr virus
EDTA: ethylenediamine tetra-acetic acid
ELISA: enzyme-linked immunosorbent assay
Env: envelope
FasL: fas ligand
FCS: fetal calf serum
Gag: group-specific antigen
Gb3: galactosylceramide
Gp120: glycoprotein 120
HAART: highly active ART
HCV: hepatitis C virus
HHV: human herpesvirus
HIV: human immunodeficiency virus
HLA: human leukocyte antigen
HSPG: heparan sulfate proteoglycan
HSV: herpes simplex virus
HTLV: human T-cell leukemia virus
IAP: inhibitor of apoptosis protein
IFN: interferon
IL: interleukin
JNK: jun NH_2-terminal kinase
LAV: lymphadenopathy-associated virus
LTNP: long-term nonprogressors
LTR: long terminal repeat

MAC: mycobacterium avium complex
MAPK: mitogen activated protein kinase
MFI: mean fluorescence intensity
MHC: major histocompatibility complex
MIP: macrophage inflammatory protein
MLR: mixed lymphocyte reaction
MMLV: moloney murine leukemia virus
mRNA: messenger RNA
NIH: national institutes of health
NK: natural killer
NSI: non-syncytia inducing
PBS: phosphate buffered saline
PBMC: peripheral blood mononuclear cells
PCR: polymerase chain reaction
PHA: Phytohemagglutinin
PI: propidium iodide
PKC: protein kinase C
PMA: phorbol myristate acetate
Pol: polymerase
RANTES: regulated upon activation, normal T-cell expressed and secreted
RNA: ribonucleic acid
RT-PCR: reverse transcriptase PCR
SCID: severe combined immunodeficiency
siRNA: short-interfering RNA
SI: syncytia inducing
SIV: simian immunodeficiency virus
STAT: signal transducer and activator of transcription
Tat: transactivating protein
TcR: T-cell receptor
TGF: transforming growth factor
Th: T-helper
TNF: tumor necrosis factor
TT: tetanus toxoid
UV: ultraviolet
Vif: viral infectivity factor
Vpr: viral protein R
VV: vaccinia virus

1. INTRODUCTION

Over the past 25 years, great strides have been made in both basic and clinical research and the understanding of the Human Immunodeficiency Virus (HIV). The HIV/ Acquired Immune Deficiency Syndrome (AIDS) pandemic is of great concern to the global population and much effort and research is still required to understand and treat the viral disease.

1.1. HUMAN IMMUNODEFICIENCY VIRUS

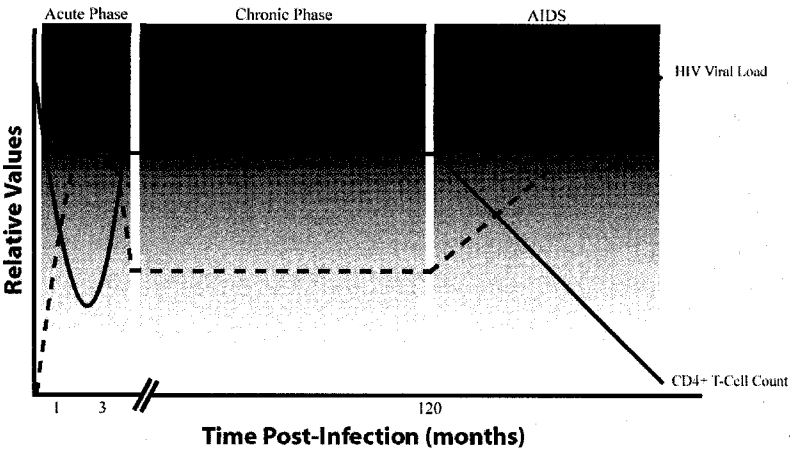
Currently, there are over 40 million people worldwide living with HIV/AIDS. Sub-Saharan Africa remains by far the worst affected region with an estimated 25 million people currently living with HIV. South-East Asia is the second worst affected region in the world. Numerous governments, organizations and researchers are currently trying to assist these afflicted regions and to ameliorate the lives of HIV-infected individuals by providing greater access to antiretroviral drugs and other medications.

HIV was first isolated and characterized in 1983 by Luc Montagnier's group of researchers [1, 2]. HIV is the etiologic agent responsible for AIDS, which is normally observed 8-10 years after the initial infection, during the later stages of viral immunopathogenesis. HIV infection is characterized with a primary burst of viral replication that is initially contained by a strong cellular and humoral response within weeks (Figure 1). However, the virus eventually escapes immune containment and AIDS onset follows.

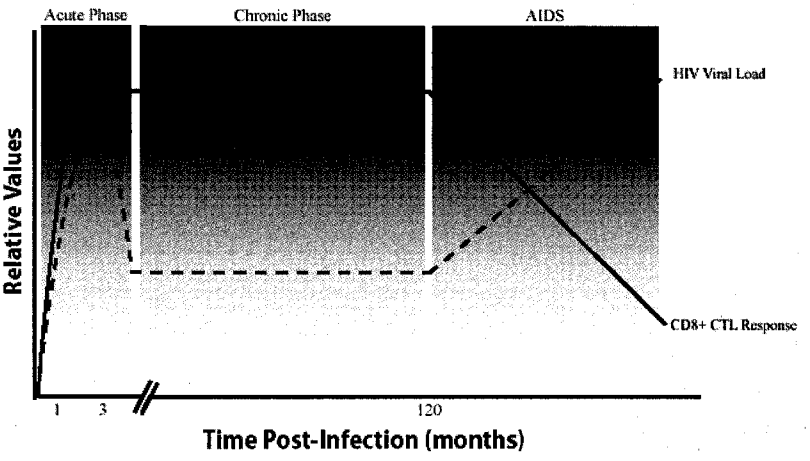
HIV is classified as a member of the *Retroviridae* family of viruses (genus *Lentivirus*). In the past, HIV has also been known as the Human T-cell Leukemia Virus (HTLV)-III, Lymphadenopathy-Associated Virus (LAV) and AIDS-associated Retrovirus

Figure 1. Relationship between HIV-1 viral load and the CD4 T-cell counts/CD8+ CTL response. ***A,*** Inverse relationship between the number of circulating CD4+ T-cells in an HIV-1 seropositive individual and the HIV-1 plasma viral load. ***B,*** During the acute phase of viral infection, there is a concurrent rise in the CTL response with that of the viral load. The sharp rise in this response during the early stages of infection is due to the initial control of viral replication by the CTLs. Both the CD8+ CTL response and the viral load maintain a set point during the chronic phase of infection that lasts approximately 8-10 years. However, as HIV-1 infection leads to AIDS progression, the virus eventually escapes immune containment and an inverse relationship between the CD8+ CTL response and HIV-1 viral load is observed. Adapted from: *Gulzar, N. and K.F. Copeland, CD8+ T-cells: function and response to HIV infection. Curr HIV Res, 2004. **2**(1): p. 23-37.*

A


Acute Phase
Chronic Phase
AIDS
HIV Viral Load
Relative Values
CD4+ T-Cell Count
1
3
120
Time Post-Infection (months)

B


Acute Phase
Chronic Phase
AIDS
HIV Viral Load
Relative Values
CD8+ CTL Response
1
3
120
Time Post-Infection (months)

(ARV). The World Health Organization has estimated that HIV/AIDS has killed approximately 25 million individuals over the last 25 years and according to current estimates and projections, HIV/AIDS spread may result in 90 million people afflicted by the virus in Africa, thus, making it one of the most destructive pandemics in modern history.

The four major routes of transmission are unprotected sexual intercourse, contaminated needles, vertical transmission or mother-to-child through breast milk. Additionally, viral infection may occur through the transfer of blood, semen, vaginal fluid or pre-ejaculate.

Two species of HIV have been found to infect humans: HIV-1 and HIV-2. HIV-1 and -2, despite sharing a similar genomic structure, have up to 25% divergence in their structural and functional genes [3]. HIV-2 was discovered subsequent to the discovery of HIV-1 and prevalence of HIV-2 is essentially confined to West Africa [4, 5]. The mortality rate associated with HIV-2 is estimated to be a third lower than that of HIV-1 [6] and HIV-2 more closely resembles Simian Immunodeficiency Virus (SIV) of sooty mangabeys and macaques [7]. On the other hand, HIV-1 is thought to have originated after jumping from wild chimpanzees to humans during the last century [8]. Since the majority of the studies presented in this thesis concern HIV-1 infection and its effects, only this viral strain will be discussed from now on.

The three earliest recorded instances of HIV-1 infection were as follows: *a.* sample taken in 1959 from an adult male living in the Democratic Republic of Congo; *b.* tissue sample from a 15-year old African-American teenager who died in St. Louis, USA in 1969; and *c.* from a Norwegian sailor who died in 1976 [9].

1.1.1. HIV-1: *Structure and Life Cycle*

HIV-1 is a spherical, enveloped virus approximately 120 nm in diameter. The virus is comprised of two copies of its single-stranded RNA genome that encodes the virus' nine genes. Of these nine genes, the *gag*, *pol* and *env* genes are essential in viral infection and replication as they contain the information needed to make the structural proteins required for new virus particles production [10-13].

Upon entry into the target cell, the viral RNA genome is transcribed to a double-stranded DNA intermediate by the virally encoded reverse transcriptase and then integrated into the host cell genome by the HIV-1 encoded integrase. Once inside the cell, two pathways are possible: the infection may become *a.* latent; or *b.* productive, where a large number of virus particles are synthesized and viral infection spreads [14].

1.1.2. HIV-1: *Clinical Pathology*

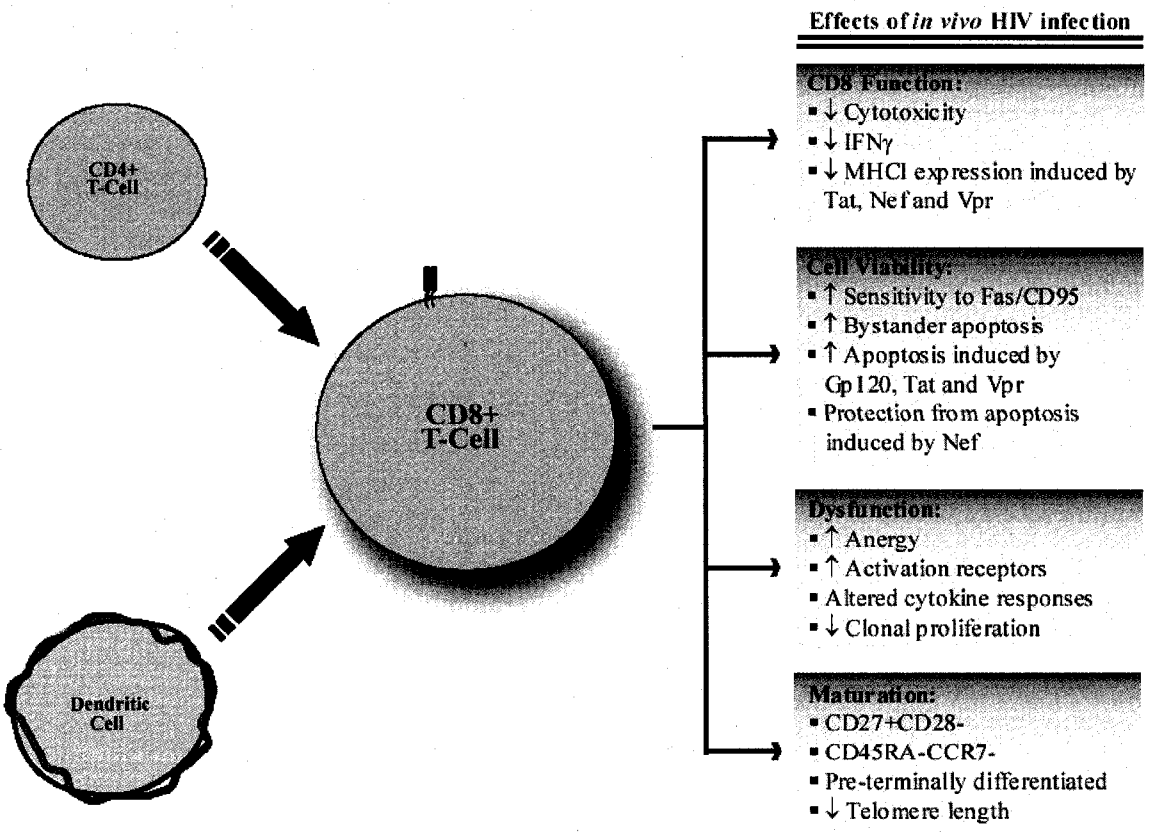
During the initial stages of HIV-1 infection (2 - 4 weeks post-exposure), approximately 30% of infected individuals develop an influenza or mononucleosis-like illness of which some common symptoms include: fever, lymphadenopathy, pharyngitis, rash, malaise, headaches and nausea. These symptoms have an average duration of approximately one month [15]. Subsequent to the appearance of these symptoms, a state of clinical latency begins due to the strong initial immune response. Clinical latency, also termed the asymptomatic phase, can last up to 10 years. However, near the end stages of disease, immune control is lost and AIDS develops. Common illnesses observed during this stage of clinical disease due to the failure of host immune control include: oral candidiasis, tuberculosis, herpesvirus reactivation, B-cell lymphomas and Kaposi's sarcoma.

Pneumocystosis and cryptococcosis, and in the final stages of AIDS, reactivation or infection with Cytomegalovirus (CMV) and Mycobacterium Avium Complex (MAC) are more prominently observed.

1.1.3. HIV-1: *Pathogenesis*

At a cellular level, infection with HIV-1 sets into motion a progressive decline in the number of circulating CD4+ T-cells in an infected individual and also results in the development of the immunodeficiency that is characteristic of AIDS. In fact, the CD4 cell-surface molecule was the first receptor identified for HIV-1 [16] and the presence of this molecule, along with co-receptors that are involved in viral attachment and entry, determines susceptibility to viral binding and infection *in vivo* for CD4+ T-cells, macrophages and Dendritic Cells (DC). Furthermore, during the course of infection, there is a polarization of the cell-mediated and humoral responses. This polarization has been linked to changes in cytokine secretion [17]. T-helper (Th)-1 cells are known to secrete Interleukin (IL)-2 and Interferon (IFN)-γ which favor cell-mediated responses. Th-2 cells are characterized by IL-4, IL-5 and IL-10 secretion and favor humoral immune responses [18]. HIV-1 infection has been characterized by a shift from a Th-1 to a Th-2 cytokine pattern [19, 20]. With the imbalance of cytokine production, CD4+ T-cell destruction and the appearance of autoimmune diseases, viral pathogenesis results in the dysfunction of the host immune system and AIDS onset [21-23] (Figure 2). AIDS is associated with opportunistic infections [24] that manifest themselves due to the impairment of the infected individual's immune system by the virus [25].

Figure 2. The effects of *in vivo* HIV-1 infection on CD8+ T-cell responses. CD4+ T-cells and DCs provide the appropriate help and co-stimulatory signals to CD8+ T-cells for effective maturation and generation of lytic activity. During the course of infection, the abilities of CD4+ T-cells and DCs are hindered, thereby impairing the proper maturation of the CD8+ T-cells. In addition, CD8+ T-cell responses are altered in HIV-1 infection resulting in detrimental effects on the function, cell viability and maturation of these cells. Adapted from: *Gulzar, N. and K.F. Copeland, CD8+ T-cells: function and response to HIV infection. Curr HIV Res, 2004. 2(1): p. 23-37.*


CD4+
T-Cell
CD8+
T-Cell
Dendritic
Cell
Effects of *in vivo* HIV infection
CD8 Function:
▪ ↓ Cytotoxicity
▪ ↓ IFNγ
▪ ↓ MHCI expression induced by Tat, Nef and Vpr
Cell Viability:
▪ ↑ Sensitivity to Fas/CD95
▪ ↑ Bystander apoptosis
▪ ↑ Apoptosis induced by Gp120, Tat and Vpr
▪ Protection from apoptosis induced by Nef
Dysfunction:
▪ ↑ Anergy
▪ ↑ Activation receptors
▪ Altered cytokine responses
▪ ↓ Clonal proliferation
Maturation:
▪ CD27+CD28-
▪ CD45RA-CCR7-
▪ Pre-terminally differentiated
▪ ↓ Telomere length

It is currently thought that the use of antiviral drugs and vaccines would be the most effective route in the control and elimination of HIV-1. However, in order to design effective drugs and vaccines, a greater understanding of the effects of viral infection on the immune system and its components is required. While it has been clearly established that the major cellular target of HIV-1 is the CD4+ T-cell population, there has been much recent evidence demonstrating the susceptibility of CD8+ T-cells to HIV-1 infection both *in vivo* [26-36] and *in vitro* [26-31]. CD8+ T-cells are an important component of the cellular immune response and these cells provide a major immunological defence against HIV-1 infection. In healthy individuals, CD8+ Cytotoxic T-lymphocytes (CTL) recognize and lyse cells infected with foreign pathogens through a Major Histocompatibility Class (MHC)-I foreign antigen-recognition system [32]. In HIV-1 infected individuals, the appearance of HIV-1 specific CD8+ CTLs in the peripheral blood corresponds to a decrease in plasma viraemia within the first few weeks of HIV-1 infection [33]. Some of these studies and the complex interplay between antiviral CD8+ T-cell functions and strategies for the subversion of the host immune response by HIV-1 will be discussed in the next few sections.

1.2. CD8+ T-CELL FUNCTION

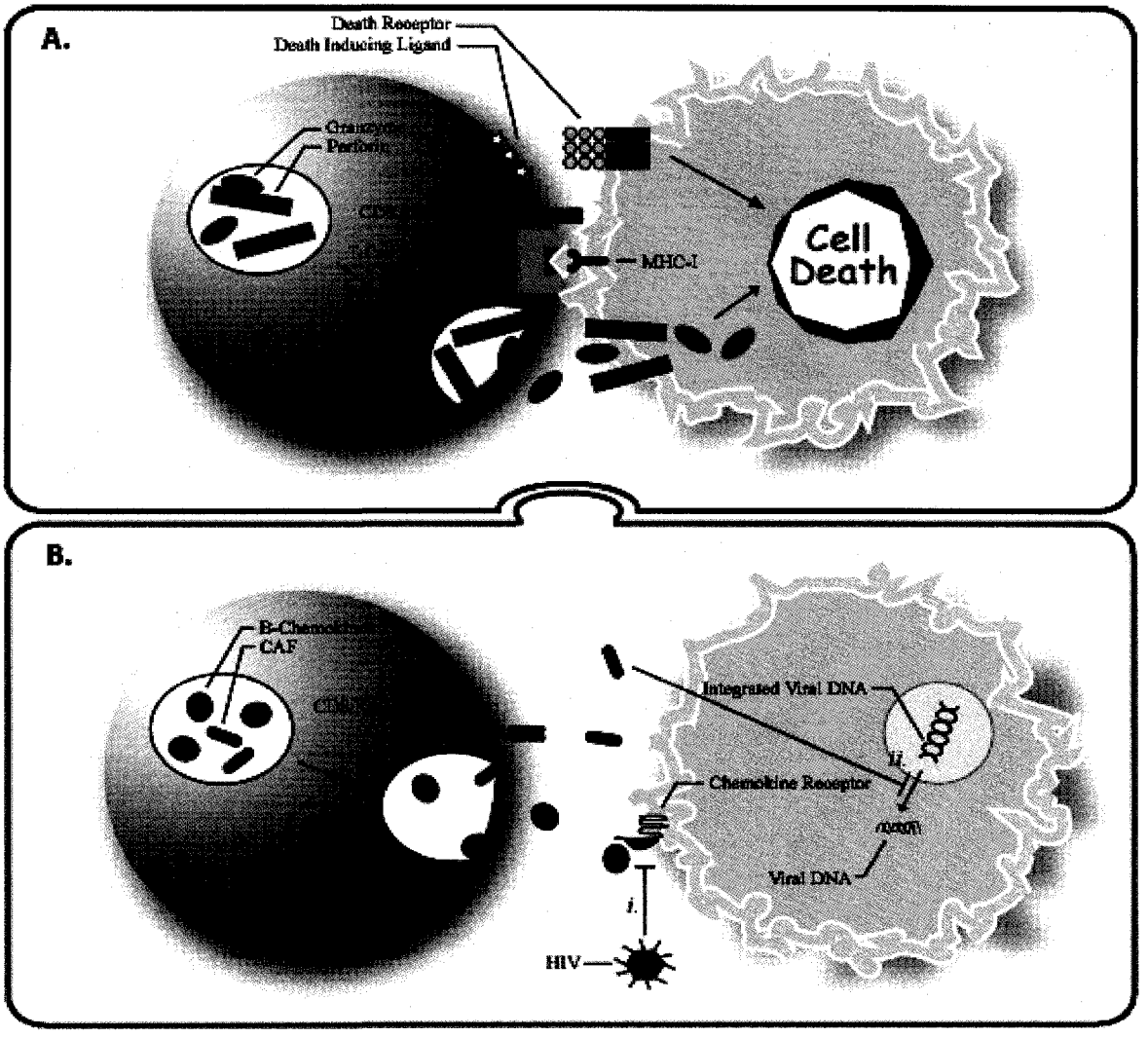
HIV-1 is able to infect numerous types of immune cells, such as T-cells, B-cells, cells of the monocyte/macrophage lineage and DCs [34]. The main cellular targets of HIV-1 are CD4+ T-cells, a subset of T-lymphocytes that play a key role in the orchestration of the host immune response to foreign pathogens. During the course of HIV-1 infection, the number of CD4+ T-cells inversely correlates with plasma viral load [35, 36] (Figure 1).

CD8+ T-cells, another subset of T-lymphocytes, play a key role in the cell-mediated response in the immune system as well. These immune cells, also referred to as CTLs, protect the host from foreign organisms through both lytic and non-lytic means (Figure 3). The CTL response also eliminates transformed tumor cells and regulates the immune response [37].

1.2.1. The Lytic Pathways

The predominant mechanism by which CTLs lyse infected cells is through secretion of perforin and granzymes [38]. CTL recognition of the target (infected) cell occurs at the immunological synapse formed between the T-cell Receptor (TcR), CD8 receptor and associated signaling molecules. The initiation of this pathway results in the release of cytolytic granules into the extracellular space between the CTL and the target cell. CTL secretion of the granule contents, consisting of perforin (a pore-forming protein) and granzymes (serine proteases), into the close proximity of the target cell results in the swift death of the target cell [39]. Studies have shown that exocytosis of these two secretory proteins is MHC-I restricted, calcium-dependent and antigen-specific [32]. A second lytic pathway, albeit less predominantly used, is the calcium-independent Fas-mediated pathway. This pathway can also be used by CD4+ T-cells [40]. In this lytic route, expression of Fas Ligand (FasL) by the CTL occurs after TcR engagement with the target cell [41]. FasL engagement leads to target cell death through the initiation of the apoptosis pathway by interaction with the Fas/CD95 receptor expressed on the surface of the susceptible target cell. This pathway is principally involved in the regulation of immune responses [42]. Of interest is the recent study that hinted at the contribution of Fas-mediated cell death to the granule

Figure 3. CD8+ T-cell activities in response to HIV-1 infection. ***A,*** The lytic pathways. An infected cell displays an 8-11 amino acid peptide loaded within its MHC-I molecules. CD8+ CTL recognition of the infected cells occurs through interactions between the CTL CD8 cell-surface molecule, the T-cell receptor and the MHC-I molecule of the infected cell. After CTL recognition, the infected cell can be cleared by two mechanisms. The secretion of perforin and granzymes from cytoplasmic granules results in the delivery of the granzymes into the infected cell, leading to cellular lysis. The other mechanism occurs when a death-inducing ligand expressed by the CTL binds to a death receptor on the surface of the infected cell. The predominant ligand expressed by CD8+ CTLs is FasL, which binds to the Fas/CD95 receptor displayed on the infected cell surface. ***B,*** The non-lytic pathways. Upon viral infection, CD8+ T-cells secrete β-chemokines (MIP-1α, MIP-1β, RANTES) and CAF. *i.* The binding of β-chemokines to the HIV-1 co-receptors (CXCR4, CCR5) abrogates viral binding and entry. *ii.* CAF activity results in the suppression of HIV-1 LTR-mediated transcription. The nature and how CAF suppresses HIV-1 transcription is still an enigma. Adapted from: *Gulzar, N. and K.F. Copeland, CD8+ T-cells: function and response to HIV infection. Curr HIV Res, 2004. 2(1): p. 23-37.*


A.
Death Receptor
Death Inducing Ligand
Granzyme
Perforin
CD8
TCR
MHC-I
Cell Death
B.
B-Chemokine
CAF
CD4
Integrated Viral DNA
ii.
Chemokine Receptor
Viral DNA
i.
HIV

exocytosis-mediated pathway attributed to CD8+ T-cells. Kojima *et al.* revealed by immunoelectron microscopy that FasL was localized to the outer membrane of the cytoplasmic granules during degranulation [43]. These findings indicate that these two lytic pathways may be more intertwined than previously thought.

1.2.2. The Non-Lytic Pathways

In addition to their CTL abilities, the CD8+ T-cell Non-cytotoxic Antiviral Response (CNAR) can produce and secrete a number of soluble HIV-1 inhibitory factors that can block intracellular viral replication in CD4+ T-cells and other cellular subsets [44]. This pathway does not directly eliminate virally infected cells like the perforin/granzyme and FasL-mediated routes. It however does play a large role in the control of viral replication, especially in the context of HIV-1 infection [45-47]. The first group of suppressive factors secreted by CD8+ T-cells that inhibited HIV-1 replication was identified by Cocchi *et al.* [48]. These factors were identified as the β-chemokines: Macrophage Inflammatory Protein (MIP)-1α, MIP-1β and Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES). These factors inhibit HIV-1 replication by binding to their cognate chemokine receptors, thereby blocking access to co-receptors that are critical in viral binding and entry into cells [49].

Blocking viral entry is not the sole mechanism by which CD8+ T-cells abrogate viral replication. Recent studies have shown that soluble factors produced by CD8+ T-cells are potent suppressors of HIV-1 Long Terminal Repeat (LTR)-mediated gene expression in human CD4+ T-cells [50-54]. In these studies, the use of antibodies to the aforementioned β-chemokines (MIP-1α, MIP-1β and RANTES) did not abrogate suppression of LTR-

mediated gene expression, thereby indicating the presence of a novel soluble factor [50, 51]. This soluble factor was described by Walker *et al.* as the CD8+ Antiviral Factor (CAF) [44]. Much controversy currently exists as to the nature of CAF (summarized in [47]) [55, 56]. Recent studies have elucidated the role that IL-16, macrophage-derived chemokine, IFNs, antithrombin III and α-defensins 1-3 may play a role in the activity of CAF [57-61]. Conflicting reports indicate that CAF is a protein of approximately 30 kDa that is pH and heat stable [47], while others suggest that the antiviral action of CAF is due to the activity of one or more cytokines or chemokines acting in concert [45]. What is known about the function of CAF in HIV-1 infection, apart from its ability to act on LTR-mediated transcription, is that it does not block HIV-1 entry into susceptible cells [50], does not hinder provirus integration [62] and nor does it inhibit HIV-1 reverse transcription [56, 63]. In addition, the activity of CAF is not MHC restricted unlike that of the CTL response [55, 64]. Chang *et al.* proposed that the action of CAF occurs through its binding to one or more of its receptors, thus activating intracellular signaling pathways and Signal Transducer and Activator of Transcription (STAT)-1 phosphorylation. The resultant phosphorylation thereby suppresses HIV-1 transcription through the LTR [45]. Due to the nature of CAF being neither HIV-1 antigen specific [63] or solely produced by CD8+ T-cells [45], it has been hypothesized that the CAF activity may be an innate, rather than an adaptive immune response.

In addition, almost all CD8+ T-cells secrete IFN-γ and Tumor Necrosis Factor (TNF)-α, which induce a potent antiviral state in cells during the course of infection [65-68]. Memory CD8+ T-cells provide a ready source of IFN-γ [69], which is augmented by the addition of IL-12 [70]. Recent results demonstrated an increase in IFN-γ production,

following the *in vitro* infection of the CD8+ T-cell population [71]. Conversely, in a patient cohort, the number of cells producing IFN-γ decreased in late stage HIV-1 infected patients, though it was temporarily enhanced during Antiretroviral Therapy (ART) [72]. Both the lytic and non-lytic activities of CD8+ T-cells have been reported to correlate strongly with delayed disease progression in HIV-1-infected individuals [73-75]. While there is an early increase in the number and function of HIV-1 specific CTLs, it has been well established that in chronic infection, the decline in the CD8+ T-cell response accompanies progression to symptomatic disease [33, 76] (Figure 1). The significance and importance of CD8+ T-cells in viraemic control was elucidated in studies with Long-Term Nonprogressors (LTNP) and SIV [77-80]. CD8+ T-cells from LTNPs of HIV-1 infection maintain higher numbers, greater functionality and more persistent HIV-1 specific CTL responses than those of progressors [76, 81].

1.3. SUSCEPTIBILITY OF CD8+ T-CELLS TO HIV-1 INFECTION

HIV-1 is able to infect numerous cell types of the immune system as well as non-immune cells. Early in the course of infection, HIV-1 infects DCs that line the mucosal membranes of the individual. From here, the virus binds to and infects cells of the monocyte/macrophage lineage. The virus is said to be M-tropic at this stage of disease. With progression to disease, the virus becomes T-tropic and the monocyte/macrophage cells serve as viral reservoirs that circulate throughout the lymphatic system, where they serve to disseminate the virus to T-lymphocytes through chronic, active non-lytic infection [82]. More recently, mucosal depletion of T-cells in the gut is recognized as an early, irreversible change before the onset of immune disease [83-85]

1.3.1. HIV-1 Infection of CD4+ T-Cells

CD4+ T-cells are the predominant T-lymphocytes affected by viral infection. CD4+ T-cells play a crucial role in the immune protection of an individual by orchestrating the immune response. During the latter course of infection, the number of circulating CD4+ T-cells in an individual dramatically decreases and the viral load inversely correlates with the CD4+ T-cell count [86, 87] (Figure 1). Much evidence currently supports the notion that the CD4+ T-cells are depleted by various apoptotic mechanisms during viral pathogenesis [22, 35, 88-94]. HIV-1 has a direct lytic effect on CD4+ T-cells and has been shown to decrease CD4+ T-cell numbers through bystander-mediated apoptosis (for a review, see [89]). HIV-1 enters CD4+ T-cells through binding of the viral glycoprotein gp120 to the CD4 receptor found on the surface of these cells [95]. Soon after the discovery of CD4 as a primary receptor for HIV-1, the role of co-receptors in efficient viral binding, fusion and entry was elucidated [96]. To date, there have been over 10 identified co-receptors for HIV-1 [49]. These co-receptors were found to be of the G protein-coupled receptor superfamily, of which CXCR4 and CCR5 are the most predominantly used by the virus [95]. M-tropic strains are known to use CCR5, while T-tropic strains use CXCR4 [97]. The role of these co-receptors in HIV-1 entry is to facilitate a conformational change in the envelope protein when gp120 interacts with the co-receptor, leading to the extension, insertion and fusion of gp41 into the target cell membrane [97]. Work done by Steffens *et al.* in 2003 exhibited the co-localization of CD4 and CCR5, thereby postulating that this co-localization serves to amplify the interactions between the co-receptor, CD4 and gp120 of the virus [98]. The presence of lipid rafts has also been posited to aid in viral adsorption and penetration with target cells [99-101]. Lipid rafts are organized areas of the cell membrane that are rich in sphingolipids

and cholesterol and serve to enhance viral binding by facilitating co-localization of target cell receptors and viral envelope glycoproteins.

1.3.2. HIV-1 Infection of CD8+ T-Cells

The cause of CD8+ T-cell dysfunction and depletion during HIV-1 infection *in vivo* still remains unclear. As will be discussed further on, this phenomenon is generally attributed to the lack of CD4+ T-cell help or to toxic effects induced by the secretion of soluble viral proteins in the cellular milieu. It has been demonstrated that the presence of CD4+ T-cells and Antigen Presenting Cells (APC) are critical in CD8+ T-cell maturation and function [102]. In addition, antigenic stimulation of CD8+ T-cells may result in a state of anergy and possibly cell death [103]. HIV-1 production and replication in infected individuals has been shown to induce an accompaniment of chronic unresponsiveness in the immune system.

The potential immunosuppressive effects of HIV-1 infection aside, work done by Potter *et al.* and others have implicated CD8+ T-cells as a reservoir of circulating HIV-1 infected cells [28, 104]. Direct infection of CD8+ T-cells may also explain the abnormalities and dysfunction seen in this subset during AIDS pathogenesis. HIV-1 infected CD8+ T-cells have been found in the peripheral blood [105] and lungs [106] of HIV-1 seropositive individuals. However, with the use of quantitative Polymerase Chain Reaction (PCR), Douek *et al.* showed that only a minority of CD8+ T-cells are infected *in vivo* [107]. The same study also demonstrated that the CD8+ T-cells that were HIV-1 specific did not serve as cellular targets of the virus. On the other hand, evidence also exists that CD8+ T-cells serve as targets for viral infection by HIV-1. Unintegrated HIV-1 DNA can be detected in

CD8+ T-cells [30] and the infected CD8+ T-cell population serves as a reservoir for HIV-1 proviral sequences in the peripheral blood late in viral pathogenesis [105]. Over the past couple of years, numerous theories and studies have been proposed to explain HIV-1 entry into CD8+ T-cells. One such study involved the selection of CD8-tropic variants of HIV-1 that were syncytium inducing and cytopathic for CD8+ T-cells [26, 29]. A few of these viral isolates from HIV-1 infected individuals were shown to be able to use the CD8 cell-surface molecule as a receptor [29]. Studies have shown that CD8+CD4- lymphocyte lines were able to harbor productive HIV-1 infection [28, 108]. In addition, Akimoto *et al.* showed that the envelope glycoprotein, gp105, of HIV-2 was able to bind CD8 molecules on the surface of T-cells [109]. They could not however demonstrate the binding of gp120 of HIV-1 to the CD8 receptor and to date, no studies have shown the direct interaction of gp120 with the CD8 receptor. Yet, this does not discount the evidence that HIV-1 may enter cells which do not express CD4. Binding and entry may occur through interactions with G protein-coupled receptors [110-112] or other receptors, such as galactosylceramide (Gb3) [113-115] found on the surface of target cells. The chemokine receptors CXCR4 and CCR5 are both expressed on the surface of isolated primary CD8+ T-cells [71]. Fukada *et al.* also demonstrated that CCR5 is expressed on memory and effector populations of CD8+ T-cells, but not on naïve CD8+ T-cells [116]. Cell-to-cell transfer of viral particles has also been postulated as mode of HIV-1 spread and entry into CD8+ T-cells [31, 117]. Lastly, a number of investigators have shown that the expression of the CD4 molecule on CD8+ T-cells increased subsequent to activation [118-124]. These aptly termed $CD4^{dim}CD8^{bright}$ T-cells were found to be susceptible to HIV-1 infection. These results suggest that HIV-1 may infect CD8+ T-cells

through receptor-mediated pathways, thereby disrupting the functions of the memory and effector subsets of CD8+ T-cells that are vital in immune clearance.

1.4. THE EFFECT OF HIV-1 INFECTION ON CD8+ T-CELL FUNCTION

CD8+ T-cells form a critical component of the cellular immune response during viral infection. Many viruses have adopted strategies to evade and hinder the CTL response [125-145]. The role of CD8+ T-cells in the context of HIV-1 infection is to suppress viral replication and to aid in the clearance of virally infected cells. They do so by the secretion of soluble factors (CAF, MIP-1α, MIP-1β, RANTES) and by the direct lysis of infected cells (secretion of perforin and granzymes). Conflicting studies demonstrate the relationship between the CTL response and viral load. One such study showed an inverse relationship between the percentage of CTL that are HIV-1 specific and the viral load [146]. However, this relationship has not been observed in other studies [147, 148]. In a study of HIV-1 LTNPs, heightened responses to the Hepatitis C Virus (HCV) were maintained in addition to their HIV-1 specific responses [149-152]. During the asymptomatic phase of HIV-1 pathogenesis, infected individuals exert a strong HIV-1-specific cytolytic response. It has been estimated that the proportion of circulating CD8+ T-cells that are specific for HIV-1 antigens is as high as 18% during established infection [153, 154]. However, as infection progresses to the symptomatic phase, the cytolytic response diminishes and these individuals usually develop AIDS if ART is not properly administered [76].

In order for a virus to survive, it must adopt numerous strategies to evade the host immune response. Some of the strategies utilized by viruses in immune evasion are their ability to hide from immunity by remaining dormant and thus minimizing antigen

expression, sequestration into areas of low immune surveillance, failure of antigen display and antigenic variation [155-157]. Similarly, HIV-1 has developed numerous methods to escape and hinder the activities of CD8+ T-cells (Figure 2).

1.4.1. Antigenic Variation

During the course of synthesis and assembly of viral proteins, epitope peptides are presented in MHC-I molecules at the surface of the infected cell. Here, the CTL can recognize the loaded peptide and lyse the infected cell within five hours [158]. As long as the HIV-1 infected cells are eliminated rapidly from the immune system, the chances for antigenic variation of viral epitopes is minimized. However, with the high rate of mutation inherent in the function of the HIV-1 reverse transcriptase, as well as the high level of replication due to the impairment of the CTL function by infection, selection of escape mutants is easily favoured [36]. It has also been reported that numerous HIV-1 infected individuals who generate a strong CTL response early in infection can generate antigenic variants that are not recognized by CTLs during the latter stages of disease, thereby circumventing the CTL response [159-161]. Price *et al.* provided evidence of the selective pressure exerted by CTL on the viral population in acute HIV-1 infection. This study demonstrated the positive selection of proviral sequences encoding variants within a Nef CTL epitope that were able to evade CTL recognition [162]. McMichael *et al.* have stated that the selection of mutants by CTLs is one of the hallmarks of HIV-1 infection [46]. Thus, under the pressure of the host immune response, HIV-1 selects for epitopes that contain mutated amino acids that are not recognized by CTL, provided such mutations are not detrimental for virus function [163, 164].

1.4.2. Evasion of CD8+ T-Cell Recognition

HIV-1 encodes various proteins that aid the virus in hiding from the immune response of the host. One such protein encoded by the virus is the Nef protein. Nef is a 27-34 kDa protein that has multiple functions including: enhancement of viral infectivity [165, 166], down-modulation of CD4 expression on the surface of infected cells [167-171], as well as various effects on cellular signal transduction pathways [172]. Another function of Nef is the down-regulation of MHC-I molecules from the surface membrane of various immortalized cell lines and T-lymphocytes [173-176]. The specificity and activity of CD8+ T-cells occurs through interactions between its TcR and with both MHC-I molecules and the loaded 8-11 amino acid peptide of the target cell [68]. Proteins encoded by HIV-1 have different effects on the MHC class I subtypes. It was discovered that Nef modulates the expression of Human Leukocyte Antigen (HLA)-A and HLA-B molecules on the surface of infected cells, but does not down-regulate HLA-C or HLA-E, Natural Killer (NK) cell inhibitory receptors [177]. By down-regulating HLA-A and HLA-B surface molecules but not HLA-C and HLA-E molecules, HIV-1 is able to evade both the CTL response exerted by CD8+ T-cells (adaptive immunity) and the response from NK cells (innate immunity) [68, 164]. Other identified HIV-1 accessory genes that have also been implicated in playing a role in MHC-I down-regulation include the *vpu* and *tat* genes [178, 179]. In contrast to its ability to evade the lytic activity of CD8+ T-cells, HIV-1 does not have an effect on the non-lytic antiviral activity of these cells, as found by Tomiyama *et al.* [175]. Through the use of a virus strain encoding a Nef protein that failed to down-regulate MHC-I molecules but retained the other functions of Nef, the researchers discovered the production of the β-chemokines and IFN-γ was not impaired [131].

1.4.3. Cytopathic Effects of HIV-1

A more direct consequence of HIV-1 infection is the cytopathic effect the virus exerts on CD8+ T-cells. During the course of infection, there is an inversion of the CD4+:CD8+ T-cell ratio that occurs due to the loss of CD4+ T-cells [180]. In addition, the absolute numbers of CD8+ T-cells present in the immune system begins to decline during the late stages of infection [181]. Similar to the cell death studies done with CD4+ T-cells, much debate currently exists as to the ways by which CD8+ T-cells are depleted during *in vivo* HIV-1 infection [182-190] (for a review of the apoptotic strategies of HIV-1, see [191]). Recently, Mueller *et al.* found that HIV-1 specific CD8+ T-cells are three-fold more sensitive to Fas/CD95-induced apoptosis when compared to CMV-specific CD8+ T-cells [192]. They also found that HIV-1 infected macrophages can induce apoptosis of these CD8+ T-cells, thereby demonstrating the pro-apoptotic effect HIV-1 has on bystander cells [89, 91, 94, 193-195]. Furthermore, the appearance of macrophages at sites of inflammation and activation has also been shown to induce apoptosis in CD8+ T-cells [196]. In contrast, the work of Derby *et al.* concluded that the antigen-dependent death of CD8+ CTL was Fas-independent, TNF-α receptor 2-mediated, caspase-dependent and resulted in a decrease in Bcl-2, an anti-apoptotic protein [197]. Herbein *et al.* reported that CD8+ T-cells were depleted during *in vitro* infection by the interaction of gp120 and CXCR4 found on the surface of these cells [198]. Turnover of CD8+ T-cells during infection increased due to gp120/CXCR4 interactions was also supported by the work done by Blanco *et al.* [199].

HIV-1 encoded proteins have been shown to have pro-apoptotic properties, and not only kill infected and bystander T-cells, but also create a state of chronic immune activation that is responsible for the anergic effects and clonal deletion of immune cells that occurs

during infection [191] (Table I). The HIV-1 protease was found to be able to induce lysis in both yeast and mammalian cells with drastic alterations in membrane permeability [200]. The HIV-1 encoded Tat protein is essential for replication by activating the viral LTR and thus enhancing transcription. The pro-apoptotic effects of Tat include its ability to up-regulate FasL [201]. This effect was enhanced by the activation of cyclin-dependent kinases [202]. Bartz and Emerman found that Tat was able to induce apoptosis and increase the sensitivity of cells to apoptotic stimuli by the up-regulation of caspase 8 [203], an initiator caspase of the apoptotic pathway. Signaling through the CD4 receptor is thought to play a major role in CD4+ T-cell depletion because of the high affinity between gp120 and CD4 [90, 204]. Work done by LaBonte *et al.* showed, however, that CD4 is not inherently required for the induction of cell death [205]. They found that the HIV-1 envelope glycoprotein was able to induce cell death in CD4-negative cells expressing CCR5. In addition, cross-linking of gp120 on T-cells, followed by signaling through the TcR results in apoptosis of the T-cells [90]. Gp120 has also been shown to down-regulate Bcl-2 [206] and activate caspase 3 [207], the central effector molecule of apoptosis.

The Vpr protein of HIV-1, which functions to arrest the cell cycle in the G2/M stage and facilitates the transport of viral preintegration complexes [208], exhibits a certain level of dichotomy in the course of infection. On the one hand, the protein is able to induce apoptosis associated with mitochondrial depolarization and caspase 9 activation in T-cells and Peripheral Blood Mononuclear Cells (PBMC) [209-211]. On the other hand, Zhu *et al.* showed that Vpr up-regulated Survivin in mammalian cell lines [212]. Survivin belongs to the family of Inhibitor of Apoptosis Proteins (IAP) and functions to inhibit apoptosis. Vpr was found to be able to transactivate the *survivin* promoter and blocking of the Vpr-induced

G2/M arrest led to the aberration of *survivin* promoter transactivation. Recently, Nef has been shown to block death receptor-mediated apoptosis pathways by blocking the function of the apoptosis-signal regulating kinase-1 [213]. Nef has also been shown to block the mitochondrial apoptosis pathway by blocking the activity of the pro-apoptotic protein Bad [214]. Thus by encoding proteins that suppress or delay apoptosis, HIV-1 can prolong production of progeny virus. During later stages of infection, HIV-1 encodes proteins that promote apoptosis, thereby facilitating the spread of progeny virus to neighbouring cells while also evading the host immune response.

Although much of the above studies were performed in immortalized cell lines and CD4+ T-cells, it is likely that the various pro- and anti-apoptotic proteins encoded by HIV-1 would exert similar effects on CD8+ T-cells during the course of productive *in vivo* infection.

1.4.4. Anergizing Effects of HIV-1

The anergic effect observed on cells of the immune system is due to the state of chronic immune activation created by HIV-1 infection. Infection has been shown to hinder responses by T-cells to recall antigens [215] and to increase the turnover rate and activation of CD8+ T-cells [216]. Activation of T-cells in the absence of a co-stimulatory signal results in a defect in IL-2 production and leads to anergy [215, 216]. Recent studies in murine [126, 128] and human [133, 143, 154, 217] viral infections have shown anergic or dysfunctional CD8+ T-cell responses. CD8+ T-cells from HIV-1 seropositive individuals respond poorly to anti-CD3 stimulation, suggesting anergy of these cells [103]. In addition, CD8+ T-cells from infected individuals display increased levels of activation antigens, are unresponsive to

Table I. HIV-1 proteins involved in the control of cell death. Adapted from: *Gulzar, N. and K.F. Copeland, CD8+ T-cells: function and response to HIV infection. Curr HIV Res, 2004. **2**(1): p. 23-37.*

Pro-Apoptotic	
Tat	Up-regulation of FasL
	Up-regulation of TRAIL
	Activation of caspase-8
	Down-regulation of BCL-2
Protease	Activation of caspase-8
	Increase in plasma membrane permeability
	Cleavage of BCL-2
Env	Synctium formation
	Up-regulation of Fas
	Activation of caspase-3
	CXCR4-dependent cell death
	Decrease in BCL-2 expression
Vpr	Cytochrome c release
	Increase in plasma membrane permeability
	Activation of caspase-9
Nef	Activation of caspases
	Up-regulation of the Fas-FasL apoptotic pathway
Anti-Apoptotic	
Env	Down-regulation of CD4 from surface of infected cells
Vpr	Up-regulation of BCL-2
	Down-regulation of BAX
Nef	Down-regulation of CD4 from surface of infected cells
	Down-regulation of MHC-I molecules from the surface of infected cells
	Inhibition of ASK-1 activity
	Inhibition of Bad activity

ASK-1, Apotosis-Signal Regulating Kinase-1; Bax, BCL-2-associated X protein; TRAIL, TNF-related apoptosis-inducing ligand

T-cell signaling and show a decreased level of clonal proliferation [218-220]. The anergic defect is partially restored by the addition of exogenous anti-CD28 and IL-2 [221]. The TcR and CD28 receptors act in concert in the activation of T-lymphocytes and function to stimulate cellular proliferation, suppress apoptosis and maintain antigen responsiveness [222, 223]. The unresponsiveness of CD8+ T-cells to anti-CD3 stimulation can be explained by the observation that the CD3 receptor is down-regulated on CD8+ T-cells during the course of infection [224, 225]. The decrease in CD3 receptor expression is also associated with the down-modulation of CD28 receptor expression on the extracellular surface [226]. The proper expression of CD3 and CD28 on the surface of T-lymphocytes is very important in T-cell responses and this down-regulation during infection may help to explain the impairment of CD8+ T-cell function and their anergic state.

HIV-1 antigen burden has a profound effect on cytokine expression which consequently affects CD8+ T-cell function. Work done by Gabe *et al.* showed that HIV-1 enhanced the secretion of the anti-inflammatory cytokine Transforming Growth Factor (TGF)-β by CD8+ T-cells, thus inhibiting the IFN-γ response to HIV-1 and unrelated antigens [227]. This demonstrates how an HIV-1 specific response can suppress both HIV-1-specific and unrelated immune responses of CD8+ T-cells. Glutathione, a thiol essential for the synthesis of DNA molecules, functions to augment IL-2 production and helps initiate antigen specific immune responses. In HIV-1 infected individuals, CD8+ T-cells with a high glutathione content are depleted, further exacerbating the CD8+ T-cell functional abnormalities [228]. The importance of IL-15 in the expansion and maintenance of the CD8+ CTL response during primary HIV-1 infection has also been studied [229-232].

1.4.5. CD8+ T-Cell Intracellular Signaling in Response to Infection

In order for CD8+ T-cells to effectively respond to growth factors and mitogens, there must be proper engagement of extracellular receptors. These receptors are coupled to protein tyrosine kinases or G proteins, as in the case of the chemokine receptors. Activation of the Mitogen Activated Protein Kinase (MAPK) results in the signaling cascade that includes Raf-1, MEK, ERK, RSK and GSK3 activation. These proteins aid in the proper activation of transcription factors that regulate such cellular processes as cell cycle, stress response, apoptosis, differentiation and proliferation [233]. STATs also play an important role in the activation of transcription factors by binding to specific promoter elements [234]. Protein Kinase C (PKC), calcium release, fibronectin binding integrin and cAMP-dependent protein kinase A all play important roles in cellular regulation as well [235, 236]. The role of the c-Jun NH_2-terminal kinase (JNK) pathway was elucidated in a series of studies that demonstrated that the inhibition of this pathway resulted in the resistance of the Double Positive (DP) precursor thymocytes to cell death [237]. Double positive CD4+CD8+ thymocytes undergo positive (recognition of peptides in the context of self-MHC) and negative (recognition of self-peptides in the context of self-MHC) selection before completing their maturation and migration from the thymus [238, 239]. Thus, the proper activation of the JNK pathway serves to ensure that the DP thymocytes undergo proper maturation to single positive CD4+ and CD8+ T-cells.

Intracellular signaling pathways differ between CD4+ and CD8+ T-cells as both cells have different functions during an immune response to an infection. CD8+ T-cells employ their cytolytic activity against pathogens, whereas CD4+ T-cells aid in the production of cytokines that exert important regulatory activity. CD4+ T-cells respond to antigen

associated with MHC-II molecules, while CD8+ T-cells respond to antigen associated with MHC-I molecules [240, 241]. Both CD4 and CD8 enhance MHC-TcR interactions to ensure proper responses to antigen presentation. Early studies by Ravichandran and Burakoff demonstrated that both the CD4 and CD8 molecules were associated with equal amounts of the protein tyrosine kinase p56lck, and that both molecules enhanced IL-2 production when cross-linked with TcR-antibodies [242]. Akimoto *et al.* demonstrated the association of p56lck with the α-chain of the CD8 molecule and found that the binding of the envelope glycoprotein of HIV-2, gp105, induced the phosphorylation of the kinase [109]. Activated p56lck interacts with other signaling molecules via its SH2 and SH3 domains, aiding in their recruitment to the TcR and hence T-cell activation. The authors found, however, that kinase activity was greater than four-fold in CD4 than in CD8 molecules when cross-linked with the TcR-antibodies. They concluded that CD8 molecules may be more dependent on PKC-mediated signaling pathways, whereas tyrosine kinase activation is more predominantly used by CD4 molecules. Recent work has shown that the heterodimeric CD8αβ was a more effective receptor than the homodimeric CD8αα and that the CD8αβ receptor associates with and activates p56lck predominantly in lipid rafts [243]. Lipid raft domains contain numerous signaling molecules such as Src family kinases, G proteins and molecules involved in Ca^{2+} influx [99]. Cholesterol depletion of these rafts resulted in the inhibition of receptor tyrosine phosphorylation and T-cell signaling [244]. Kersh *et al.* recently found that memory CD8+ T-cells have more extensive lipid rafts and more efficiently induce the phosphorylation of numerous transcription factors such as ERK, JNK and p38 [245]. Lipid rafts in memory CD8+ T-cells thus serve to aid in the transduction of downstream signaling events. Another difference between CD4 and CD8 signaling is that CD8+ T-cells in response to antigen, can

produce IFN-γ independently of IL-12. In contrast, CD4+ T-cells require IL-12 and STAT-4 activation for the production of IFN-γ via the TcR [246]. The activation of the ERK and p38 pathways is also essential for the production of IL-2 and the release of perforin/granzyme B by CD8+ CTLs [224, 247].

The binding of the HIV-1 envelope glycoprotein to the CD4 receptor was found to activate the MAPK/ERK kinase pathway and stimulated the expression of nuclear factors that resulted in the production of inflammatory proteins [248]. The aberrant expression of these proteins may contribute to enhanced HIV-1 replication, a state of chronic anergy and the impairment of the host immune system that characterizes AIDS. Ligation of CD4 by gp120 also inhibits signal transduction, protein tyrosine phosphorylation and calcium mobilization [249]. Similar results in CD8+ T-cells may be found as more studies concerning the interactions between the HIV-1 envelope glycoprotein and receptors on the surface of CD8+ T-cells are undertaken. Gp120 of HIV-1 was also able to induce tyrosine phosphorylation of the protein tyrosine kinase Pyk2 when both CXCR4 and CCR5 were present on the cell surface [250]. In addition, the envelope proteins of both HCV and HIV-1 were able to activate p38 MAPK, the tyrosine phosphatase SHP2 and stimulate the production of IL-8 [251]. Treatment of CD4+ T-cells with the envelope glycoprotein was able to inhibit the activity of JNK and ERK2, but this observation was not found in CD8+ T-cells [252]. Similar to gp120, another protein encoded by HIV-1 that is involved in the disruption of signaling pathways of T-cells is the Nef protein. Nef has been demonstrated to associate with tyrosine kinases (Hck and Lck) [253, 254] and to induce the synthesis of IL-6 [248] and IL-2 production through PI3K induction [255]. Nef is also involved in the reactivation of latently HIV-1 infected cells by signal transduction from Ras to MAPK

cascades [256] and inhibits T-cell migration by altering focal adhesion kinases [257]. In order to evade host CD8+ T-cell responses, Nef possesses the ability to down-regulate MHC-I molecules from the surface of infected cells. The down-regulation of MHC-I molecules occurs through a PACS-1 and PI3K-dependent pathway [258] and Nef induces apoptosis of cells through serine/threonine protein kinases [259]. These results demonstrate the way in which Nef affects viral pathogenesis by skewing T-cell responses. The over-expression of Tat, another HIV-1 regulatory protein, induces TNF-α and IFN-γ production [260]. Vpr is able to induce the arrest of the cell cycle in the G2 stage, thereby facilitating the transcription of HIV-1 [261]. Vpr also affects T-cell proliferation, induces apoptosis of both CD4+ and CD8+ T-cells and interferes with NF-κB mediated transcription, thereby disrupting cytokine synthesis [262]. In summary, HIV-1 is able to disrupt cellular signaling through numerous fashions, leading to the impairment of the host immune response. Thus, the disruption of cellular signaling can result in the improper maturation and function of CD8+ T-cells.

1.4.6. The Role of CD4+ T-Helper Cells in CD8+ T-Cell Function

In addition to the anergic phenotype and disrupted cell signaling, several studies looking at the impairment of CD8+ CTL function in response to HIV-1 infection found that a lack of CD4+ T-cell help was found to play a large role in CTL functional defects [263, 264]. Although CD4+ T-helper cells may not be required for the initial induction of the CTL response, they are required for the maintenance of this response during infection [265, 266]. The role of CD4+ helper T-cells in the proper maintenance of CTL responses has been elucidated in both animal [267-270] and human [136, 271-274] viral infections. Recent data has shown the association of the helper CD4+ T-cell responses and the proper functioning of

HIV-1-specific CD8+ CTL responses [263]. Indeed, the improper functioning of CD8+ T-cells during infection may be explained by the observation that HIV-1 specific CD4+ T-cells are the preferential cellular targets of the virus [275].

One of the most striking characteristics of HIV-1 infection is the progressive loss of CD4+ T-cells. However, impairment of the function of the CTLs occurs early in the course of infection [276]. In a study of HIV-1 infected individuals who had low numbers of circulating HIV-1 specific CTLs, the CD4+ T-helper cell responses were also hindered, leading to the conclusion that HIV-1 impaired the virus-specific CD4+ T-helper cell functions [102]. In addition, another study showed that in the absence of circulating CD4+ T-helper cells, HIV-1 specific CTL were impaired in their IFN-γ production even though these cells were able to circulate for prolonged periods of time [136]. In patients treated with Highly Active Anti-Retroviral Therapy (HAART) during primary infection, strong CTL and CD4+ T-cell responses were maintained during the first year of therapy [277, 278] as opposed to those individuals who were chronically infected [279]. Recently, Ostrowski *et al.* showed the correlation between low levels of CD4+CD28+ T-cells and mortality in HIV-1 infected patients [280]. Within the CD4+CD28+ subset, they illustrated a decrease in CD4+CD45RA+ naïve and CD8+CD45RO+ memory cells with viral pathogenesis, indicating improper maintenance and maturation of these cells.

An inverse relationship was found between the number of proliferating memory CD8+ T-cells and CD4+CD25+ T-cells [281]. CD4+CD25+ T-cells are regulatory cells that suppress immune responses by affecting antibody production, cytokine secretion and T-cell responses [282, 283]. Studies in murine systems have demonstrated that CD4+ T-cells aid in the interactions between CD8+ T-cells and DCs. In order for an effector and memory CD8+

T-cell to be properly generated, a naive CD8+ T-cell must be activated by a professional APC expressing the proper co-stimulatory molecules. It is believed that the principal APC for activating naive CD8+ T-cells is the DC [284]. Activated CD4+ T-cells express CD40 ligand (CD40L), which induces a signal on DCs through their CD40 receptor, allowing the DC to become a potent inducer of the CTL response. This action is probably due to the up-regulation of B7-1 and B7-2 (co-stimulatory molecules) and the induction of pro-inflammatory cytokines such as IL-12 [285-288]. In a study of HIV-1 infected patients, a CD40L trimer was able to induce CTL responses in the absence of CD4+ T-cell help, though the degree of enhancement was not as pronounced as with the presence of Th-cells [289]. In the patients with poor responses to CD40L, a dysfunction of the CD8+ memory T-cells was found, which was reversed with the addition of IL-2. In contrast to the requirement of CD4+ Th cells in the proper maintenance of CTL responses, Mintern *et al.* showed that CTL responses can be converted from CD4-dependent to CD4-independent by simply increasing the number of naïve precursor CTLs [290]. Thus, the proper function and maintenance of the CD8+ CTL response involves a complex interplay between cells of the immune system such as DC and CD4+ Th cells.

1.4.7. HIV-1 Impairment of CD8+ T-Cell Maturation

CD8+ T-cells arise from CD4+CD8+ DP precursor thymocytes that originate in the thymus. One of the hallmarks of the immune system is it's ability to generate memory. This defining aspect of the immune response is critical in controlling and recognizing viral infections. In response to an infection, CD8+ T-cells differentiate. Upon antigen recognition through MHC-I molecules, naïve CD8+ T-cells (CD8+CD45RA+CD28+CCR7+) become

activated and differentiate into memory (CD8+CD45RO+CD28+CD27+CCR7+) and effector (CD8+CD45RA+CD28-CD27-CCR7+) T-cells [291]. Once the immune response subsides and the infection is cleared, the majority of the activated CD8+ T-cells are eliminated, but a few remain circulating in the immune system. These circulating cells exist as memory cells that are pathogen-specific [292]. In addition, the memory CD8+ T-cells are able to circulate for extended periods of time because of high levels of anti-apoptotic molecules [293] and, unlike naïve CD8+ T-cells, they are able to proliferate in the absence of antigen *in vivo* [294]. In the face of infection, the repertoire of CD8+ T-cell subsets has been shown to be modulated by different viral infections [295]. After acute *in vivo* infection, CD8+ T-cell differentiation follows the pattern of naïve → effector → effector memory (lack lymph node homing receptors) → central memory (possess lymph node homing receptors) T-cells [291]. The differentiated CTLs are characterized by the secretion of IL-2, IFN-γ and TNF-α cytokines, which aid their effector and proliferation functions [296]. In HIV-1 infection, despite the appearance of virus-specific CD8+ CTL during the initial course of infection, immune containment of the virus is eventually hindered, leading to AIDS progression. It has been suggested that eradication of an infection by T-cell mediated lysis may become hampered once the infection becomes chronic as in the case of HIV-1 infection. Looking at CD8+ T-cells specific for HIV-1, Epstein-Barr Virus (EBV) and HCV, Appay *et al.* demonstrated that during chronic primary infection, CD8+ T-cells express different phenotypes at different stages of cellular differentiation according to the viral specificity [297]. This suggests that various distinct memory CD8+ T-cell populations are exhibited in different virus infections. In a patient study, Zhang *et al.* found that HIV-1 specific CD8+ T-cells are granzymeA+perforin-CD45RA-CD27+ and are not directly cytotoxic [298, 299].

They also discovered that CMV and EBV-specific cells in HIV-1 infected patients express the phenotypic markers granzymeA+CD45RA+CD27-. These findings were corroborated in a study of *ex vivo* lymphoid tissue from HIV-1 infected patients that showed a defect in the coexpression of perforin and granzyme A in CD8+ T-cell granules [300]. Though granzyme A is not deficient in HIV-1 specific CTLs, the expression of granzyme B mRNA is significantly lower in infected patients than in healthy individuals [301]. Again looking at phenotypic markers during infection, the majority of HIV-1 specific CD8+ T-cells do not express CD28 and are of the CD27+ memory phenotype [302, 303]. Both CD28 and CD27 are costimulatory molecules that provide the appropriate signals required for proper T-cell activation after TcR ligation. CD28+ cells have longer telomere lengths and therefore greater cell proliferation potential than CD28- cells [304]. In the immune system, the telomere length of memory CD8+ T-cells is shorter than that of naïve CD8+ T-cells [305]. Studies showing the adoptive transfer of human telomerase reverse transcriptase into CD8+ T-cell clones revealed that these cells have a greater proliferative life span [306, 307] and increased cytolytic activity [308]. The shortened telomere length in CD8+ T-cells of HIV-1 infected individuals has been implicated in the excessive activation and premature senescence of these cells [309, 310]. In addition, it has been reported that CD27- T-cells exert stronger cytolytic activity and contain higher levels of granzyme A and perforin than CD27+ T-cells [232, 299]. Being that the majority of HIV-1 specific CD8+ T-cells are of the CD28-CD27+ memory phenotype, they are thus undermined in their proliferation potential, perforin expression and cytolytic activity. However, another study has shown that the number of perforin expressing HIV-1 specific CD8+ T-cells circulating in the peripheral blood was inversely correlated with the blood CD4+ T-cell count [311]. Andrieu *et al.*

showed that the Nef protein also down-regulates surface MHC-I expression from DCs [312], thereby impairing CD8+ T-cell maturation. Antigen stimulation of CD8+ T-cells is also a determinant on the maturation of these cells during the course of infection. Tussey *et al.* hypothesized that during initial infection or when there is a sudden burst of viral replication, the CD8+ T-cell would primarily be of the effector subset [313]. Conversely, when viral replication was in the quiescent stage, the cells would be of the memory subset. In another patient study, Ellefieu *et al.* found that the majority of the HIV-1 specific memory CD8+ T-cells were pre-terminally differentiated (CD45RA-CCR7-), whereas CMV-specific cells were terminally differentiated (CD45RA+CCR7-) [314, 315]. Thus, during the chronic stage of viral infection where HIV-1 replication is at a minimum, the majority of CD8+ T-cells would be of the memory phenotype. However, the maturation of these memory CD8+ T-cells would be impaired, thus further compromising the activities of these cells and aiding viral escape from immune containment.

1.4.8. Cellular Immunosenescence

In response to DNA damage and telomere shortening, cells have the choice of two fates: i. they either senesce; or ii. they apoptose. Immunosenescence is characterized by the proliferative dysfunction and the general ageing of the lymphocyte population in an individual [316]. It is also characterized by increasing frequencies of memory cells previously exposed to antigens and decreasing frequencies of naïve cells that are critical in the recognition and combat of new antigen sources arising from pathogenic infections.

During the course of HIV-1 pathogenesis, the infected individual's immune system is continuously challenged and the state of chronic immune activation and inflammation

becomes generalized. These features have been characterized by high levels of activated CD4+ and CD8+ T-cells, high CD8 counts, increased levels of apoptosis as well as increased production of inflammatory cytokines [317]. Over the last couple of years, a general consensus has been reached that the phenomenon of chronic immune activation may be associated with poor prognosis and disease control in HIV-1 infected individuals [318-322].

Several alterations of CD8+ T-cell characteristics have been reported in HIV-1 infected individuals [323]. These include highly differentiated CD8+CD28- T-cells, a reduced capacity to proliferate, shortened telomere length and changes in cytokine secretion patterns [324-327]. A recent study illustrated the occurrence of replicative senescence characterized by CD57 expression, short telomere lengths and defective proliferative capacity in early differentiated CD8+ T-cells [317]. CD57 expression, a marker for cellular senescence, was also found to be expressed by a large fraction of CD8+ T_{EMRA} (CD45RA+CCR7-) cells than T_{EM} (CD45RA-CCR7-) cells [328]. Novel observations have also linked CD57 expression with the appearance of a negative co-stimulatory receptor for T-cells, PD-1. Trautman *et al.* observed that PD-1 expression was up-regulated on HIV-1 specific CD8+ T-cells and was associated with a reversible immune dysfunction [329]. Further studies illustrated that PD-1 expression was found to be low on naïve cells but increased on HIV-1 specific memory CD8+ T-cells [330]. This same study also correlated the expression of PD-1 on different virus-specific CD8+ T-cells (HIV = EBV > CMV > VV). Zhang *et al.* observed that PD-1 up-regulation on HIV-1 specific memory CD8+ T-cells was correlated with exhaustion in HIV-1 progressors but not in LTNPs [331]. Due to the functional and proliferative defects of CD8+ T-cells illustrated in the above studies, it may

be suggested that a general ageing or senescence of the lymphocyte population in HIV-1 infected individuals takes place.

1.5. RATIONALE AND RESEARCH OBJECTIVES

CD8+ T-cells, like other cellular subsets of the immune system, play a key role in controlling viral infection. The effects of HIV-1 infection on these cells must be extensively studied in order to not only have a better understanding of HIV-1 pathogenesis, but to also find strategies to control and treat infection. Ample research has been demonstrated for the productive infection of CD8+ T-cells both *in vivo* and *in vitro*. The current evidence for the prevalence of circulating CD8+HIV+ T-cells and the contribution that these cells make to the viral load remains inconclusive and controversial. Early studies of CD8+ T cells which reported low frequency of infection *in viv*o did not use quantitative or even semi-quantitative assays that are used routinely today. More recent studies using more sensitive quantitative PCR techniques have demonstrated frequent integration of HIV-1 into the CD8+ T cell genome. CD8+ T-cells play an important role in the cellular immune response to infection. Through their lytic and non-lytic activities, these cells effectively control the spread of infection. However, viruses such as HIV-1 have adopted various strategies to circumvent the CD8+ T-cell response and thus escape immune containment. Therefore, there continues to be a great need for research in understanding the effect HIV-1 infection has on CD8+ T-cell function.

Due to the observed decrease in CD8+ T-cell function during HIV-1 pathogenesis, the degree of susceptibility of CD8+ T-cells to HIV-1 infection both in an *in vivo* patient population and *in vitro* using primary blood-derived CD8+ T-cells and CD8+ T-cell clones

was ascertained. We hypothesized that CD8+ T-cells would serve as suitable targets of HIV-1 infection both *in vivo* and *in vitro* and the presence of extracellular receptors would confer susceptibility to viral adsorption and penetration. In the patient analysis, a 28 patient study was undertaken in order to examine the significance and frequency of CD8+HIV+ T-cells in HIV-1 seropositive individuals. Flow cytometric analysis using fluorochrome-conjugated antibodies, RT-PCR analysis using HIV-1_{pol} specific primers, and quantification of HIV-1 viral transcripts were all used to test the experimental objectives. The establishment of a viral reservoir of productively infected CD8+ T-cells during HIV-1 pathogenesis *in vivo* was also assayed. To investigate the susceptibility of CD8+ T-cells to HIV-1 infection *in vitro* and the functional significance of infection, the levels of HIV-1 replication and the effects of viral infection in CD8+ T-cells and T-cell subsets were examined. The susceptibility of CD8+ T-cells to T-cell tropic and macrophage tropic strains of HIV-1 was also analyzed. In addition, the frequency of HIV-1 infection in the CD8+ T-cell population as measured by an intracellular HIV-1 assay was determined. Lastly, the effect of HIV-1 infection on CD8+ T-cell subsets was examined. In addition, the mechanism by which the virus binds and enters these cells was examined by observing the interactions between CD8+ T-cell surface molecules and viral proteins in HTLV-I transformed CD8+ T-cell clones. It is hypothesized that HIV-1 positive CD8+ T-cells will be detected and will contribute to the viral load in HIV-1 infected individuals. Furthermore, *in vitro* HIV-1 infection will result in the interaction of CD8+ T-cell extracellular molecules and the virus envelope.

By examining the frequency and contribution of CD8+ T-cells to the disease outcome, a better understanding of the factors involved in CD8+ T-cell dysfunction and host immune failure may be elucidated. Future research must be directed towards blocking virus

induced alterations in CD8+ T-cell function and finding ways to maintain proper responses of these cells in the face of infection in order to combat the harmful effects and spread of HIV-1.

2. MATERIALS AND METHODS

2.1. Study Subjects

Volumes of 20 mL and 30 mL of whole blood anti-coagulated with Ethylenediamine Tetra-acetic Acid (EDTA) were obtained by venipuncture from 4 HIV-1 seronegative controls and 28 HIV-1 seropositive individuals attending the Ottawa Hospital Immunodeficiency Clinic, Ottawa Hospital, Ottawa, Canada. All samples were coded to ensure that laboratory personnel were blinded to the clinical condition and the HIV-1 status of the donor. All participants were volunteers and signed consent. This study was approved and carried out in compliance with guidelines of the Ottawa Hospital Research Ethics Board.

Clinical information regarding the subjects is presented in Table II. Subjects were selected in such a way to ensure a range of disease, from asymptomatic to advanced AIDS. Seven of the HIV-1 seropositive individuals were receiving combination ART at the time of the sampling. Study subjects were grouped based upon their CD4 counts, HIV-1 viral loads and treatment regimens. HIV-1 plasma virus levels were determined by the Chiron bDNA test (Chiron, Emeryville, CA).

2.2. Isolation of Lymphocyte Populations

Blood samples from healthy volunteers and HIV-1 seropositive individuals were collected into tubes containing EDTA by venipuncture. Samples were diluted with an equal volume of Phosphate Buffered Saline (PBS) and PBMCs were isolated by Ficoll-Hypaque (Amersham Biosciences, Baie d'Urfe, PQ) density centrifugation. Subsequent to density centrifugation, isolated PBMCs were washed twice in PBS. CD8+ and CD4+ T-cell

Table II. Clinical Characteristics of HIV-1 Seropositive Study Subjects

Patient #	Age (Years)	Absolute (count/mm^3) [a,b] CD4	Absolute (count/mm^3) [a,b] CD8	(%) [a] CD4	(%) [a] CD8	Ratio [a] CD4/CD8	Viral Load, RNA [a,c] (copies/mL)	Treatment [a]
p1	42	1,050	1,000	32.8	31.2	1.05	<50	3TC, abacavir, atazanavir
p2	40	935	2,010	27.7	59.8	0.46	4,080	naïve
p3	43	779	484	48.8	30.3	1.6	21,135	naïve
p4	56	653	992	29	44.1	0.7	36,926	naïve
p5	41	632	1,349	25.5	54.4	0.49	16,177	3TC, AZT, efavirenz
p6	35	515	1,778	18.8	65.1	0.29	3,080	naïve
p7	41	506	740	30.6	44.8	0.68	163,970	combivir, indinivir
p8	36	461	808	29.3	51.6	0.57	9,840	naïve
p9	46	453	1,649	18.1	65.6	0.28	18,800	naïve
p10	43	433	2,036	14.6	68.7	0.21	251,624	kaletra, invirase
p11	50	426	774	27.5	49.8	0.55	9,860	naïve
p12	32	425	1,848	16.3	71.1	0.23	7,860	naïve
p13	37	396	907	20.6	47.2	0.44	22,821	naïve
p14	36	396	558	32.7	46.1	0.71	1,382	naïve
p15	54	391	2,159	13.5	74.5	0.18	25,331	3TC, D4T, ritonavir, saquinavir, lopinavir
p16	52	379	620	24.8	40.5	0.61	580	3TC, ziagen, reyataz
p17	52	368	602	29.4	48.2	0.6	48,608	naïve
p18	45	367	666	31.6	57.5	0.55	5,770	naïve
p19	38	323	739	23	52.6	0.44	96,420	naïve
p20	38	294	N/D [e]	21.7	51.4	0.42	128,274	naïve
p21	39	254	608	19.5	46.8	0.4	73,770	naïve
p22	43	239	880	16	59	0.27	490,000	naïve
p23	61	182	646	12.5	44.5	0.28	4,955	naïve
p24	39	127	1,096	7.3	63	0.1	346,015	naïve
p25	28	87	360	10.8	44.4	0.24	207,200	off treatment
p26	49	41	817	3.4	68.1	N/A [d]	33,797	off treatment
p27	54	15	193	2.7	34.4	0.08	73,810	naïve
p28	46	10	281	2.4	65.3	N/A	61,028	reduced ARV doses

[a] At the time of sampling

[b] Normal range >300 cells/mm^3

[c] Assayed using Chiron bDNA Test™

[d] N/A, Not Applicable

[e] N/D, Not Determined

populations were isolated from freshly purified PBMCs through positive selection using anti-CD8 and -CD4 conjugated magnetic beads (Miltenyi, Auburn, CA) respectively, according to the manufacturer's protocol. Briefly, cells were labeled with the magnetically conjugated antibody of interest for 20 minutes at 4°C. Subsequent to labeling, cells were placed through a column inserted in the magnetic field of a MACS separator (Miltenyi) and unbound cells were washed with MACS buffer (PBS, 0.5% Bovine Serum Albumin (BSA), 2 mM EDTA, pH 7.2). After washing unbound cells, the column was removed from the separator and the labeled cells were eluted with the addition of MACS buffer. Following elution, the isolated T-cell subsets were washed twice with PBS.

The isolation of CD8+ T-cell subsets was performed using anti-CD8 Multisort conjugated magnetic beads (Miltenyi). In order to isolate the T-cell subsets, a second round of positive selection with magnetic bead-conjugated antibodies to either CD45RO+ or CD45RA+ was performed. Similarly, CD28, CD38 and HLA-DR positive and negative populations were selected using the Multisort conjugated magnetic beads.

2.3. Cell Culture

PBMCs and T-cell subsets were cultured in RPMI-10 (RPMI 1640 medium supplemented with 10% Fetal Calf Serum (FCS), 100 U/mL penicillin (Invitrogen, Burlington, ON), 100 μg/mL streptomycin (Invitrogen), and 20 U/mL Interleukin (IL)-2 (Sigma-Aldrich, St. Louis, MO)). CD8+ T-cell clones were cultured in RPMI-20 (RPMI 1640 medium supplemented with 20% FCS, 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen)). On occasion, exogenous IL-2 (Sigma-Aldrich) at a concentration of 100 U/mL was added to the cultures with the HTLV-I transformed CD8+ T-cell clones. Cells were incubated at 37°C with 5% CO_2.

2.4. Sources of Viruses

The T-cell tropic laboratory strain HIV_{IIIB} was obtained from Advanced Biotechnologies Incorporated (Columbia, MD). The macrophage tropic laboratory strain, HIV_{ADA}, was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent program (Germantown, MD). The clinical isolates of HIV-1 (used in Figure 13) were generated from the blood of HIV-1 infected individuals. Briefly, PBMCs were isolated from the peripheral blood of infected subjects by Ficoll-Hypaque density centrifugation. The CD8+ T-cell fraction was removed from the isolated PBMCs through the use of anti-CD8 conjugated magnetic microbeads. The remaining cells were subsequently cultured with HIV-negative PHA blastocytes in RPMI-10. The cultures were monitored at days 10 and 15 for HIV-1 p24 production by an Enzyme-Linked Immunosorbent Assay (ELISA) (SAIC-Frederick Inc., Frederick, MD).

The HIV-1 clinical isolates used in cell-surface molecule expression studies (92HT599, 92HT593 and 91US005) were all obtained from the NIH AIDS Research and Reference Reagent program (Germantown, MD)

The titre of the viral stock was determined by a $TCID_{50}$ assay. Briefly, 100 μL serial dilutions of the viral stock (approximately 1,500 ng/mL as determined by a HIV-1 p24 ELISA) was used to infect 250,000 MT2 cells (American Type Culture Collection (ATCC), Manassas, VA) in quadruplicate in a 96-well flat bottom plate. Subsequent monitoring for the formation of syncytia was performed over a 7-day culture period. The titre of the viral stock was determined by the Reed-Muench accumulative method. The titre of the HIV_{IIIB} viral stock used for CD8+ T-cell infections was found to be 1×10^5. Unless otherwise noted, 300 $TCID_{50}$/mL of the viral inoculum for each of the viral isolates was used to infect CD8+

T-cells and CD8+ T-cell clones at a density of 1 x 10^6 cells/mL during the course of the *in vitro* experiments.

2.5. HIV-1 Infection and Monitoring of Replication in CD8+ T-Cell Populations

Immediately after isolation and prior to infection, CD8+ T-cells were stimulated with 2.5 μg/mL of Phytohemagglutinin (PHA) (Sigma-Aldrich) for three hours followed by subsequent washes and a 1 hour incubation with 2 μg/mL polybrene (Sigma-Aldrich) in RPMI-10. Subsequently, the cells were washed, re-cultured in RPMI-10 and infected with 300 $TCID_{50}$/mL of the appropriate viral strains and isolates as previously described [332]. Three days after the infection, the cells were extensively washed to remove any extracellular virus and debris and were re-cultured in RPMI-10 and this time point was considered the zero time point in the infection assays. In order to determine the levels of HIV-1 replication and production, cell-free supernatants were taken at days 5, 10 and 15 (or as indicated in the results and figures) post-infection from the infected cell cultures. Virus replication was assessed by HIV-1 production as measured by an ELISA (SAIC-Frederick Inc.). In order to normalize our results, supernatants were taken from cultures of CD8+ T-cells derived from several different individuals. The supernatants were assessed for HIV-1 p24 production by an ELISA and only experiments which resulted in p24 values deemed suitable enough for a productive infection were reported.

In order to assess productive infection, the reverse transcriptase inhibitor, Azidothymidine (AZT) (NIH AIDS Research and Reference Reagent program) was used to treat the cells at a concentration of 10 μM for one hour prior to infection and was not used further throughout the time course.

2.6. Nucleic Acid Extraction and Detection of Productive HIV-1 Infection by RT-PCR Analysis

Total RNA was isolated from 3 x 10^6 cells using the GeneElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). First strand cDNA was reverse transcribed with a $pd(N)_6$ primer and Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase using the First-Strand cDNA synthesis kit (Amersham Biosciences) according to the manufacturer's protocol. RT-PCR reactions were set up for each cDNA towards the *gag* region of HIV-1 (p24 forward: 5'-ATAGAGGAAGAGCAAAACAAAA-3'; p24 reverse: 5'-CAAAATTACCCTATAGTGCA-3'). Each reaction was conducted in a total volume of 25 µL containing 2.5 µL of 2 mM dNTP (Amersham Biosciences), 1.25 µL of 50 mM $MgCl_2$ (Amersham Biosciences), 1 µL of each primer (from a 100 mM stock) and 1 U of Taq DNA polymerase (Amersham Biosciences). A total of 5 µL of each cDNA was used in the RT-PCR analysis. RT-PCR amplification was performed for 35 cycles of: denaturing at 94°C for 60 seconds, annealing at 55°C for 60 seconds and extension at 72°C for 60 seconds. This was followed by a final extension for 10 minutes at 72°C. RT-PCR amplicons were run at 80 V on a 1% agarose gel containing 0.5 µg/mL of ethidium bromide and visualized under Ultraviolet (UV) light.

For studies concerning the HIV-1 seropositive study subjects, productive infection was monitored by a nested RT-PCR for the HIV-1 polymerase gene. CD4+, CD8+ T-cell and unfractionated PBMC populations were isolated from study subjects by magnetic bead separation. Cells were cultured and cell-free supernatants were collected at 48, 72 and 120 hour time-points. Purification and isolation of HIV-1 viral RNA from cell-free supernatants was performed using the QIAamp viral RNA mini kit (Qiagen, Mississauga, ON) according to the manufacturer's protocol. The RT-PCR reactions contained 5 µl of 10X PCR buffer

(Perkin Elmer, Waltham, MA), 200 μM of each dNTP (Perkin Elmer), 1 μM of each primer, 1 U of AmpliTaq DNA Polymerase (Perkin Elmer) and 2.25 mM $MgCl_2$ (Perkin Elmer) per 50 μl reaction. The outer primer pair was HPOL4235 (5′-CCCTACAATCCCCAAAGTCAAGG-3′) and HPOL4538 (5′-TACTGCCCCTTCACCTTTCCA-3′), and 12.5 μl of sample was used in the first round reaction. The inner primer pair was HPOL4327 (5′-TAAGACAGCAGTACAAATGGCAG-3′) and HPOL4481 (5′-GCTGTCCCTGTAATAAACCCG-3′), and 2 μl of the first round products were added to the second round. Both first and second rounds were amplified for 35 cycles of 30 second incubations at 96, 65 and 72°C. The second round PCR products were visualized by gel electrophoresis in 1% agarose for 30 minutes at 100 V, followed by ethidium bromide staining and UV transillumination of the gel.

2.7. *in situ* RT-PCR Detection of HIV-1

The protocol described by Muratori *et al.* [333] was followed with some minor modifications. PBMCs were isolated from whole blood by Ficoll-Hypaque (Amersham Biosciences) and carefully washed with PBS. Approximately 1 x 10^6 cells were resuspended in 1 mL of ice-cold 10% formal saline and kept at 4°C for 2 hours with gentle mixing. After centrifugation and further washing, cells were resuspended in 1 mL of 1% TritonX-100 and incubated at 4°C for 15 minutes with constant shaking. Cells were centrifuged again and a final wash and resuspension with 200 uL of 0.1 M glycine was performed using a 1 mL syringe with a 26 gauge needle. Cells were adjusted to a final concentration of 10^7 cells/mL and were frozen in small aliquots of 2 x 10^6 – 5 x 10^6 cells and stored at -70°C for up to a month.

Prior to *in situ* RT-PCR analysis, freshly thawed cells were prepared for cDNA synthesis using the Clontech Advantage RT-for-PCR kit (Clontech, Mountain View, CA) as per the manufacturer's protocol. Prior to cDNA synthesis, cells were treated with DNAse I (New England Biolabs, Ipswich, MA). DNAse I treated cells were resuspended in 20 µl of RT buffer containing: 5X reaction buffer (50 mM Tris-HCl, pH 8.3; 74 mM KCl; 3 mM MgCl2), dNTP mix (0.5 mM each dGTP, dATP, dTTP and dCTP), RNAse inhibitor (1 U/µL), 200 U of cloned MMLV and 20 pmol of the HIV-1_{gag} specific antisense primer (5'-GTTCCTGAAGGGTACTAGTAGT-3').

After the RT step, cells were spun down and washed with ice-cold PBS. Each reaction was conducted in a total volume of 25 µL containing 2.5 µL of 2 mM dNTP (Amersham Biosciences), 1.25 µL of 50 mM $MgCl_2$ (Amersham Biosciences) and 1 U of Taq DNA polymerase (Amersham Biosciences). Each reaction contained 1 uL of the FITC-conjugated HIV-1_{gag} specific primers (forward: 5'-/56-FAM/ATAGAGGAAGAGCAAAACAAAA-3'; reverse: 5'-/56-FAM/TTCCTGAAGGGTACTAGTAGT-3'). A total of 5 µL of each cDNA was used in the RT-PCR analysis. RT-PCR amplification was performed for 30 cycles of: denaturing at 94°C for 60 seconds, annealing at 55°C for 60 seconds and extension at 72°C for 60 seconds. This was followed by a final extension for 7 minutes at 72°C.

Between 2 - 4 x 10^3 cells were placed on a glass slide and counterstained with Hoechst and CD8-ECD/Texas Red (Serotec, Raleigh, NC) at antibody concentrations of 1:100 and 1:20 respectively. Images were captured on a Zeiss Axiophot (Carl Zeiss Inc., Toronto, ON) microscope equipped with a UV source and Hoechst and Texas Red detection filters.

2.8. HIV-1 Viral Quantitation

CD4+ and CD8+ T-cell populations were isolated from study subjects by magnetic bead separation. Cells were cultured and cell-free supernatants were collected at 48, 72 and 120 hour time-points. Purification and isolation of HIV-1 viral RNA from cell-free supernatants was performed using the QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's protocol. The nucleic acid amplification test for the quantitation of HIV-1 RNA in the cell-free supernatants was performed using the COBAS AMPLICOR HIV-1 Monitor Test as per the manufacturer's protocol (Roche Molecular Systems Inc., Branchburg, NJ). The test can quantitate HIV-1 RNA over the range of 50 – 750, 000 copies/mL. Results were normalized to the number of cells cultured and present at the time of sampling in each individual T-cell population.

2.9. Flow Cytometric Analysis

Flow cytometric analysis was gated on the live cell populations. The level of infection of T-cell subsets was assessed by both two- and three-color flow cytometry. Measurement of the levels of intracellular HIV-1 staining was performed as follows: 1×10^6 cells were permeabilized with the Fix & Perm cell permeabilization kit (Caltag Laboratories, Burlingame, CA) according to the manufacturer's protocol. Cells were labeled with 10 µL of the appropriate fluorochrome-conjugated monoclonal antibodies CD8-ECD (Serotec), CD4-PC5 (Serotec) and HIV-1_{gag}-FITC (Coulter clone KC57, Beckman Coulter, Fullerton, CA) prior to flow cytometry analysis. The KC57 antibody identifies HIV-1 core antigens (p55, p39, p33 and p24). Results were assessed on an Epics ALTRA system (Beckman Coulter) after gating on lymphocytes based on their forward and side scatter properties. Analysis was performed using Expo32 software (Applied Cytometry Systems, Sacramento,

CA) and was based on a minimum of 10, 000 events. Values are expressed as the mean fluorescence intensity.

For the analysis of the purity of the isolated populations of CD8+ T-cells, the following fluorochrome-conjugated monoclonal antibodies were used: CD8-PE (Serotec), CD4-FITC (Serotec), CXCR4-PE (BD Biosciences, San Diego, CA), CCR5-PE (BD Biosciences), IgG-FITC (BD Biosciences), CD3-FITC (BD Biosciences), CD56/16-PE (BD Biosciences), CD45RA-PE (BD Biosciences), CD45RO-FITC (Immunotech, Fullerton, CA), CD28-FITC (BD Biosciences), CD38-FITC (BD Biosciences) and HLA-DR-FITC (BD Biosciences). For flow cytometric analysis, the cells of interest were washed twice in PBS and once in flow binding buffer (PBS, 0.1% NaN_3, 2% BSA). The cells were subsequently resuspended at a concentration of 1 x 10^6 cells per 100 µL of flow resuspension buffer and were labeled with 10 µL of the appropriate antibody for 30 minutes at room temperature. Prior to flow cytometric analysis, the cells were fixed in 200 µL of PBS/4% paraformaldehyde at 4°C for 5 minutes.

Receptor expression during the course of HIV-1 infection was examined in positively selected cells using CD8-PE (Serotec), CD4-FITC (Serotec), CXCR4-PE (BD Biosciences) and CCR5-PE (BD Biosciences) conjugated monoclonal antibodies as previously described [334, 335]. In order to determine the surface expression of both CD45RA and CD45RO in the CD8+CD4- and CD8+CD4+ populations, cells were examined by flow cytometry at day 5 post-infection after staining with antibodies directly conjugated to FITC.

2.10. DNA Fragmentation Assay

Prior to performing the DNA fragmentation assay, isolated CD4+ and CD8+ T-cells were either left uninfected, or infected with 300 $TCID_{50}$/mL of HIV_{IIIB} for 7 days in culture.

Subsequently, cells were lysed and treated with 20 μg/mL RNase A and 200 μg/mL proteinase K for 2 hours at 45°C. Fragmented DNA was phenol/chloroform extracted and ethanol precipitated. Jurkat T-cells (ATCC) were treated with 10 μg/mL of Camptothecin (CPT) (Sigma-Aldrich) prior to lysis as a positive control for apoptosis induction. Samples were loaded and run on a 1% agarose gel at 70 volts for 2 hours. Visualization of the DNA was performed with ethidium bromide.

2.11. Mixed Lymphocyte Reaction Assay

A heterologous Mixed Lymphocyte Reaction assay (MLR) was performed to assess the cytotoxic abilities of CD8+ T-cells. Peripheral blood-derived CD8+ T-cells were either left uninfected, or infected with 300 $TCID_{50}$/mL of HIV_{IIIB} for 7 days and used as responders/effectors against heterologous CD4+ T-cells (targets). CD8+ T-cells, both uninfected and infected, were treated with 50 ng/mL of interleukin-15 (IL-15) one day prior to performing the experiment. Previous studies in our lab illustrated the enhanced IFN-γ production and cytotoxicity of CD8+ T-cells in the presence of IL-15. Prior to co-culture, target CD4+ T-cells were labeled with 3.0 mM of 3,3'-dihexyloxacarbocyanine Iodide ($DiOC_6(3)$). Effectors and targets were incubated together at various ratios at 37°C for 3.5 hours. Prior to analysis, co-cultures were labeled with Propidium Iodide (PI) and lysis was measured by flow cytometry.

2.12. Lymphocyte Proliferation Assay

Lymphocyte proliferation was measured by the XTT based colorimetric assay (Roche Laboratories, Meylan, France) based on the reduction of a tetrazolium salt (XTT) into an orange formazan salt by active mitochondria [336].

Proliferation assays of primary CD4+ and CD8+ T-cells, PBMCs, H9 cells and CD8+ T-cell clones to HIV-1 infection was performed in 96 well dishes. Cells were either left uninfected or infected with 300 $TCID_{50}$/mL of HIV_{IIIB} and the proliferation index was determined at the indicated time points. Approximately 450,000 cells were seeded in triplicate in 100 μL of culture medium per well of a 96-well flat bottom plate.

In the memory CD8+ T-cell studies, CD8+CD45RO+ T-cells were positively isolated from the PBMCs of a healthy volunteer by magnetic labeling. For proliferation tests, approximately 450,000 cells were seeded in triplicate in 100 μL of culture medium (RPMI-10 supplemented with 20 U/mL of IL-2) per well of a 96-well flat bottom plate. Cells were incubated in the presence of each of the following antigens: 3 μg/mL αCD3/anti-CD28 (Sigma-Aldrich); 10 ng/mL PMA (Sigma-Aldrich); 2 U/mL Tetanus Toxoid (TT) (Sigma-Aldrich); and 5 μg/mL PHA. On days 2-3 post-antigenic stimulation, cells were labeled with 50 μL of the XTT labeling reagent as per the manufacturer's protocol. Proliferation was assessed by measuring the absorbance at 492 nm (with a reference wavelength at 690 nm) 18 hours post-labeling.

2.13. HTLV-I Immortalization of Primary Human CD8+ T-Cells

Immortalization assays were performed as previously described [337]. Briefly, 1 x 10^6 MT2 cells (ATCC) were gamma-irradiated with 10, 000 rad and co-cultured with 2 x 10^6 CD8+ T-cells isolated from the peripheral blood of a healthy individual in 24-well culture plates. Cells were cultured for two weeks in RPMI-20 (RPMI 1640 medium supplemented with 20% FCS, 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), and 20 U/mL IL-2 (Sigma-Aldrich)).

After 2 weeks of co-culture, transformed CD8+ T-cells were isolated by three consecutive rounds of positive selection using the Miltenyi miniMACS system (Miltenyi Biotec) as per the manufacturer's protocol. Positively selected cells were then subjected to three consecutive rounds of limiting dilution.

2.14. RT-PCR and PCR Amplification of the HTLV-I *tax* Gene

Genomic DNA and cDNA were isolated and purified from MT2 cells (ATCC), untransformed CD8+ T-cells and HTLV-I immortalized CD8+ T-cell clones (Tri8.X) by the QIAmp DNA isolation kit (Qiagen) and Clontech Advantage RT-for-PCR kit (Clontech) according to the manufacturer's instructions respectively.

Sample analysis for the detection of the HTLV-I *tax* gene was conducted by nested RT-PCR in a total volume of 50 μL containing 400 μM dNTPs (Amersham Biosciences) and 2 μM of primers (*first round,* AV45: 5'-GGACGCGTTRTCRGCTC-3', AV46: 5'-KGGRGAIAGYTGGTAKAGGTA-3'; *second round,* AV49: 5'-CCCTCCTTCCTCCAGGCCAT-3', AV80: 5'-GGTCTGGAAAAGACAGGGTTG-3'). A total of 12.5 μL of each cDNA was used in the RT-PCR analysis. RT-PCR amplification was performed for: (a) *first round,* 45 cycles of: denaturing at 94°C for 30 seconds, annealing at 50°C for 60 seconds and extension at 72°C for 45 seconds. This was followed by a final extension for 10 minutes at 72°C. (b) *second round,* 25 cycles of: denaturing at 94°C for 30 seconds, annealing at 50°C for 60 seconds and extension at 72°C for 60 seconds. This was followed by a final extension for 10 minutes at 72°C. DNA analysis of the HTLV-I *tax* gene was performed using the AV45 and AV46 primers. The second round PCR products were visualized by gel electrophoresis in 1% agarose for 30 minutes at 100 V, followed by ethidium bromide staining and UV transillumination of the gel.

2.15. RT-PCR Detection of Cell-Surface Molecule Expression in CD8+ T-Cell Clones

Total RNA was isolated from CD8+ T-cell clones using Trizol reagent (Invitrogen) as per the manufacturer's protocol. Prior to RT-PCR analysis, isolated RNA was prepared for cDNA synthesis using the Clontech Advantage RT-for-PCR kit (Clontech) as per the manufacturer's protocol. Each reaction was conducted in a total volume of 50 μL containing 10 μL of 2 mM dNTP (Amersham Biosciences), 2 μL of 50 mM $MgCl_2$ (Amersham Biosciences) and 1 U of Taq DNA polymerase (Amersham Biosciences). Each reaction contained 1 uL of the appropriate primers pairs: CD8 (forward: 5'-GATCGGACCTGGAACCTG-3'; reverse: 5'-GGCACGAAGTGGCTGAAGTA-3'); CD4 (forward: 5'-CCAAGGGGTAAAAACATACA-3'; reverse: 5'-CTTGTTCTTCAGGTCAAAGG-3'); CXCR4 (forward: 5'-GCCCTCCTGCTGACTATTCC-3'; reverse: 5'-ACTGTGGTCTTGAGGGCCTT-3'); CCR5 (forward: 5'-TGGTCCTGCCGCTGCTTG-3'; reverse: 5'-TGCTCCCCATGTGATCGG-3'); and β-actin (forward: 5'-GAAACTACCTTCAACTCCATC-3'; reverse: 5'-CGAGGCCAGGATGGAGCCGCC-3'). A total of 10 μL of each cDNA was used in the RT-PCR analysis. RT-PCR amplification was performed for 30 cycles of: denaturing at 94°C for 60 seconds, annealing at 55°C for 60 seconds and extension at 72°C for 60 seconds. This was followed by a final extension for 7 minutes at 72°C. The PCR products were visualized by gel electrophoresis in 1% agarose for 30 minutes at 100 V, followed by ethidium bromide staining and UV transillumination of the gel.

2.16. Cell-Surface Molecule Antibody Blocking Studies

Prior to HIV-1 infection, CD8+ T-cell clones were pre-treated with the appropriate antibodies for 2-3 hours at 37°C. Cells were subsequently infected with 300 $TCID_{50}$/mL of the HIV_{IIIB} laboratory strain where required. CD8+ T-cell clones were cultured in RPMI-10 supplemented with 100 U/mL IL-2. Cells were assessed at various days post-infection. HIV-1 viral production was measured from cell-free supernatants using an ELISA. Monoclonal antibodies utilized were the anti-human CD8 (BD Biosciences), anti-human CD4 (BD Biosciences), anti-human CXCR4 (BD Biosciences) and anti-mouse IgG1 (BD Biosciences).

2.17. Sequencing of Viral Envelope

Full length envelope (env) coding regions were amplified by nested PCR from genomic DNA using outer (forward: 5′-CTGGAAGCATCCAGGAAGTCAGCC-3′ and reverse: 5′-GTCCCCAGCGGAAAGTCCCTTGTA-3′) and inner (forward: 5′ GAGACAGTGGCAATGAGAGTGAAGG-3′ and reverse: 5′ CTTTTTGACCACTTGCCACCCATCTT-3′) primers. Amplified PCR fragments (2.6 kb) were purified and sequenced from both DNA strands using a set of 16 primers slightly modified as necessary as described previously [26] by cycle sequencing on an ABI 377 DNA Sequencer. Sequence assembly and comparisons were performed with Lasergene (DnaStar, Madison, Wisconsin) as well as with NCBI Blast server. The envelopes of the CD8+ T-cell clone derived isolates was compared to the published canonical T-cell tropic laboratory strain, HXB2 (HIV_{IIIB}) *(Genbank accession number NC_001802).*

2.18. Statistical Analysis

Data are expressed as the mean ± SEM. Differences in measured variables between experimental and control groups were assessed using the Student's *t* test or Spearman's Rank Correlation (as indicated in the text). Statistical significance was accepted at $p < 0.01$.

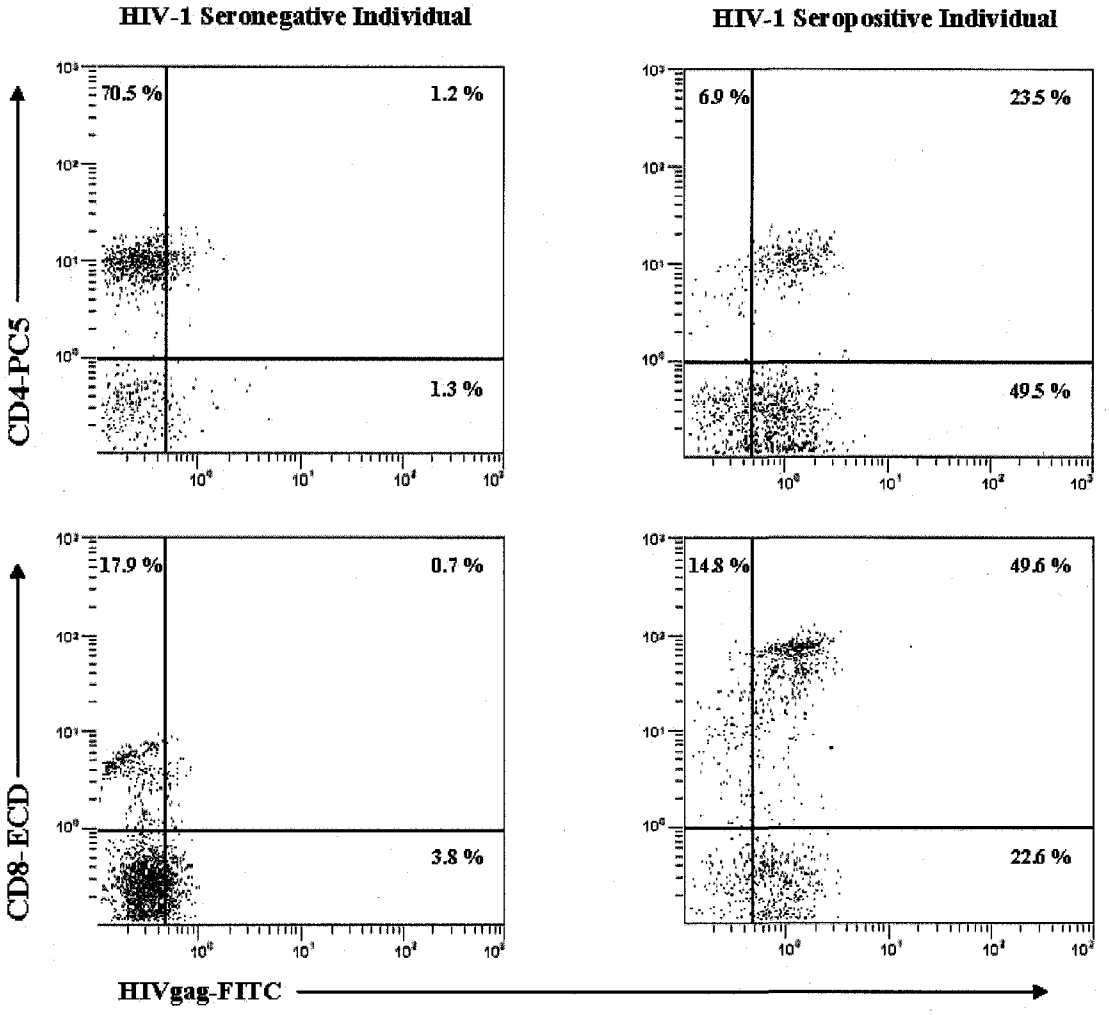
3. RESULTS

3.1. *ex vivo* ANALYSES OF HIV-1 INFECTION OF CD8+ T-CELLS: SIGNIFICANCE AND FREQUENCY

3.1.1. Frequency of HIV-1 Infected CD8+ T-Cells in the PBMCs of HIV-1 Seropositive Individuals

This study included 28 HIV-1 seropositive individuals and 4 HIV-1 seronegative controls. Subjects were chosen for this study based upon their clinical status, varying viral loads, CD4 counts and treatment regimens. Table II summarizes the clinical information of the individuals included in this study. The frequency of circulating CD4+ and CD8+ T-cells that were HIV-1 infected in the PBMCs of seropositive individuals was assessed. PBMCs were isolated from the peripheral blood of patients (p1 to p28) and healthy controls (c1 to c4). Intracellular expression of HIV-1_{gag} was ascertained by two- and three-color flow cytometry. Anti-CD4, -CD8 and -HIV fluorochrome-conjugated antibodies were used to detect expression levels in the T-cell populations of the subjects. Flow cytometric analysis of the patient samples showed that there was a higher percentage of CD4+HIV-1_{gag}+ than CD4+HIV-1_{gag}- T-cells in the HIV-1 infected individuals (Figure 4, *top right panel*; 23.5% vs. 6.9% respectively). A similar result was also found when CD8+ T-cells in the study subjects were examined (Figure 4, *bottom right panel*). It was observed that 49.6% of cells expressed the CD8+HIV-1_{gag}+ phenotype compared to 14.8% CD8+HIV-1_{gag}- cells in the representative HIV-1 seropositive individual. The detection of both CD8+HIV-1_{gag}+ and CD4+HIV-1_{gag}+ expressing cells was found in all 28 HIV-1 seropositive individuals, albeit

Figure 4. Detection of intracellular HIV-1 expression in the PBMC populations of HIV-1 seropositive individuals. HIV-1 expression was assessed by flow cytometry in the unfractionated PBMC population from HIV-1 seropositive patients and healthy controls. Subsequent to fixation and permeabilization, PBMCs were stained with anti-CD4 (*top panels*), -CD8 (*bottom panels*) and -HIV-1_{gag} fluorochrome-conjugated antibodies. Results from one representative HIV-1 seropositive individual (p6) and a healthy control (c2) are illustrated. Numbers in the quadrants represent the percentage of gated cells in each quadrant. A minimum of 10, 000 events was assessed for each analysis.


HIV-1 Seronegative Individual
HIV-1 Seropositive Individual
CD4-PC5
CD8-ECD
HIVgag-FITC
70.5 %
1.2 %
1.3 %
6.9 %
23.5 %
49.5 %
17.9 %
0.7 %
3.8 %
14.8 %
49.6 %
22.6 %

at different frequencies (Table III). In contrast, the appearance of these cells were not found in the healthy controls.

Figure 5 and Table III summarize the flow cytometric results. It was found that in the majority of patient samples that were analyzed, a significantly higher Mean Fluorescence Intensity (MFI) of CD8+HIV-1_{gag}+ than CD4+HIV-1_{gag}+ T-cells in the lymphocyte population was observed (Figure 5A; 36.9% ± 10.0% vs. 26.4% ± 13.1% respectively, $p < 0.01$, Student's *t*-test, two-tailed). This phenomenon was shown in 22 of our 28 patients studied (Figure 5B). Figure 5B illustrates the percentages of CD4+ and CD8+ T-cell infection within each HIV-1 seropositive study subject. The difference between these two populations within an individual patient is shown by a line connecting the two data points. Only 6 of the study subjects (p2, p3, p4, p16, p17 and p18) exhibited a higher level of HIV-1 infection of their circulating CD4+ T-cells than their CD8+ T-cells (Table III). The level of CD8+ T-cell infection in the HIV-1 seropositive individuals varied with a high reported in patient p28 (58.8% CD8+HIV-1_{gag}+) and a low observed in patient p27 (15.2% CD8+HIV-1_{gag}+). A similar variance was observed in the CD4+ T-cell population of the study samples (high: p4, 59.3% CD4+HIV-1_{gag}+; low: p28, 2.4% CD4+HIV-1_{gag}+).

3.1.2. Existence of $CD4^{dim}CD8^{bright}$ T-Cells in the PBMC Population of HIV-1 Seropositive Subjects

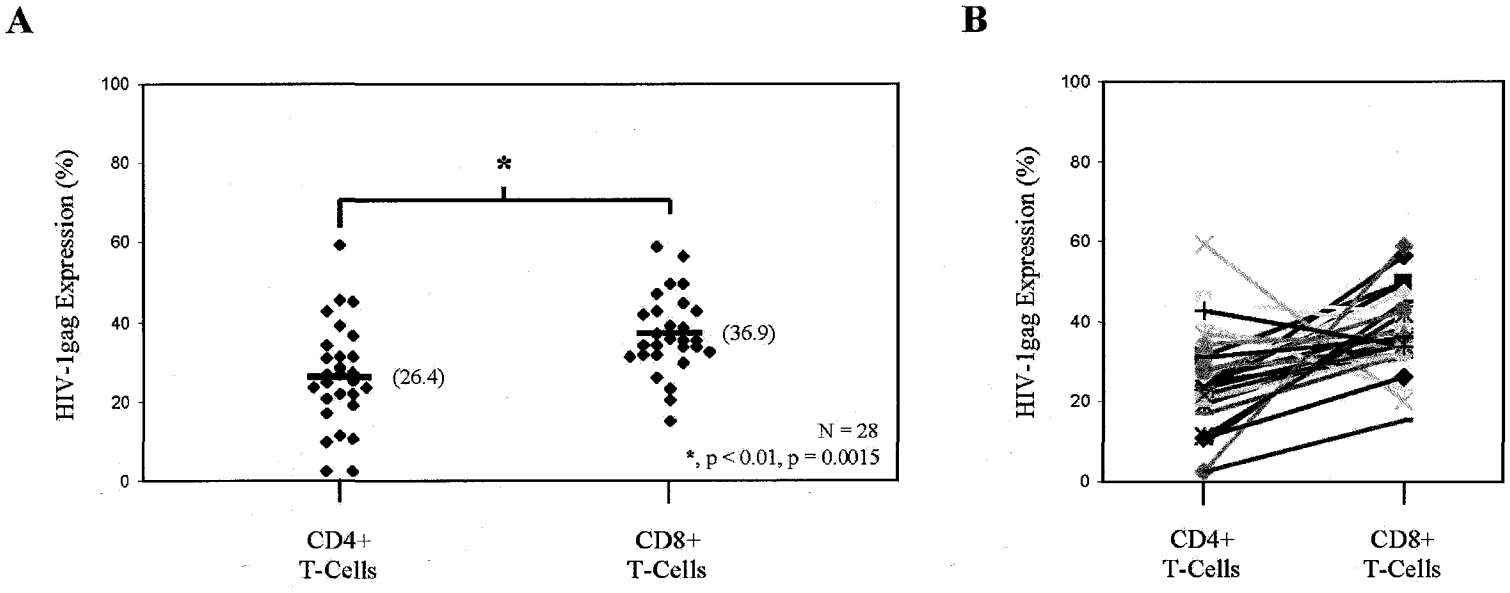
As it has been reported elsewhere, activation of CD8+ T-cells may result in the expression of the CD4 cell-surface molecule on these cells, giving rise to the appearance of T-cells with the $CD4^{dim}CD8^{bright}$ phenotype [27, 106, 118-123, 338-341]. These $CD4^{dim}CD8^{bright}$ circulating T-cells may serve as a potential target for HIV-1 infection both *in vivo* [106, 120, 122, 123, 339, 342] and *in vitro* [27, 119, 122, 124]. With these studies in

Table III. Frequency of HIV-1 Infected CD8+ T-Cells *ex vivo*

Patient #	*Absolute (count/mm^3) [a,b]*		*Viral Load, RNA [a,c]*	*HIV-1$_{gag}$-expressing Cells (%)*		$CD4^{dim}CD8^{bright}$HIV-1$_{gag}$ Cells
	CD4	*CD8*	*(copies/mL)*	*CD4*	*CD8*	*(%)*
p1	1,050	1,000	<50	10.7	26.2	N/D
p2	935	2,010	4,080	45.6	39.2	0.1
p3	779	484	21,135	36.5	33.6	N/D
p4	653	992	36,926	59.3	20.2	N/D
p5	632	1,349	16,177	31.4	49.7	0.3
p6	515	1,778	3,080	23.5	49.6	N/D
p7	506	740	163,970	25	31.2	N/D
p8	461	808	9,840	25.3	38.5	N/D
p9	453	1,649	18,800	26.8	47.1	N/D
p10	433	2,036	251,624	22.1	56.6	0.4
p11	426	774	9,860	27	34.1	N/D
p12	425	1,848	7,860	19.3	35.5	N/D
p13	396	907	22,821	28.5	31.7	0.7
p14	396	558	1,382	31	35.7	N/D
p15	391	2,159	25,331	23.5	35.4	N/D
p16	379	620	580	39.1	23	1.1
p17	368	602	48,608	42.8	33.9	N/D
p18	367	666	5,770	45	42.7	0.8
p19	323	739	96,420	31.2	29.7	N/D
p20	294	N/D[e]	128,274	21.9	34.3	0.8
p21	254	608	73,770	34.2	37	1.3
p22	239	880	490,000	27.3	42.7	1.5
p23	182	646	4,955	20.6	32.4	0.6
p24	127	1,096	346,015	17	31.7	0.6
p25	87	360	207,200	11.4	41.8	1
p26	41	817	33,797	9.6	44.9	0.4
p27	15	193	73,810	2.3	15.2	N/D
p28	10	281	61,028	2.4	58.8	1
Controls						
c1	N/D	N/D	N/A	0	0	0
c2	N/D	N/D	N/A	0	0	0
c3	N/D	N/D	N/A	0	0	0
c4	N/D	N/D	N/A	0	0	0

[a]At the time of sampling
[b]Normal range >300 cells/mm^3
[c]Assayed using Chiron bDNA Test™
[d]N/A, Not Applicable
[e]N/D, Not Determined

Figure 5. Frequency of CD4+ and CD8+ T-cell HIV-1 infection in HIV-1 seropositive individuals. Each dot represents data from a study subject (see Table III). ***A,*** Flow cytometric results of the percentage of CD4+HIV-1_{gag}+ and CD8+HIV-1_{gag}+ T-cell populations from the HIV-1 seropositive individuals were plotted and graphed. Mean is indicated by horizontal line and values in parentheses. Significance was assayed by Student's *t*-test and statistical difference was accepted at $p < 0.01$ and denoted by an (*). **B,** Individual differences between CD8+ and CD4+ T-cell HIV-1 infection frequencies is illustrated by the horizontal line joining the two populations.

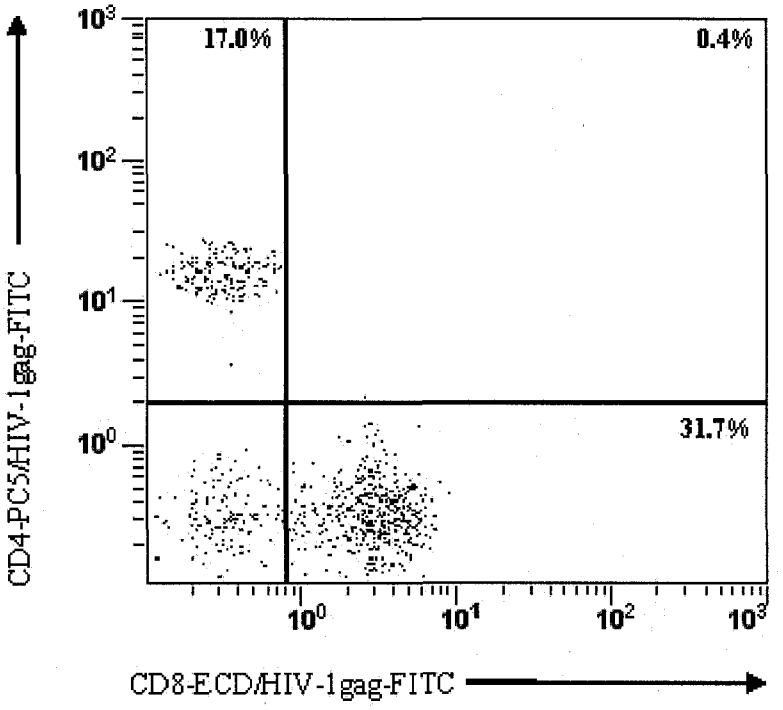

A
B
HIV-1gag Expression (%)
100
80
60
40
20
0
*
(26.4)
(36.9)
N = 28
*, p < 0.01, p = 0.0015
CD4+
T-Cells
CD8+
T-Cells

mind, the percentage of these DP cells in the study population and the amount that these DP cells contribute to the levels of CD8+ T-cell infection observed in the HIV-1 seropositive individuals was examined (Table III). Three-color flow cytometric analysis was performed on a number of study samples to determine the frequency of DP cells in HIV-1 seropositive individuals. Cells were gated on those that demonstrated HIV-1_{gag} expression, and therefore, only Gag-positive T-cells were examined for DP expression. Subsequently, cells were assessed for their CD8 and CD4 expression using fluorochrome-conjugated antibodies. Figure 6 illustrates the results from a representative analysis of a patient sample (p24). From the analyses, it was observed that the majority of the samples analyzed showed less than 1.5% of the cells that exhibited the $CD4^{dim}CD8^{bright}$ phenotype (Table III). The existence of DP cells that were HIV-1_{gag} negative was not found in the analyses of the HIV-1 seropositive individuals. None of the HIV-1 seronegative controls showed HIV-1_{gag} expression and hence, no DP cells were observed in this population. However, one of the controls (c2) did show minimal DP expression in their PBMC population, indicating the existence of these cells is not HIV-1 specific [343]. From these results, it can be inferred that the levels of CD8+HIV-1_{gag}+ T-cells that were observed in the study subjects was not due solely to the presence of HIV-1 infected $CD4^{dim}CD8^{bright}$ cells.

3.1.3. The Effects of CD4 Counts, Viral Loads and Treatment on the Frequency of CD8+HIV-1_{gag}+ T-Cells

In the patient studies, the percentage of CD8+HIV-1_{gag}+ T-cells circulating in the PBMC population of HIV-1 seropositive individuals was found to be significantly higher than that of CD4+HIV-1_{gag}+ cells ($p < 0.01$, Figure 5; Table III). The question of whether the clinical characteristics of the study subjects would have an effect upon CD8+ T-cell

Figure 6. Three-color flow cytometric analysis of CD4+HIV-1_{gag}+ and CD8+HIV-1_{gag}+ T-cell populations. Subsequent to fixation and permeabilization, PBMCs were stained with anti-CD4, -CD8 and -HIV-1_{gag} fluorochrome-conjugated antibodies. Results are representative of patient p24. Numbers in the quadrants represent the percentage of gated cells in each quadrant. A minimum of 10, 000 events were assessed for each analysis.

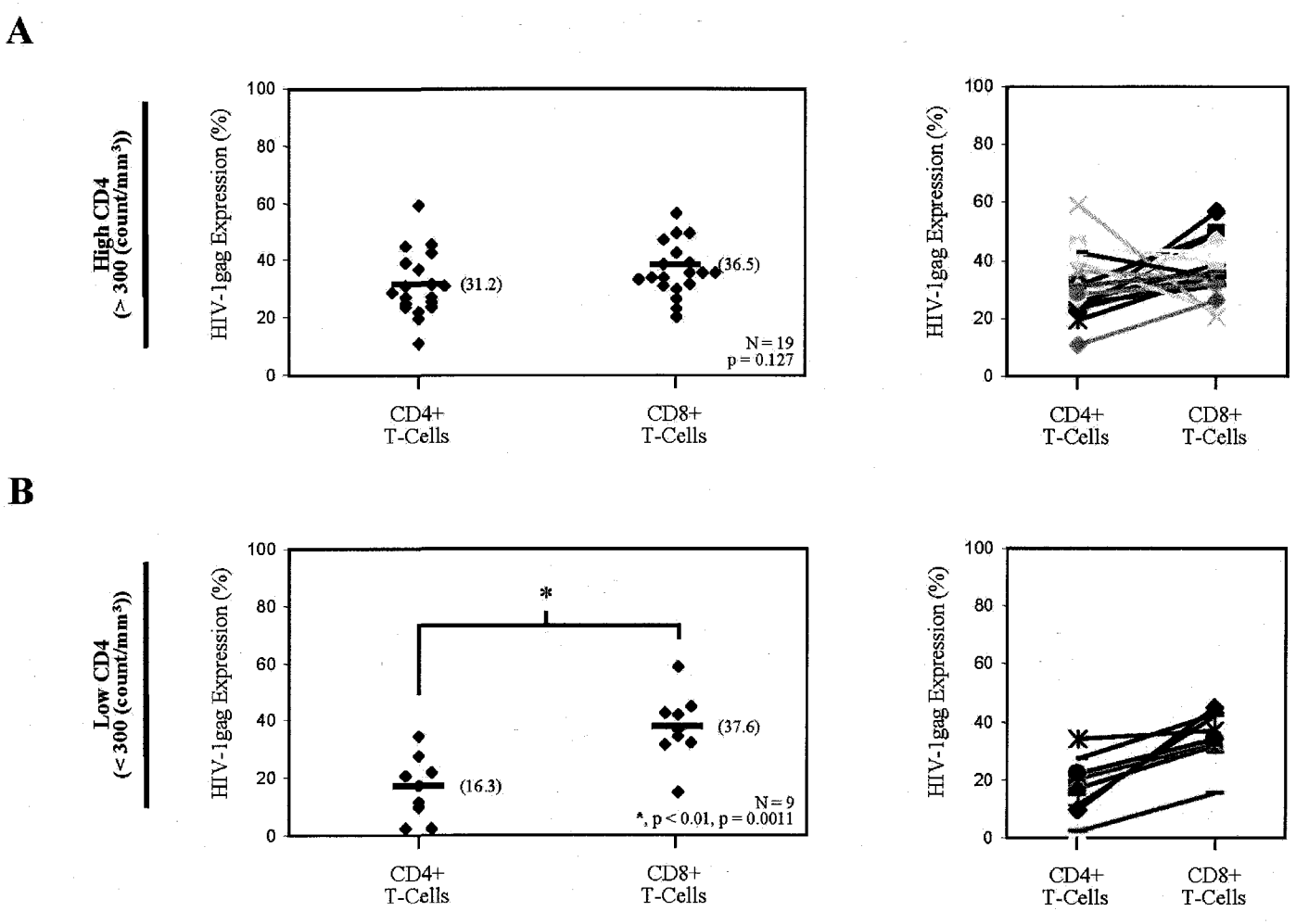

17.0%
0.4%
31.7%
10^3
10^2
10^1
10^0
10^0
10^1
10^2
10^3
CD4-PC5/HIV-1gag-FITC
CD8-ECD/HIV-1gag-FITC

infection frequency was addressed next. Subjects were selected for this study based upon their differing clinical status. In the analysis, HIV-1 seropositive individuals were divided based upon their CD4 counts, viral loads and whether they had received ART at the time of sampling.

Subjects were first divided based upon their CD4 counts into two groups: *a.* those with high CD4 counts (> 300 counts/mm^3; Figure 7A); and *b.* those with low (< 300 counts/mm^3; Figure 7B). Similar to the overall results (Figure 5), the percentage of CD8+HIV-1_{gag}+ expressing cells was higher than those that expressed CD4+HIV-1_{gag}+ in both populations. In the 19 patients that were classified as having high CD4 counts, only 7 study subjects demonstrated higher levels of CD4+ T-cell infection than CD8+ T-cells (Figure 7A, *right panel*). However, from the results shown in Figure 7A (*left panel*), the percentages of CD8+HIV-1_{gag}+ and CD4+HIV-1_{gag}+ cells were almost statistically equivalent (36.5% ± 9.4% vs. 31.2% ± 11.3% respectively). In the subjects classified as having low CD4 counts (n = 9), none of the subjects analyzed showed a higher degree of CD4+ T-cell infection than their CD8+ T-cell counterparts (Figure 7B, *right panel*). In fact, the percentage of CD8+HIV-1_{gag}+ T-cells was significantly higher than the percentage of CD4+HIV-1_{gag}+ T-cells found (Figure 7B, *left panel*; 37.6% ± 11.8% vs. 16.3% ± 10.9%, $p < 0.01$, Student's *t*-test, two-tailed).

Patients were also divided based upon their viral loads: *a.* those with high viral loads (> 50, 000 copies/mL); and *b.* those with low viral loads (< 50, 000 copies/mL) at the time of sampling. In both groups, the mean percentage of CD8+HIV-1_{gag}+ T-cells was higher than the CD4+HIV-1_{gag}+ T-cells (Figure 7C, D). These results showed statistical significance in the study subjects classified with high viral loads (Figure 7C, *left panel*; $p < 0.01$, Student's *t*-test, two-tailed). Even the patient (p1) with undetectable viral loads (< 50 copies/mL)

Figure 7. Frequency of CD4+ and CD8+ T-cell HIV-1 infection in HIV-1 seropositive individuals based upon their clinical profiles. Flow cytometric results of the percentage of CD4+HIV-1_{gag}+ and CD8+HIV-1_{gag}+ T-cell populations from the HIV-1 seropositive individuals were plotted and graphed (see Table I and II). Each dot represents data from a study subject. Mean is indicated by a horizontal line and values in parentheses. Significance was assayed by Student's *t*-test and statistical difference was accepted at $p < 0.01$. HIV-1 seropositive individuals were sub-divided based upon their clinical profiles. ***A,*** high CD4 counts vs. ***B,*** low CD4 counts; ***C,*** high viral load vs. ***D,*** low viral load; and those ***E,*** treatment naïve (or off treatment) vs. ***F,*** treatment experienced. *Right panels,* Individual differences between CD8+ and CD4+ T-cell HIV-1 infection frequencies is illustrated by the horizontal line joining the two populations.


A
High CD4
(> 300 (count/mm³))
HIV-1gag Expression (%)
100
80
60
40
20
0
(31.2)
(36.5)
N = 19
p = 0.127
CD4+
T-Cells
CD8+
T-Cells
HIV-1gag Expression (%)
CD4+
T-Cells
CD8+
T-Cells
B
Low CD4
(< 300 (count/mm³))
HIV-1gag Expression (%)
*
(16.3)
(37.6)
N = 9
*, p < 0.01, p = 0.0011
CD4+
T-Cells
CD8+
T-Cells
HIV-1gag Expression (%)
CD4+
T-Cells
CD8+
T-Cells

C
High Viral Load
(>50,000 (copies/mL))
HIV-1gag Expression (%)
100
80
60
40
20
0
(19.5)
(37.9)
*
N = 10
*, p < 0.01, p = 0.0031
CD4+
T-Cells
CD8+
T-Cells
D
Low Viral Load
(<50,000 (copies/mL))
HIV-1gag Expression (%)
(30.3)
(36.3)
N=18
p = 0.103
CD4+
T-Cells
CD8+
T-Cells

E
Treatment Naïve
(or Off Treatment)
HIV-1gag Expression (%)
100
80
60
40
20
0
(27.9)
(35.8)
N = 21
p = 0.026
CD4+
T-Cells
CD8+
T-Cells
F
Treatment
HIV-1gag Expression (%)
100
80
60
40
20
0
(22.0)
(40.1)
N=7
p = 0.028
CD4+
T-Cells
CD8+
T-Cells

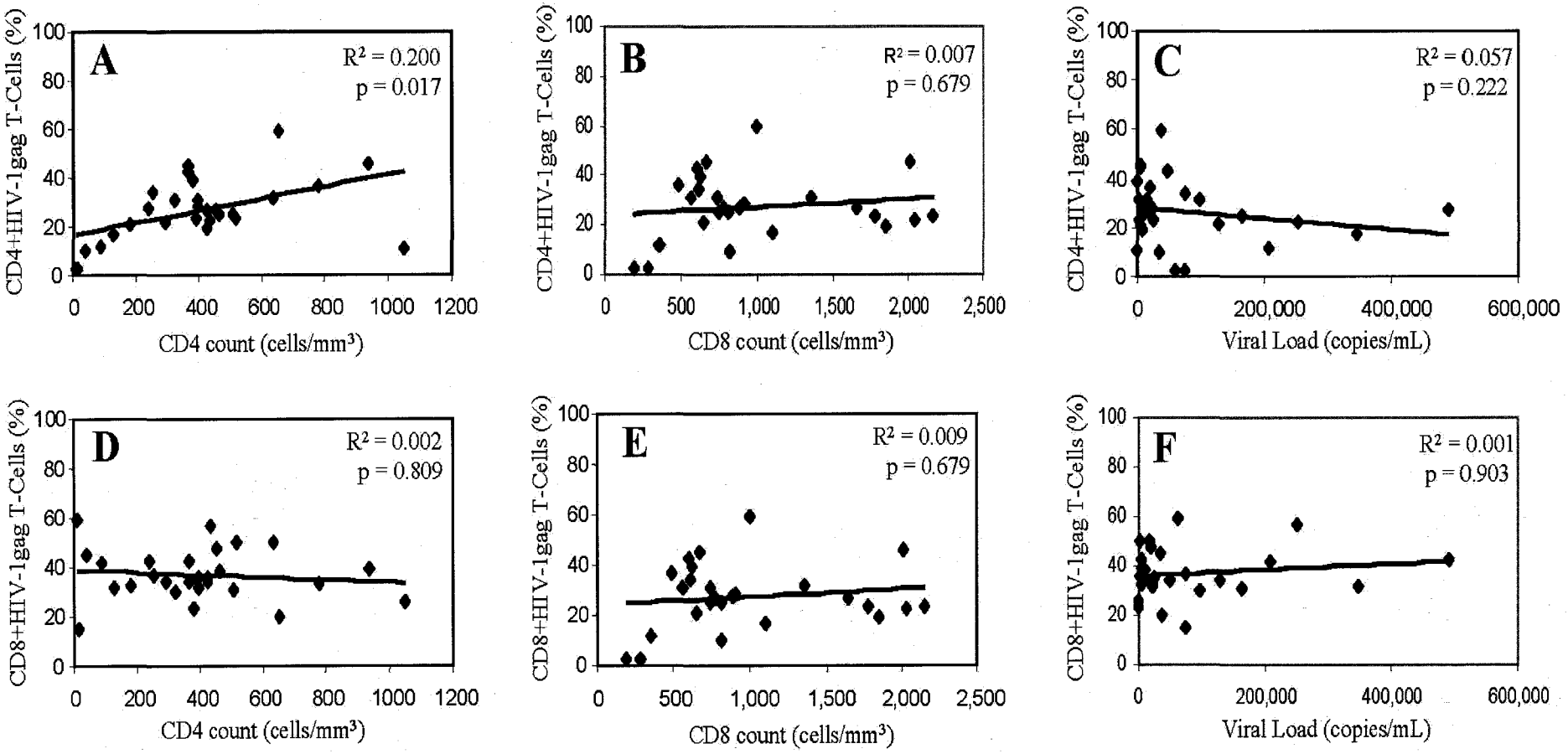
showed a higher frequency of HIV-1 infected CD8+ T-cells (26.2%) than infected CD4+ T-cells (10.7%).

Lastly, patients were classified based upon whether they had received HIV-1 therapy or not at the time of sampling. The majority of patients that volunteered for this study had not received treatment (n = 19) or had been off ART at the time of sampling (n = 2). Similar to the results found when patients were divided based upon their viral loads and CD4 counts, irrespective of whether or not the patient had received treatment, both groups exhibited a higher frequency of CD8+ T-cell infection (Figure 7E, F, *left panels*). However, statistical significance was not observed in both study populations. In patients receiving ART (Figure 7F, *right panel*), all of the subjects showed lower levels of CD4+ T-cell infection than CD8+ T-cell infection.

3.1.4. Correlation Between Disease Progression and CD8+ T-Cell Infection

The results clearly demonstrated that the levels of CD8+ T-cell infection were consistently elevated in comparison to the levels of CD4+ T-cell infection regardless of the clinical status of the study subjects (CD4 counts, viral loads, ART). It was hypothesized that the frequency of HIV-1 infected CD8+ T-cells would increase *in vivo* as the disease progresses (lower CD4 counts, higher viral loads) and as the primary targets for HIV-1, CD4+ T-cells, were depleted. The results from the flow cytometric analysis of the study subjects (the percentages of CD4+HIV-1_{gag}+ and CD8+HIV-1_{gag}+ expressing cells) were correlated and graphed with the correspondent CD4 counts, CD8 counts and viral loads (Table II). Figure 8A-F illustrates the results from the analysis. The only trend that was observed is shown in Figure 8A. This graph illustrates that the number of cells expressing the CD4+HIV-1_{gag}+ phenotype increases as the CD4 counts increase. However, this

Figure 8. Correlation between frequency of CD4+ and CD8+ T-cell HIV-1 infection and disease progression (CD4- , CD8- counts and viral loads). Each dot represents data from a study subject (see Table II). The solid line represents the line-of-best-fit for predicting correlation between the two variables ascertained in each plot. Correlation between CD4+HIV-1_{gag}+ T-cells in HIV-1 seropositive individuals and ***A,*** CD4 counts; ***B,*** CD8 counts; and ***C,*** viral load. Correlation between CD8+HIV-1_{gag}+ T-cells in HIV-1 seropositive individuals and ***D,*** CD4 counts; ***E,*** CD8 counts; and ***F,*** viral load. Significance was assayed by Spearman's rank correlation and statistical difference was accepted at $p < 0.01$. R^2, Spearman's rank correlation coefficient.

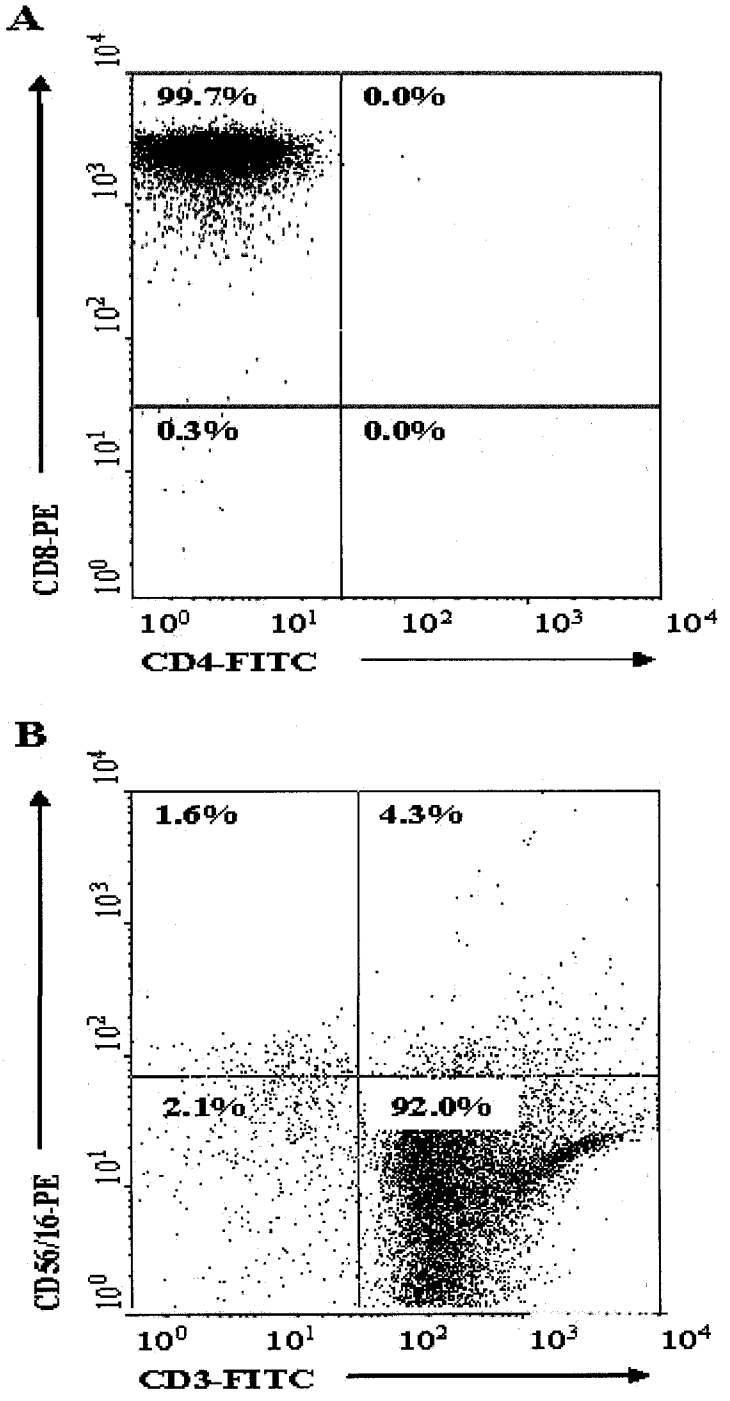

A
R² = 0.200
p = 0.017
CD4+HIV-1gag T-Cells (%)
CD4 count (cells/mm³)
B
R² = 0.007
p = 0.679
CD4+HIV-1gag T-Cells (%)
CD8 count (cells/mm³)
C
R² = 0.057
p = 0.222
CD4+HIV-1gag T-Cells (%)
Viral Load (copies/mL)
D
R² = 0.002
p = 0.809
CD8+HIV-1gag T-Cells (%)
CD4 count (cells/mm³)
E
R² = 0.009
p = 0.679
CD8+HIV-1gag T-Cells (%)
CD8 count (cells/mm³)
F
R² = 0.001
p = 0.903
CD8+HIV-1gag T-Cells (%)
Viral Load (copies/mL)

association did not show statistical significance ($p > 0.01$, Spearman's rank correlation). No correlation was observed between the frequency of circulating CD8+HIV-1_{gag}+ cells in the HIV-1 seropositive individuals and their clinical profiles (Figure 8D-F). Thus, it seems that the frequency of HIV-1 infected CD8+ T-cells in an individual is not dependent upon disease status.

3.1.5. Productive Infection of CD8+HIV-1_{gag}+ T-Cells *ex vivo*

An important question to address when examining the results is, are the CD8+HIV-1_{gag}+ T-cells productively infected *in vivo*? In other words, are these cells actively producing virus or do these cells serve as a latent reservoir? To address this question, CD4+, CD8+ and unfractionated PBMC populations from 5 HIV-1 seropositive individuals and one HIV-1 seronegative individual were isolated. It was hypothesized that due to the high frequencies of CD8+HIV-1_{gag}+ cells observed in the study populations, that these cells would be able to support productive HIV-1 replication. Cell isolation was performed using magnetically conjugated antibodies and cell purity was confirmed by flow cytometry. Analysis of the magnetically separated CD8+ T-cells demonstrated the substantial purity of the cell population, as greater than 99% of the cells expressed the CD8 cell-surface molecule (Figure 9A, upper left quadrant). Of greater concern was the presence of contaminating CD4+ T-cells in the selected CD8+ population. Analysis of CD8+ T-cell purity also revealed that the number of contaminating CD4+ T-cells was negligible in the total cell population (Figure 9A, lower right quadrant). The proportion of Natural Killer (NK) cells present in the CD8+ T-cell population was also examined by the detection of CD56+/CD16+ cells in the CD8+CD3+ T-cell population. As examined by flow cytometry, the proportion of contaminating NK cells in the population was less than 1.5% (Figure 9B).

Figure 9. Purity of selected CD8+ T-cell populations. ***A,*** Positively selected CD8+ T-cells were stained with the fluorochrome-conjugated antibodies CD8-PE and CD4-FITC and examined for purity by flow cytometry. ***B,*** CD8+ T-cells were stained with CD3-FITC and CD56/16-PE to examine the NK cell proportion of the CD8+ T-cell population. Numbers in the quadrants represent the percentage of gated cells in each quadrant. A minimum of 10, 000 events was assessed for each analysis.

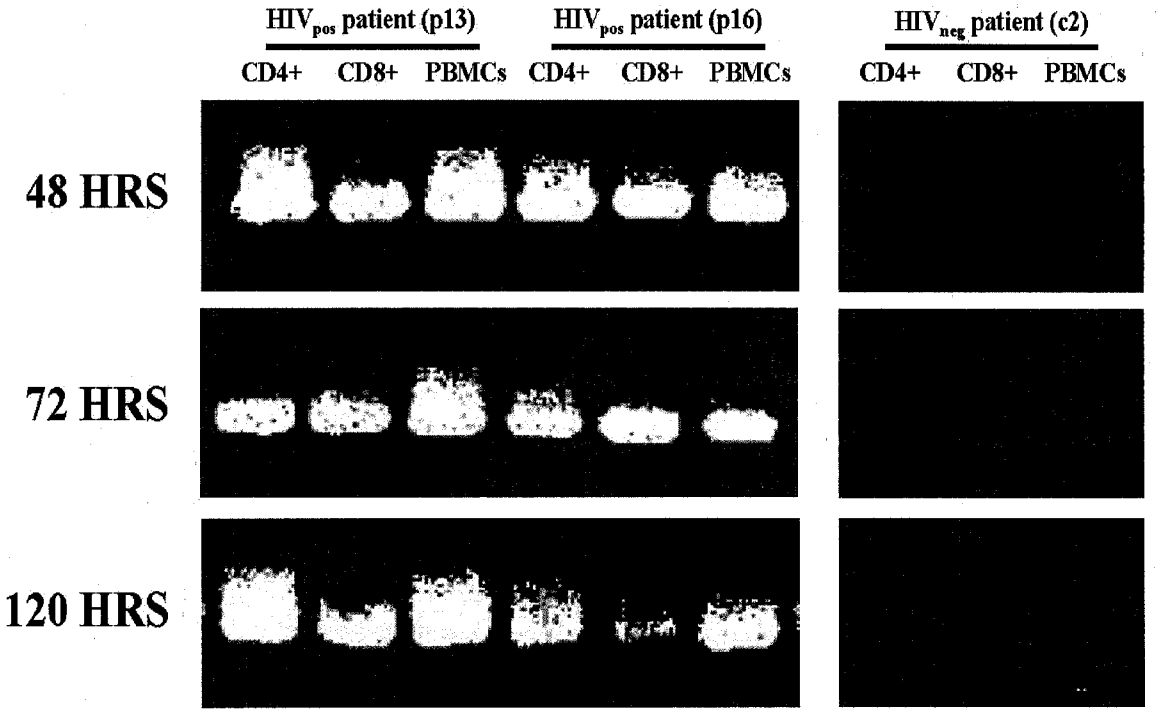

A
99.7%
0.0%
0.3%
0.0%
CD8-PE
CD4-FITC
B
1.6%
4.3%
2.1%
92.0%
CD56/16-PE
CD3-FITC

Isolated T-cells and unfractionated PBMC populations were cultured and cell-free supernatants were collected at 48, 72 and 120 hour time points. Viral RNA was isolated from the cell-free supernatants, subjected to nested RT-PCR amplification and assayed for the HIV-1 polymerase gene (HIV-*pol*). Representative results from two HIV-1 seropositive individuals (p13 and p16) and one HIV-1 seronegative control (c2) are shown in Figure 10. RT-PCR analysis shows the presence of a HIV-*pol* specific band in the supernatant for both isolated CD4+ and CD8+ T-cell populations and unfractionated PBMCs for the HIV-1 seropositive study subjects over the 120 hour time course. No HIV-*pol* specific band was found in the supernatants of the control subject. Though the results were not quantitative, one can infer from Figure 10, that the bands seen in the CD8+ T-cell lanes for patients p15 and p16 were of less intensity than those found in the corresponding CD4+ T-cell and unfractionated PBMC lanes. The intensity of the CD8+ T-cell specific HIV-*pol* band also diminished over the 120 hour culture period. The band observed at the 120 hour time point in the CD8+ T-cell lane of p16 shows an almost undetectable signal. DNA contamination in the cell-free supernatant samples was verified by PCR analysis. No HIV-*pol* specific DNA band was observed in the samples by PCR analysis, thus eliminating virally infected cells as a source of contamination in our analyses of virus present in the supernatants.

3.1.6. Contribution of CD8+HIV-1_{gag}+ T-Cells to the HIV-1 Viral Load *ex vivo*

In order to further address the question of productive HIV-1 infection of CD8+ T-cells from HIV-1 seropositive individuals, cell-free supernatants were collected from CD4+ and CD8+ T-cell populations as above. HIV-1 RNA in cells suggests productive infection, but does not prove that infectious virus is released. Thus, viral RNA was subsequently isolated from the supernatant samples and HIV-1 RNA levels were quantified

Figure 10. Productive HIV-1 infection of CD8+ T-cells *ex vivo*. Isolated CD4+, CD8+ T-cells and unfractionated PBMC populations were cultured and cell-free supernatants were collected at 48, 72 and 120 hours. Productive infection of the lymphocyte populations was assayed by RT-PCR analysis and denoted by the presence of a HIV-1 polymerase specific band. Illustrated are results from two HIV-1 seropositive individuals (p13 and p16) and one healthy control (c2).


HIV$_{pos}$ patient (p13)
HIV$_{pos}$ patient (p16)
HIV$_{neg}$ patient (c2)
CD4+ CD8+ PBMCs CD4+ CD8+ PBMCs CD4+ CD8+ PBMCs
48 HRS
72 HRS
120 HRS

from cell-free supernatants using the COBAS AMPLICOR HIV-1 Monitor Test as per the manufacturer's protocol. Results are summarized in Table IV and Figure 11. Values obtained were normalized to T-cell numbers in culture at the time of sampling. Based upon the lower intensity bands shown in the CD8+ populations in Figure 10, CD8+ T-cells were expected to be low-level producers of HIV-1 *ex vivo*. As expected, isolation and quantitation of HIV-1 RNA from both CD4+ and CD8+ T-cell populations of the HIV-1 seropositive study subjects that were sampled was successful (Table IV). However, in all 5 patients that were assayed, viral production from the CD8+ T-cells was 2-3 logs lower than that of their correspondent CD4+ T-cell population (Figure 11). In fact, the levels of HIV-1 RNA produced by the isolated CD8+ T-cells in all but one of the study subjects examined exhibited a decrease in viral production over a 120 hour time course, corroborating the HIV-*pol* RT-PCR results found in Figure 10. Only patient p25 showed an increase in HIV-1 RNA production in their CD8+ T-cell population from 2.1×10^3 copies/mL at 48 hours to 4.5×10^3 copies/mL at 120 hours. This patient had the highest reported viral load at the time of sampling and the lowest CD4 count of the study subjects that we analyzed. Conversely, 3 of the 5 patients showed an increasing trend of HIV-1 RNA production in their isolated CD4+ T-cell populations. No HIV-1 transcripts were found in the supernatant of the control subject (data not shown). A value for the 48 hour time point of p16 was not recorded due to an error in the sample analysis. No obvious trend was observed between the levels of CD4+ T-cell or CD8+ T-cell HIV-1 RNA production and the clinical characteristics of the patients. Thus, CD8+HIV-1_{gag}+ T-cells are productively infected *ex vivo* and can actively replicate HIV-1, albeit at very low levels.

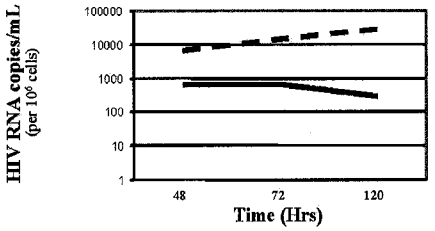
Table IV. Productive HIV-1 Infection of CD8+ T-Cells

Patient #	*Absolute (count/mm^3) [a,b]*		*Viral Load, RNA [a,c]*	*HIV-1 $_{gag}$-expressing Cells (%)*		*HIV-1 RNA (copies/mL) (x 10^3)*	
	CD4	*CD8*	*(copies/mL)*	*CD4*	*CD8*	*CD4*	*CD8*
p3	779	484	21,135	36.5	33.6	6.7 (48 hrs) 14.3 (72 hrs) 322.9 (120 hrs)	0.6 (48 hrs) 0.6 (72 hrs) 0.3 (120 hrs)
p13	396	907	22,821	28.5	31.7	409.1 (48 hrs) 476.3 (72 hrs) 1150.0 (120 hrs)	3.3 (48 hrs) 1.7 (72 hrs) 1.6 (120 hrs)
p16	379	620	580	39.1	23	94.9 (48 hrs) 175.0 (72 hrs) 60.1 (120 hrs)	B/D[d] (48 hrs) 2.1 (72 hrs) 1.1 (120 hrs)
p21	254	608	73,770	34.2	37	64.3 (48 hrs) 208.9 (72 hrs) 45.7 (120 hrs)	0.5 (48 hrs) 0.4 (72 hrs) 0.3 (120 hrs)
p25	87	360	207,200	11.4	41.8	264.4 (48 hrs) 210.7 (72 hrs) 322.9 (120 hrs)	2.1 (48 hrs) 3.9 (72 hrs) 4.5 (120 hrs)
Controls							
c2	N/D	N/D	N/A	1.2	0.7	B/D (48 hrs) B/D (72 hrs) B/D (120 hrs)	B/D (48 hrs) B/D (72 hrs) B/D (120 hrs)

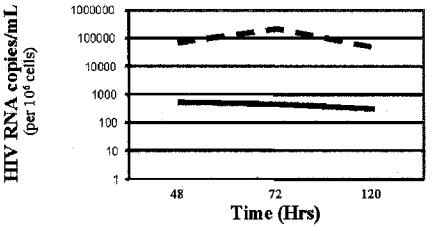
[a]At the time of sampling
[b]Normal range >300 cells/mm^3
[c]Assayed using Chiron bDNA Test™
[d]B/D, below detection

Figure 11. Contribution of HIV-1 infected CD8+ T-cells to the viral load. Cell-free supernatants were isolated at 48, 72 and 120 hours from the CD4+ and CD8+ T-cell populations of the indicated HIV-1 seropositive individuals. Viral RNA was quantified using the COBAS AMPLICOR HIV-1 Monitor Test. Quantification of HIV-1 RNA isolated from the CD4+ T-cell population (*dashed line*) and CD8+ T-cell population (*solid line*) are shown (also see Table III). Results were normalized to the number of cells present at the time of sampling in each individual T-cell population.

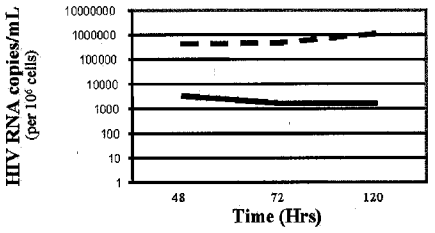
p3



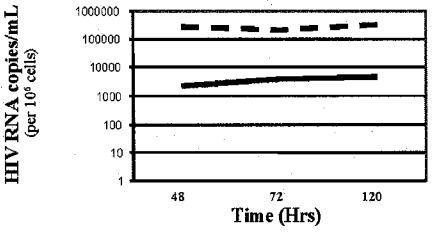
p21



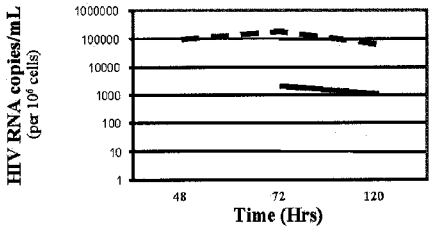
p13



p25



p16

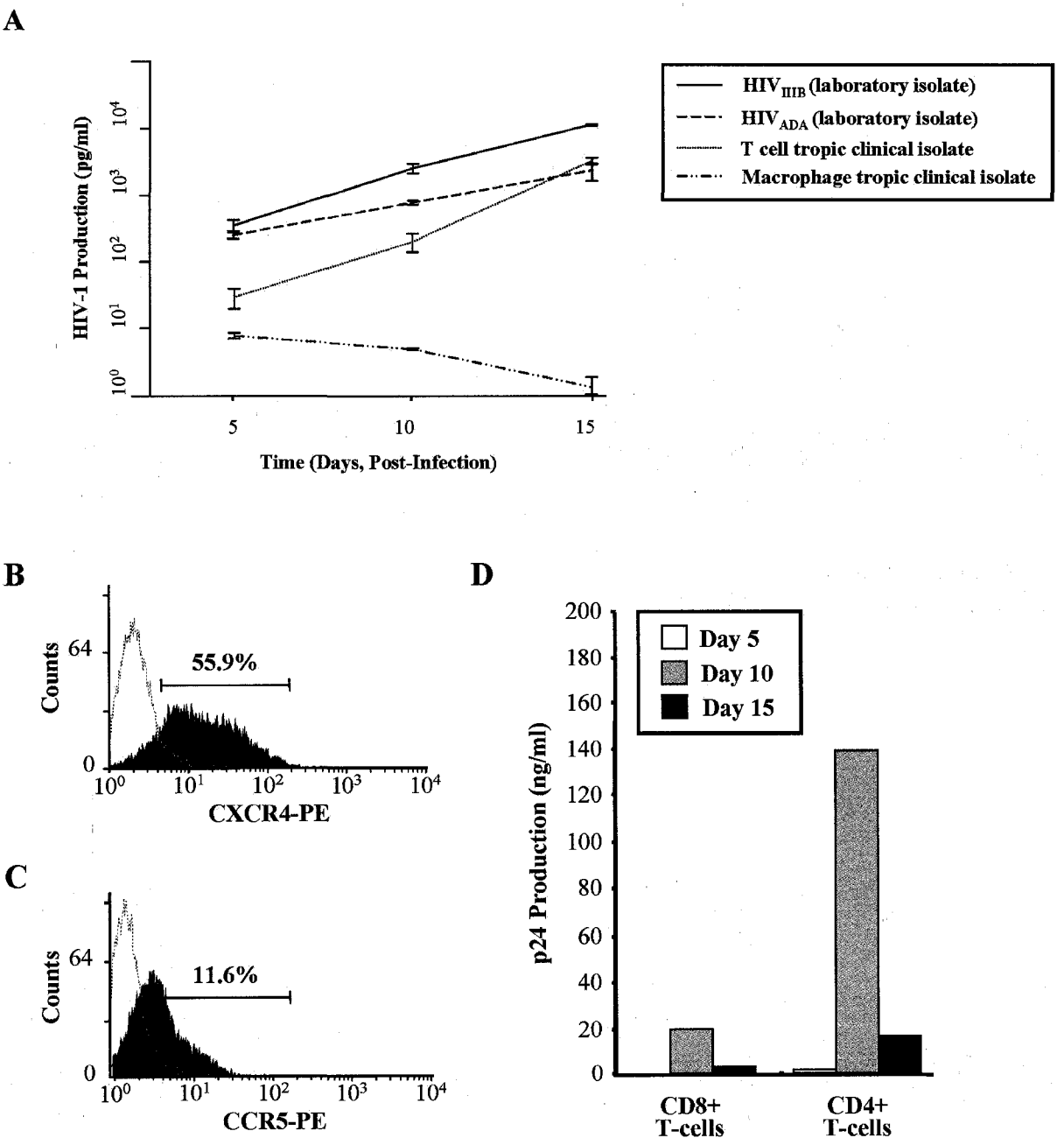


3.2. *in vitro* HIV-1 INFECTION OF CD8+ T-CELLS: SUSCEPTIBILITY AND EFFECTS ON CD8+ T-CELL FUNCTIONS

3.2.1 Susceptibility of Primary Blood-Derived CD8+ T-Cells to HIV-1 Infection *in vitro*

CD8+ T-cells were separated from PBMCs of healthy individuals by positive selection through the use of magnetic beads and selection purity was assessed by flow cytometry similar to the studies undertaken in Section 3.1.5. After establishing the purity of the selected CD8+ T-cell population and demonstrating the absence of any contaminating CD4+ T-cells (Figure 9), the ability of CD8+ T-cells to support HIV-1 infection was ascertained. Unless otherwise noted, primary CD8+ T-cells were infected immediately after isolation as described in the Material and Methods. *In vitro* infection of primary CD8+ T-cells was performed with T-cell tropic (also termed X4-tropic due to its co-receptor usage) and macrophage tropic (also termed R5-tropic) strains of HIV-1, along with Non-Syncytia Inducing (NSI) and Syncytia Inducing (SI) clinical isolates of the virus. The CD8+ T-cells were infected with an equal amount of virus, allowing the infection sensitivity of the CD8+ T-cells for the viral isolates and strains to be compared. Figure 12A shows a 15 day time course of virus replication of the two laboratory strains and the two clinical isolates of HIV-1 in positively selected CD8+ T-cells. As measured by a HIV-1 p24 ELISA, the T-cell tropic strain, HIV_{IIIB}, maintained the highest levels of viral production in the CD8+ T-cell population. The levels of virus replication for the HIV_{IIIB} strain reached a peak at day 15 post-infection (Figure 12A). The replication of the T-cell tropic clinical isolate was also supported in CD8+ T-cells, although to lower levels than the laboratory strain. Similarly, CD8+ T-cells also supported moderate levels of replication of the macrophage tropic laboratory strain, HIV_{ADA}, but replication of the macrophage tropic clinical isolate was poor.

Figure 12. Infection of CD8+ T-cells by HIV-1. ***A,*** Selected CD8+ T-cells were infected with 300 $TCID_{50}$/mL of the T-cell tropic and macrophage tropic laboratory strains and clinical isolates of HIV-1. HIV-1 p24 production was measured by an ELISA at the indicated time points with cell-free supernatants from cultures. Results show the mean HIV-1 production and SEM of three independent experiments. ***B,*** CXCR4 receptor expression on CD8+ T-cells as measured by flow cytometry. One representative of three experiments is shown. The mean fluorescence of CXCR4 receptor and SEM of three independent experiments is 55.9% ± 2.1. ***C,*** CCR5 receptor expression on CD8+ T-cells. One representative of three experiments is shown. The mean fluorescence of CCR5 receptor and SEM of three independent experiments is 11.6 ± 1.0. CD8+ T-cells were also stained with negative controls (isotype control, dashed line). ***D,*** Positively selected CD4+ and CD8+ T-cells were infected with 300 $TCID_{50}$/mL of HIV_{IIIB}. HIV-1 production was measured by an ELISA at the indicated time points from cell-free supernatants.


A
HIV-1 Production (pg/ml)
10^0
10^1
10^2
10^3
10^4
5
10
15
Time (Days, Post-Infection)
HIV_{IIIB} (laboratory isolate)
HIV_{ADA} (laboratory isolate)
T cell tropic clinical isolate
Macrophage tropic clinical isolate
B
Counts
64
0
55.9%
10^0
10^1
10^2
10^3
10^4
CXCR4-PE
C
Counts
64
0
11.6%
10^0
10^1
10^2
10^3
10^4
CCR5-PE
D
p24 Production (ng/ml)
200
180
160
140
120
100
80
60
40
20
0
Day 5
Day 10
Day 15
CD8+ T-cells
CD4+ T-cells

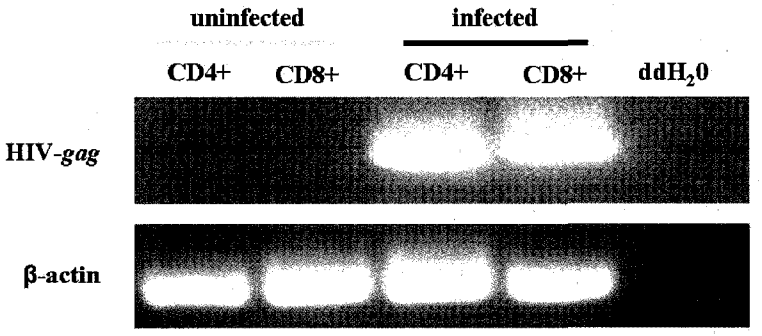
In contrast to the viral kinetics of the T-cell and macrophage tropic laboratory strains and the T-cell tropic clinical isolate, the levels of HIV-1 production for the macrophage tropic clinical isolate declined over the 15 day time course. Input virus was not the cause of the low levels of viral production observed by this clinical isolate as CD8+ T-cells were extensively washed 3 days post-infection and day 0 of the infection assay was denoted at this point. Based on the above results, the HIV_{IIIB} strain was used in subsequent experiments due to the ability of CD8+ T-cells to support high levels of replication of this virus.

Most HIV-1 strains require CXCR4 or CCR5 as a co-receptor for viral entry, along with CD4 expression. These viral strains have been classified into distinct phenotypes: SI or NSI based on their ability to use the CXCR4 or CCR5 co-receptors respectively [97]. The surface expression of the chemokine receptors on primary CD8+ T-cells before infection was examined by flow cytometry. It was observed that the CXCR4 chemokine receptor was significantly expressed on the surface of the CD8+ T-cells (55.9 % ± 2.1, Figure 12B). The expression of the CCR5 chemokine receptor was only moderately expressed on the CD8+ T-cell-surface (11.6% ± 1.0, Figure 12C). Comparison of viral replication kinetics was assessed between CD8+ and CD4+ T-cells over a 15 day time course (Figure 12D). CD8+ T-cells were found to support approximately 10-fold lower levels of HIV-1 replication than CD4+ T-cells *in vitro*.

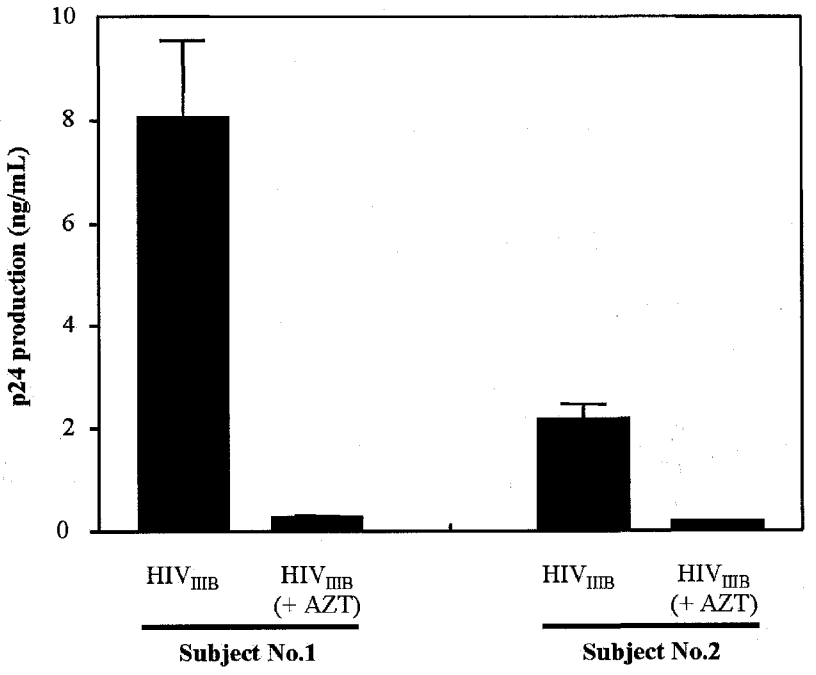
Productive infection was demonstrated by RT-PCR analysis. Only HIV-1 infected CD8+ T-cells, and CD4+ T-cells, showed that viral infection was productive as both T-cell populations showed a HIV-1 *gag*-specific band when RT-PCR analysis was performed (Figure 13A). Productive infection was also demonstrated in Figure 13B where the presence of AZT abrogated HIV-1 replication in CD8+ T-cells. Differences in the levels of viral production were also observed between the two subjects. Subject 2 supported lower levels of

Figure 13. Productive HIV-1 infection of CD8+ T-cells *in vitro*. ***A,*** RT-PCR analysis of HIV-*gag* transcripts in the uninfected and infected populations of isolated CD4+ and CD8+ T-cells. Isolated lymphocyte populations were left uninfected or infected with 300 $TCID_{50}$/mL of HIV_{IIIB} for 7 days prior to RT-PCR analysis. Detection of β-actin was run as a loading control. Parallel samples were run without reverse transcriptase as a control for DNA contamination of the samples (data not shown). ***B,*** Infection of CD8+ T-cells is sensitive to AZT. Infection was performed in the absence and presence of AZT and HIV-1 production was measured by an ELISA for the HIV-1 gag protein, p24. Cells were treated with 10 μM of AZT for one hour prior to infection. CD8+ T-cells from two uninfected individuals were infected with 300 $TCID_{50}$/mL of HIV_{IIIB} laboratory strain. The results shown are the mean and SEM of three independent experiments.

A


uninfected
infected
CD4+
CD8+
CD4+
CD8+
ddH20
HIV-gag
β-actin

B


10
8
6
4
2
0
p24 production (ng/mL)
HIVIIIB
HIVIIIB (+ AZT)
HIVIIIB
HIVIIIB (+ AZT)
Subject No.1
Subject No.2

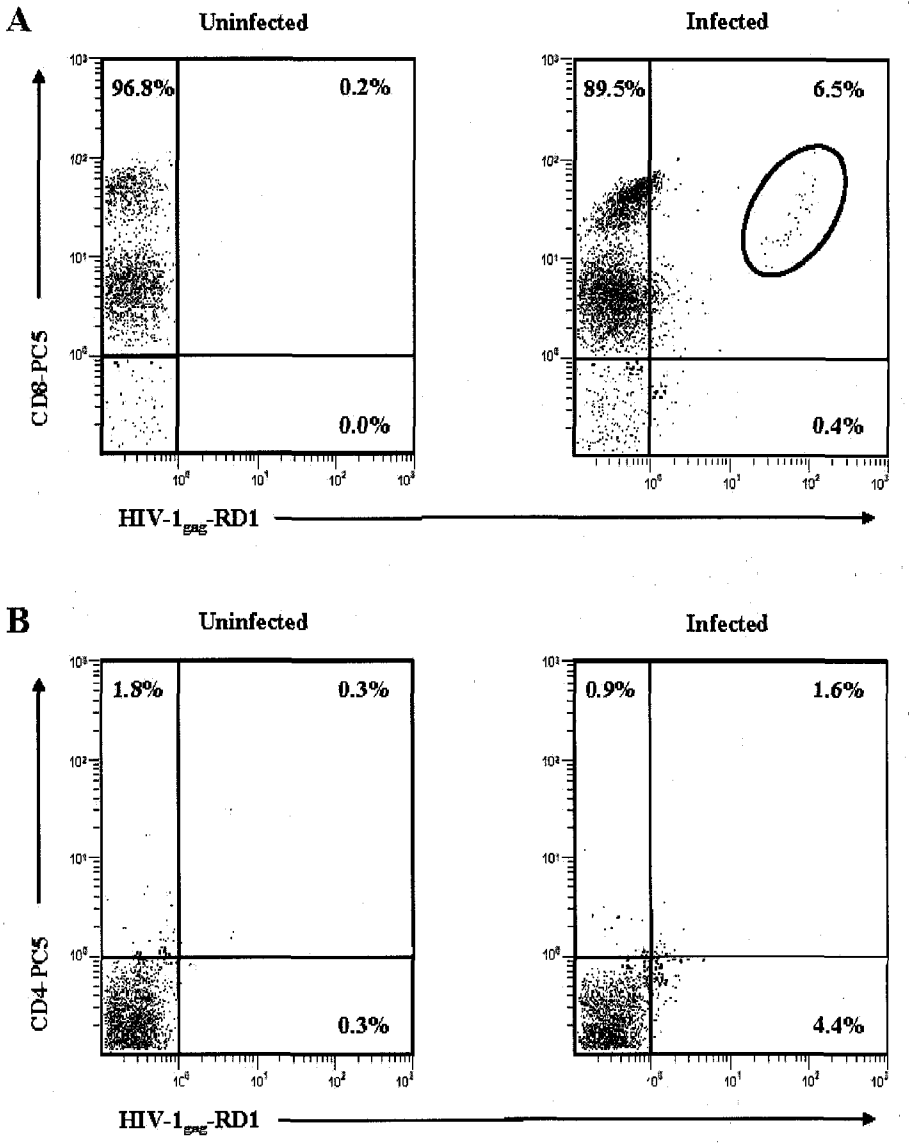
HIV-1 production in their isolated CD8+ T-cells in the absence of AZT indicating the differences in viral replication that are supported by CD8+ T-cells from various individuals. Furthermore, productive HIV-1 infection was also illustrated by *in situ* RT-PCR analysis of *in vitro* HIV-1 infected PBMCs (Figure 14). A novel method for determining the histological localization of low-copy DNA/RNA targets has been the development of the *in situ* amplification process [344]. The use of this technique has allowed researchers to combine the sensitivity of PCR with the specificity of *in situ* hybridization. Recent applications of this technique have illustrated its use in assaying HIV-1 infection. Its use included the detection of HIV-1 transcripts at low-copy numbers in target cells and in the identification of novel cellular reservoirs of the virus [345, 346]. This application was used to determine the presence of infected CD8+ T-cells that may only be detected at very low levels or persist as a latent infection, in the *in vitro* HIV-1 infected PBMC population (Figure 14). In order to minimize any false-positives or non-specific results, cells were subjected to RNase-free DNase I digestion prior to amplification. Though the results are not quantitative, the presence of cells that were positive for both CD8 (as denoted by the CD8-Texas Red stain) and HIV-1 *gag* specific transcripts (for a better illustration of this observation, see Figure 14, *right panels*) were observed. These results indicate that productively HIV-1 infected CD8+ T-cells can be found in the *in vitro* infected PBMC population.

To further determine whether CD8+ T-cells could support HIV-1 infection, flow cytometry was performed to detect the presence of HIV-1 p24 intracellularly in this population. As shown in Figure 15A (circle, upper right quadrant), 6.5% of the CD8+ T-cells were infected intracellularly with HIV-1. As a control for the contribution of CD8+CD4+ cells to the 6.5% of total infected cells, the isolated and infected CD8+ T-cells were stained additionally with a CD4 antibody and intracellular HIV-1 expression was again

Figure 14. *in situ* RT-PCR analysis of HIV-1 infected PBMCs. PBMCs were left uninfected or infected for 7 days with the HIV_{IIIB} laboratory viral strain. Cells were subsequently fixed and permeabilized with 10% formal saline and 1% Triton X-100 respectively. RT-PCR analysis was performed on cDNA samples of the uninfected and infected PBMCs. Briefly, samples were loaded and RT-PCR conditions were: 1. 95°C for 2 minutes; 2. 30 cycles of *(a)* 94°C for 1 minute, *(b)* 55°C for 1 minute, *(c)* 72°C for 1 minute; 3. final extension for 7 minutes at 72°C using FITC-conjugated HIV-*gag* primers (forward: 5'-/56-FAM/ATAGAGGAAGAGCAAAACAAAA-3'; reverse: 5'-/56-FAM/TTCCTGAAGGGTACTAGTAGT-3'). Cells were adhered to microscope coverslides and stained with the DAPI and CD8-Texas Red/ECD conjugated antibodies prior to fluorescence microscopy analysis. *Right panel,* Enlargement of cells indicated by arrows indicating the presence of HIV-1 infected CD8+ T-cells in the PBMC population

infected
DAPI
CD8-TexasRed
HIVgag-FITC
Merge
DAPI
CD8-TexasRed
HIVgag-FITC
Merge
uninfected
infected

Figure 15. Detection of intracellular HIV-1 expression in CD8+ T-cells. CD8+ T-cells were infected with 300 $TCID_{50}$/mL of HIV_{IIIB} or left uninfected. ***A,*** Intracellular p24 was examined on the CD8+ T-cell population by flow cytometry at day 15 post-infection with antibodies to HIV-1_{gag} (p24-RD1) and CD8. In the infected population, the number in the upper right quadrant represents the number of gated cells in the circle as to minimize any false-positives. ***B,*** To determine the contribution of CD4+ cells to *in vitro* CD8+ T-cell infection, intracellular HIV-1 expression was examined in the CD8+ T-cell population by flow cytometry at day 15 post-infection with antibodies to HIV-1_{gag} and CD4. Numbers in the quadrants represent the percentage of gated cells in each quadrant. A minimum of 10, 000 events was assessed for each analysis.

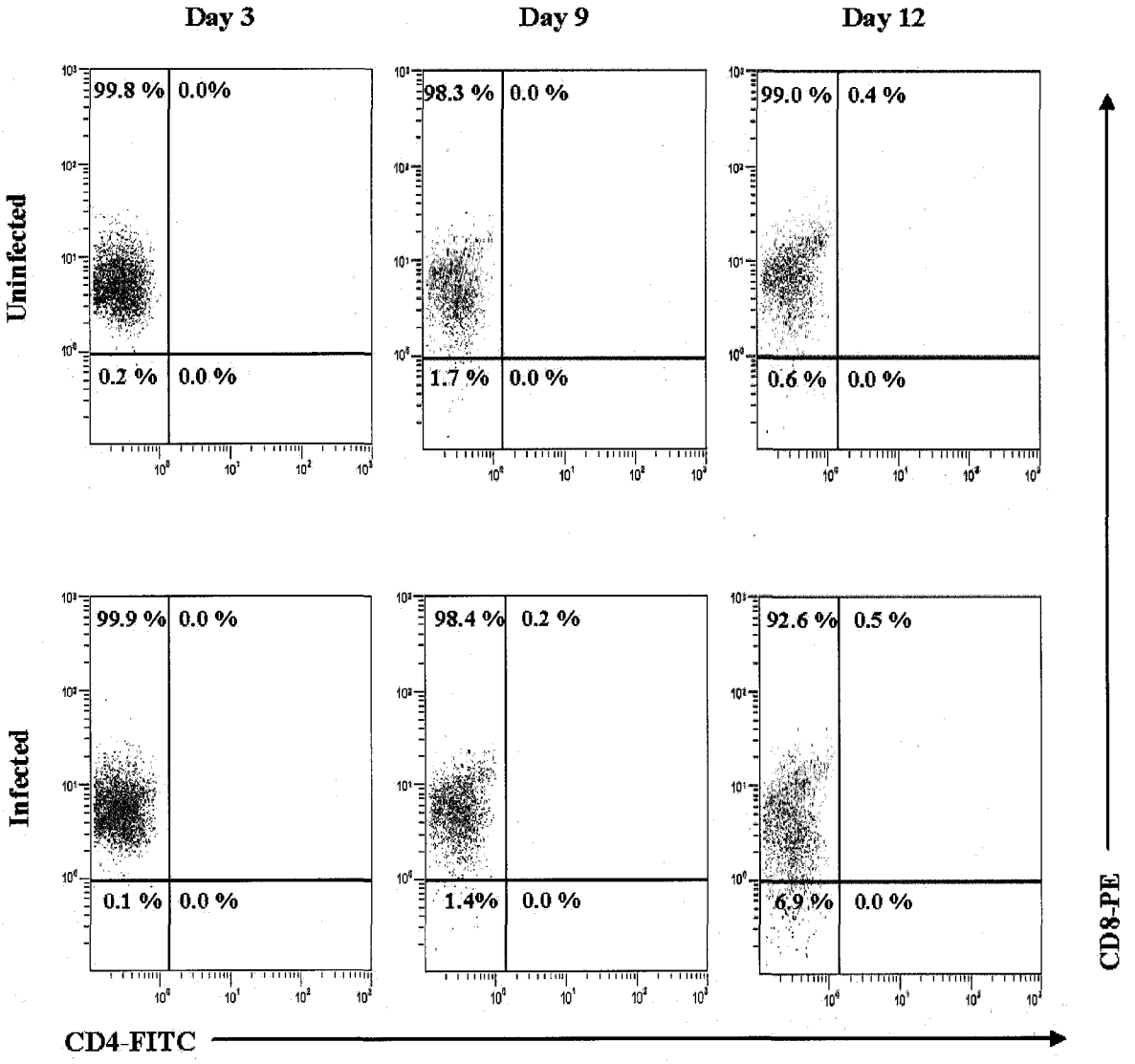

A
Uninfected
Infected
96.8%
0.2%
0.0%
89.5%
6.5%
0.4%
CD8-PC5
HIV-1gag-RD1
B
Uninfected
Infected
1.8%
0.3%
0.3%
0.9%
1.6%
4.4%
CD4-PC5
HIV-1gag-RD1

measured by flow cytometry (Figure 15B). From the results, approximately 1.6% of the CD8+CD4+ cells contributed to the total levels of the CD8+ T-cell infection obtained in Figure 15A. Two distinct populations of CD8+ T-cells were observed in the flow cytometric analysis based upon the expression levels of the CD8 molecule. These populations, that have been observed by others, differ in their surface expression of the CD8 cell-surface molecule and have been aptly termed $CD8^{high}$ and $CD8^{low}$ expressing populations [347, 348]. A similar experiment illustrated that approximately 20 - 25% of CD4+ T-cells stained intracellularly for HIV-1 (data not shown).

3.2.2. Expression of the CD4 Cell-Surface Molecule on the Surface of CD8+ T-Cells During Infection

It has recently been suggested that HIV-1 enters CD8+ T-cells through the up-regulation of the CD4 cell-surface molecule on the surface of these cells during the course of infection [124]. To examine the possibility of CD4 up-regulation in the *in vitro* infection system used throughout the studies, CD8+ T-cells were infected and monitored for CD4 expression by flow cytometry over a 12 day time course (Figure 16). Interpretation of the flow cytometry data showed very distinct populations. Analysis of both the uninfected and infected CD8+ T-cell populations showed that CD4 expression on these cells reached a maximum of 0.4% and 0.5%, respectively, on day 12 of culture. This suggests that cell culture conditions, not HIV-1 infection *per se*, resulted in this observed minimal up-regulation of CD4 on the surface of CD8+ T-cells. However, the overall majority of CD8+ T-cells were of the single-positive phenotype and the contribution of DP cells was negligible.

Figure 16. CD4 cell-surface molecule up-regulation on the surface of CD8+ T-cells. Uninfected and HIV-1 infected CD8+ T-cells were examined at the indicated time points by flow cytometry for the expression of the CD8 and CD4 cell-surface molecules. CD8+ T-cells were infected with 300 $TCID_{50}$/mL of HIV_{IIIB} and cell-surface expression of CD8 and CD4 was assessed at days 3, 9 and 12 by flow cytometry. Numbers in the quadrants represent the percentage of gated cells in each quadrant. A minimum of 10, 000 events were assessed for each analysis.


Day 3
Day 9
Day 12
Uninfected
Infected
99.8 %
0.0%
0.2 %
0.0 %
98.3 %
0.0 %
1.7 %
0.0 %
99.0 %
0.4 %
0.6 %
0.0 %
99.9 %
0.0 %
0.1 %
0.0 %
98.4 %
0.2 %
1.4%
0.0 %
92.6 %
0.5 %
6.9 %
0.0 %
CD4-FITC
CD8-PE

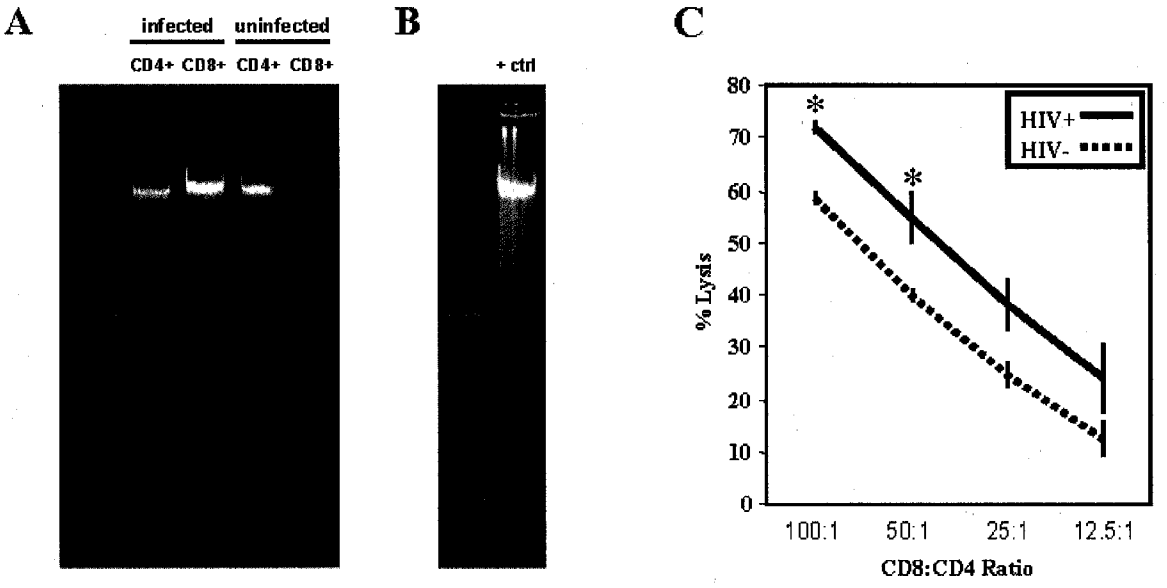
3.2.3. The Effect of HIV-1 Infection on CD8+ T-Cell Viability and Function

The failure of CD8+ T-cells to control viral replication during HIV-1 pathogenesis suggests that CD8+ CTLs are impaired both in lysing HIV-1 infected cells and suppressing viral replication. This failure may arise from the resistance of HIV-1 infected targets to CTL recognition [131, 160, 349], from a lack of effector function by the CTLs themselves [224] or to the direct cytopathic effects of the virus itself [183, 185, 186, 350-352].

Over the past few years, it has been documented that HIV-1 infection results in the induction of the apoptosis signaling cascade in CD8+ T-cells through various mechanisms [89, 92, 103, 198, 199, 353-355]. Apoptosis induction results in activation of the caspase cascade and ends with the DNA fragmentation and degradation of the cell [356]. Therefore, the ability of HIV-1 to induce the cell death of CD8+ T-cells was examined by analyzing DNA fragmentation in the infected T-cell population. It was observed that *in vitro* HIV-1 infected CD4+ and CD8+ T-cells showed a laddering/smear that is characteristic of DNA degradation compared to their uninfected counterparts when their DNA was isolated and electrophoresed (Figure 17A). As a positive control, Jurkat T-cells were treated with CPT, a known inducer of apoptosis [357]. CPT-treatment of Jurkat cells resulted in the same DNA fragmentation pattern of HIV-1 infected CD4+ and CD8+ T-cells (Figure 17B), indicating the cytopathic effects of HIV-1 infection in CD8+ T-cells.

The CTL function of CD8+ T-cells was also assessed in the face of HIV-1 infection by a MLR. A two-way heterologous MLR was performed in order to assess the cytotoxic killing ability of HIV-1 infected CD8+ T-cells (Figure 17C). Effector CD8+ T-cells (*uninfected,* dashed line; *infected,* solid line) were mixed with CD4+ T-cells (targets) from a heterogenic individual at the various indicated ratios and lysis was measured by PI release

Figure 17. The effects of HIV-1 infection on CD8+ T-cell viability and function. ***A,*** DNA fragmentation of CD8+ T-cells in response to HIV-1 infection. Cells were infected with 300 $TCID_{50}$/mL of the HIV_{IIIB} laboratory strain. Infected and uninfected CD4+ and CD8+ T-cells were isolated and lysed 7 days post-infection/culture. Cellular lysates were loaded on a 1% agarose gel and electrophoresed at 70 V for 2 hours. DNA was visualized by ethidium bromide staining. ***B,*** Jurkat cells treated with 10 μg/mL of camptothecin, an apoptosis inducer. ***C,*** Non-specific lytic function of CD8+ T-cells. Infected and uninfected CD8+ T-cells (effectors) were incubated for 3.5 hrs at 37°C with 3.0 mM DiO stained heterologous CD4 T-cells (targets) at the indicated effector:target ratios. After incubation, effector:target co-cultures were stained with propidium iodide and percent lysis of target cells was measured by flow cytometry. *solid line,* lytic abilities of HIV-1 infected CD8+ T-cells; *dashed line,* lytic abilities of uninfected CD8+ T-cells. A minimum of 10, 000 events were assessed for each analysis. Statistical significance of $p < 0.01$ is denoted by an (*) and assayed by Student's *t*-test.


A
infected
uninfected
CD4+ CD8+ CD4+ CD8+
B
+ ctrl
C
HIV+
HIV-
% Lysis
0
10
20
30
40
50
60
70
80
100:1
50:1
25:1
12.5:1
CD8:CD4 Ratio

through flow cytometric analysis. Exogenous IL-15 was added to both uninfected and infected CD8+ T-cell populations prior the MLR analysis based upon previous studies done in the lab that demonstrated the cytokine as being a strong inducer of IFN-γ production. Results illustrate that there is an enhancement of the non-specific cytotoxicity of CD8+ T-cells in response to infection. This enhancement increases as the effector:target ratios increase and is statistically significant at the 100:1 and 50:1 ratios ($p < 0.01$, Student's *t*-test, two-tailed).

3.2.4. Memory CD8+ T-Cells Support Higher Levels of HIV-1 Replication

Subsets of CD8+ T-cells have been shown to be significant in the control of HIV-1 infection and disease progression. It has been postulated that higher levels of CD8+CD38+ T-cells may be associated with poor prognosis and were found to be elevated in individuals who have progressed to AIDS [318, 358-360]. In contrast, high levels of CD8+HLA-DR+ cells were observed in asymptomatic individuals [361-363]. In studies with CD8+ CTLs, HLA-DR expression was found to correlate with proliferation and CD28 expression [304]. Based on the possibility that HIV-1 infection may be involved in hindering the function and activities of various key CD8+ T-cell subsets, the susceptibility of the aforementioned subsets to *in vitro* HIV-1 infection was examined. The CD8+ T-cell subsets were positively selected from the CD8+ T-cell population by the use of magnetic beads and were subsequently infected with the T-cell tropic laboratory strain HIV_{IIIB}, as the use of this strain resulted in the greatest productive infection of CD8+ T-cells (Figure 12A). Viral production was measured in the CD8+ T-cell subset cultures over a 10 day time course by a HIV-1 p24 ELISA. The negative fractions obtained from the subset isolation were also assessed for HIV-1 production. Interestingly, the CD8+CD28+, CD8+CD38+ and CD8+HLA-DR+

subsets all supported very low levels of HIV-1 replication (Figure 18A). In fact, the levels of viral production decreased over the course of *in vitro* infection in these subsets. Figure 18A also showed that 2.5 - 4 fold higher levels of replication were supported in the CD8+CD28-, CD8+CD38- and CD8+HLA-DR- populations, compared to the positive fractions.

Susceptibility of the memory and naïve CD8+ T-cell populations was also examined in response to HIV-1 infection with the HIV_{IIIB} strain. The CD8+CD45RA- and CD8+CD45RO+ memory T-cell populations both supported approximately 5 - 10 times higher levels of HIV-1 replication than the CD8+CD45RA+ and CD8+CD45RO- naïve populations (Figure 18B). When comparing the CD8+ T-cell subsets, the memory CD8+ T-cell subset supported high levels of HIV-1 replication and production (Figure 18A).

3.2.5. Proliferation of Memory CD8+ T-Cells in Response to Infection

HIV-1 infection has been reported to affect the frequency and function of circulating CD8+ T-cells, thereby hindering the ability of these cells to eradicate viral infection and maintain T-cell mediated immunity. Based upon these observations, numerous studies have documented the effects of HIV-1 infection on the CTL response [364]. It is with these studies, concerning the response of CD8+ T-cells during *in vitro* infection, that the novel idea of the effect of HIV-1 infection on the CD8+CD45RO+ memory T-cell subset was set out to be examined. In order to better define the effect of HIV-1 infection on the function and abilities of the CD8+ T-cell memory subset, CD8+CD45RO+ T-cell proliferation in response to antigenic stimulation was measured. Antigenic stimulation encompassed both mitogens and antigens in the experimental assays. The CD8+CD45R0+ subset was isolated through two consecutive rounds of magnetic bead separation. As assessed by flow cytometry and shown in Figure 19A, the majority of the isolated CD8+ T-cells were of the

Figure 18. HIV-1 replication in CD8+ T-cell subsets. ***A,*** CD28, CD38 and HLA-DR positive and negative populations were infected with HIV-1 and cultured. Cell-free supernatant samples were taken post-infection at the indicated time points for measurements of HIV-1 production by an ELISA. Results are the mean and SEM of three independent experiments. ***B,*** HIV-1 preferentially replicates in the CD8+CD45RO+ memory T-cells. CD45RO (memory) positive and negative populations and CD45RA (naïve) positive and negative populations were infected with 300 $TCID_{50}$/mL of HIV_{IIIB} and cultured. Cell-free supernatants were taken at days 5 and 10 post-infection for HIV-1 measurement by a p24 ELISA. Results are representative of two independent experiments.

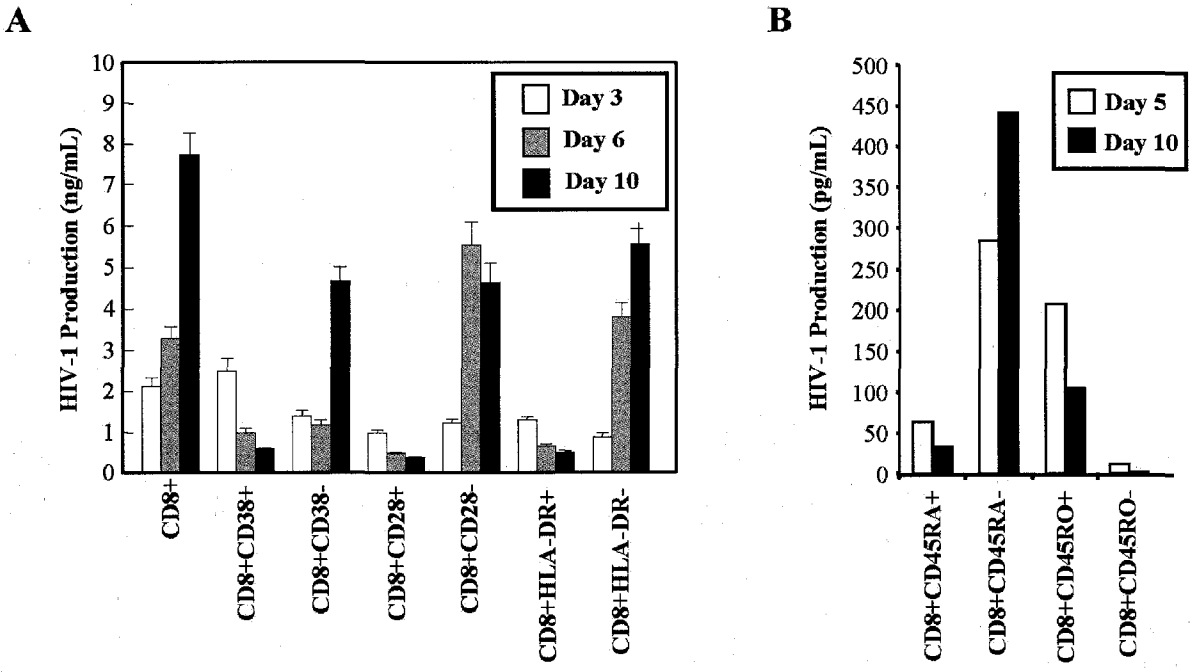
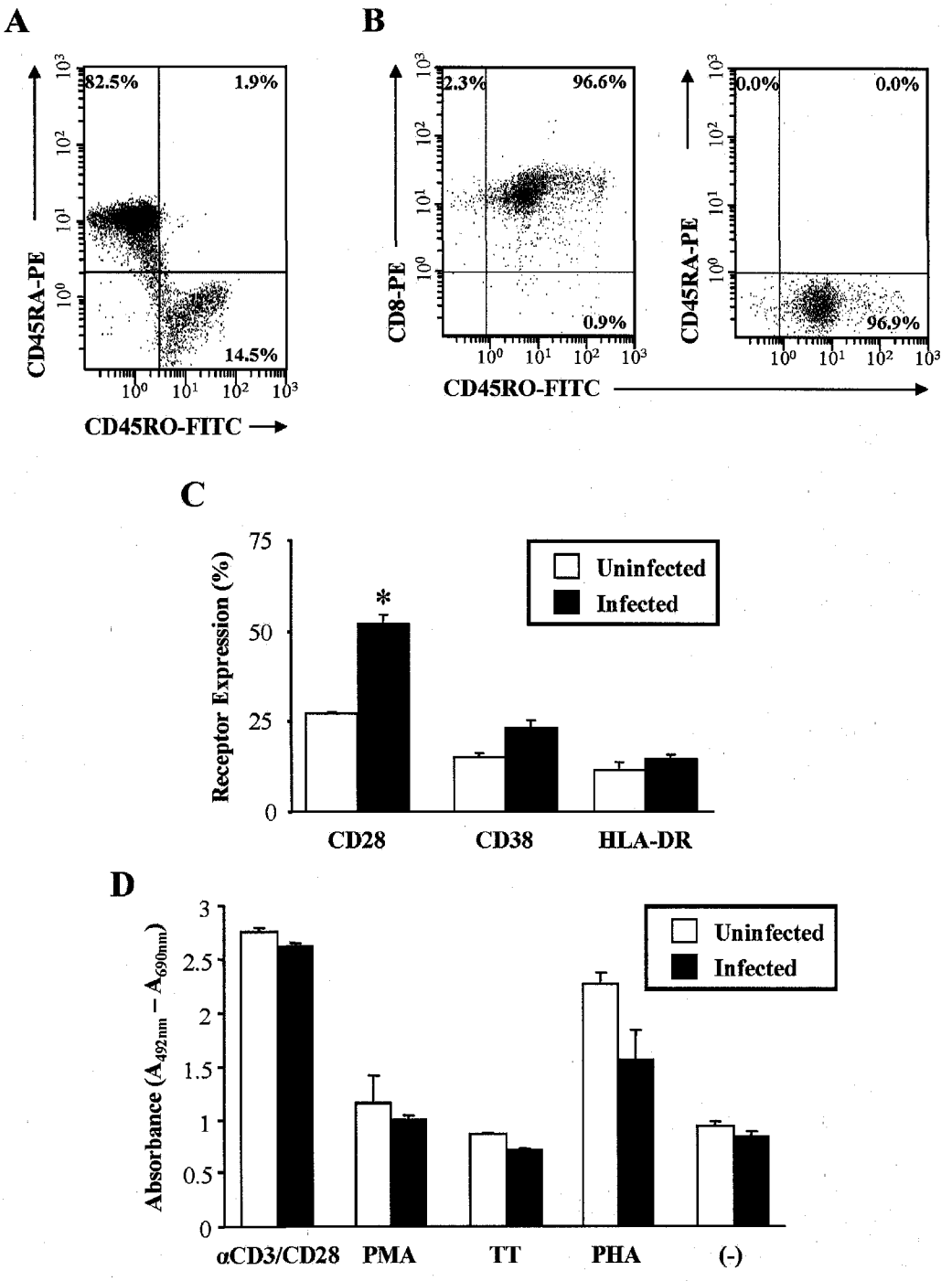

A
HIV-1 Production (ng/mL)
0
1
2
3
4
5
6
7
8
9
10
Day 3
Day 6
Day 10
CD8+
CD8+CD38+
CD8+CD38-
CD8+CD28+
CD8+CD28-
CD8+HLA-DR+
CD8+HLA-DR-
B
HIV-1 Production (pg/mL)
0
50
100
150
200
250
300
350
400
450
500
Day 5
Day 10
CD8+CD45RA+
CD8+CD45RA-
CD8+CD45RO+
CD8+CD45RO-

Figure 19. Proliferative response of HIV-1 infected CD8+CD45RO+ memory T-cells in response to antigenic stimulation. ***A,*** Positively selected CD8+ T-cells were stained with CD45RO-FITC and CD45RA-PE to determine the percentage of memory cells in the isolated CD8+ T-cell population. ***B,*** The purity of the selected CD8+CD45RO+ memory T-cells was assessed by flow cytometry and staining with CD45RO-FITC, CD45RA-PE (right histogram) and CD8-PE (left histogram). Numbers in the quadrants represent the percentage of gated cells in each quadrant. ***C,*** Expression of the activation markers CD28, CD38 and HLA-DR in uninfected (□) and infected (■) CD8+CD45RO+ memory T-cells. Results are the mean fluorescence and SEM of three independent experiments as measured by flow cytometry. Statistical significance of $p < 0.01$ is denoted by an (*). ***D,*** Lymphocyte proliferative ability of uninfected (□) and infected (■) CD8+CD45RO+ memory T-cells as measured by the XTT based colorimetric assay. Memory T-cells were infected (or left uninfected) for 7 days prior to antigen stimulation. Cells were stimulated with αCD3/CD28, Phytohemagglutinin (PHA), Phorbol Myristate Acetate (PMA), Tetanus Toxoid (TT) or left unstimulated (-). Proliferation was measured at days 2-3 post-antigenic stimulation by labeling with an XTT reagent during the last 18 hours of culture. Results are the mean and SEM of three independent experiments. Data is expressed as the mean absorbance ($A_{492nm} - A_{690nm}$) and SEM.


A
82.5%
1.9%
14.5%
CD45RA-PE
CD45RO-FITC
B
2.3%
96.6%
0.9%
CD8-PE
CD45RO-FITC
0.0%
0.0%
96.9%
CD45RA-PE
C
Receptor Expression (%)
75
50
25
0
*
Uninfected
Infected
CD28
CD38
HLA-DR
D
Absorbance ($A_{492nm} - A_{690nm}$)
3
2.5
2
1.5
1
0.5
0
Uninfected
Infected
αCD3/CD28
PMA
TT
PHA
(-)

naive subset (82.5% CD8+CD45RA+ naive T-cells; 14.5% CD8+CD45RO+ memory T-cells). The purity of our selected CD8+CD45RO+ memory T-cell population was also assessed. The selected memory T-cell population was devoid of any contaminating CD8+CD45RA+ naïve T-cells (Figure 19B, right histogram) and was composed of approximately 97% pure cells of the CD8+CD45RO+ phenotype (Figure 19B, left histogram).

Lymphocyte proliferation was examined by utilizing the XTT based colorimetric assay. The assay is based on the cleavage of the yellow tetrazolium salt (XTT) to form an orange formazan dye by metabolically active cells [336] and measured by absorbance. Prior to antigen stimulation, the CD8+CD45RO+ memory T-cells were infected (or left uninfected) for 7 days. Before measuring proliferation, the activation state of the memory cells was assessed before and after infection (Figure 19C). Based upon the flow cytometry results, the mean fluorescence of CD28 expression nearly doubled (uninfected, 27.3% ± 0.3; infected, 52.3% ± 2.4) during the course of HIV-1 infection and this was significant (p-value, 0.0026). There were marginal increases in CD38 and HLA-DR expression in response to infection. To determine the proliferative capacity of the memory subset, cells were stimulated for 2-3 days with various antigens both in the presence and absence of HIV-1 infection. Based upon the results, it was found that minimal differences in *in vitro* lymphocyte proliferation between infected and uninfected CD8+CD45RO+ memory T-cells in response to antigenic stimulation (Figure 19D) were observed. Overall, HIV-1 infection resulted in a slightly reduced rate of proliferation in the CD8+CD45RO+ memory subset. Antigenic stimulation with PHA resulted in a greater than 2-fold increase in proliferation in the uninfected memory T-cell population. However, the presence of PHA resulted in an approximately 30% reduction in the cleavage of XTT in the HIV-1 infected population,

indicating a decrease in proliferation. Conversely, αCD3/CD28 stimulation resulted in a nearly 3-fold increase in the absorbance values in both the uninfected and infected CD8+CD45RO+ memory T-cell populations.

3.3. MODULATION OF CELL-SURFACE MOLECULES ON CD8+ T-CELL CLONES IN RESPONSE TO *in vitro* HIV-1 INFECTION

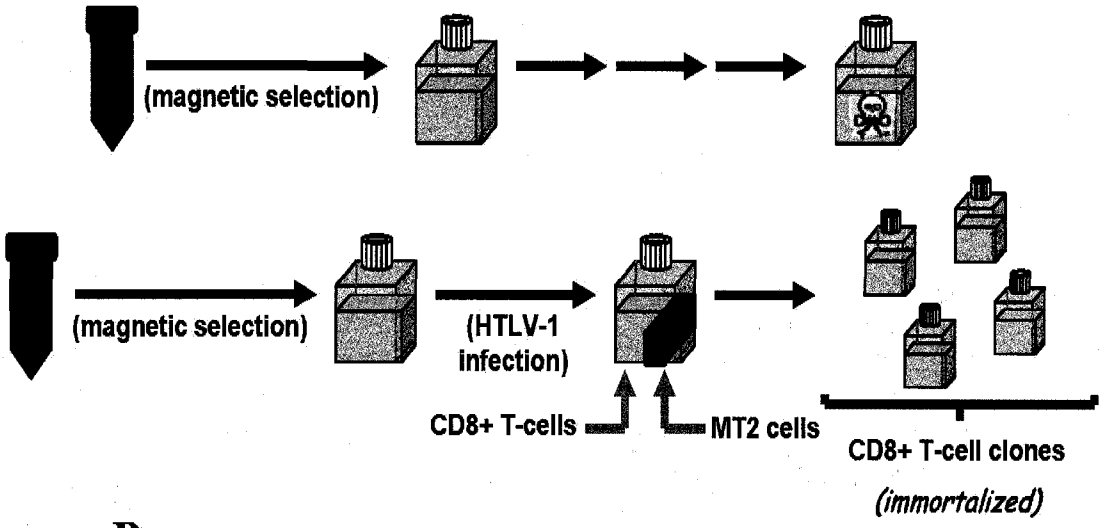
3.3.1. HTLV-I Transformation of Primary Blood-Derived CD8+ T-Cells

After successfully examining the frequency, significance and susceptibility of CD8+ T-cells to both *ex vivo* and *in vitro* HIV-1 infection, attention was focused on the mechanism(s) involved in CD8+ T-cell mediated viral entry. Before examining the mechanism(s), a set of immortalized CD8+ T-cell clones was established in order to provide a ready source of CD8+ T-cells (without the constant need of freshly isolated primary CD8+ T-cells for every experiment) and in order to normalize the experimental results. HTLV-I transformation of isolated primary CD8+ T-cells from a healthy volunteer resulted in the generation of a set of CD8+ T-cell clones. Primary CD8+ T-cells were co-cultured with MT2 cells, a productively HTLV-I infected CD4+ T-cell line, and transformed cells were isolated and propagated by limiting dilution (Figure 20A). The set of resultant CD8+ T-cell clones were termed Tri8.X (where X denotes the CD8+ T-cell clone isolate).

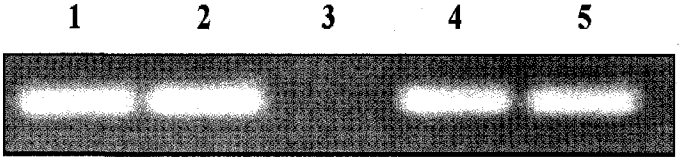
PCR analysis of the CD8+ T-cell clones for the HTLV-I *tax* gene indicated that the CD8+ T-cell clones had all stably integrated the HTLV-I genome (Figure 20B). MT2 cells, used as a source of HTLV-I virus and as a control for PCR analysis, also showed a band specific for HTLV-I *tax*. Incorporation of the HTLV-I genome into target cells may result in either a silent or productive infection [365]. A silent infection is characterized by the integration of the HTLV-I genome into the host cell without any detectable HTLV-I encoded mRNA. Conversely, a productive infection is characterized by viral mRNA transcription and production. RT-PCR analysis for the HTLV-I *tax* gene product resulted in the appearance of a HTLV-I *tax*-specific band only in the MT2 cell population (Figure 20C).

Figure 20. HTLV-I transformation of primary blood-derived CD8+ T-cells. ***A,*** Isolated CD8+ T-cells from a healthy individual were transformed with HTLV-I. CD8+ T-cells were co-cultured with irradiated MT2 cells in RPMI-10. After 2 weeks of co-culture, transformed CD8+ T-cells were isolated by positive selection using the Miltenyi miniMACS system and then subjected to three consecutive rounds of limiting dilution. ***B,*** Expression of the HTLV-I genome in CD8+ T-cell clones. PCR analysis of the *tax* gene of HTLV-I in MT2 cells (lanes 1 and 2) and the CD8+ T-cell clone Tri8.D6b.D7 (lanes 4 and 5). ***C,*** RT-PCR analysis of the *tax* gene of HTLV-I in untransformed CD8+ T-cells (lane 1), MT2 cells (lane 2) and the CD8+ T-cell clones Tri8.A, Tri8.E and Tri8.F (lanes 3, 4, and 5 respectively). ***D,*** Hypothetical model of the activation of the HIV-1 LTR by the HTLV-I Tax and HIV-1 Tat proteins.

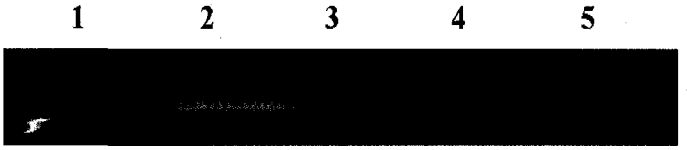
A


(magnetic selection)
(magnetic selection)
(HTLV-1 infection)
CD8+ T-cells
MT2 cells
CD8+ T-cell clones
(immortalized)

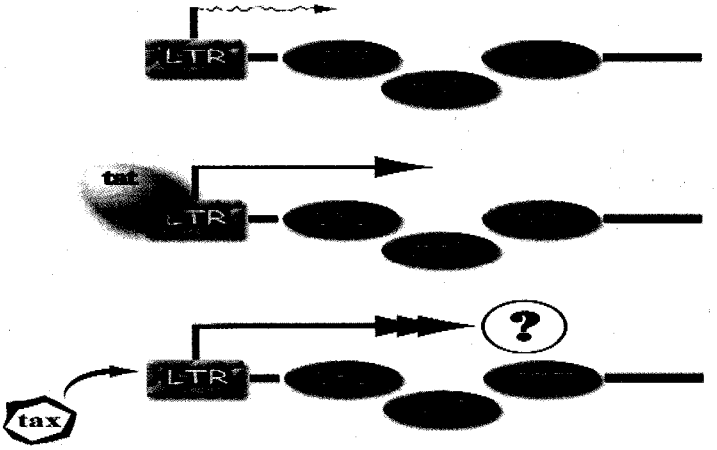
B


1
2
3
4
5

C


1
2
3
4
5

D


LTR
tat
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These results indicate that the immortalization process resulted in the generation of CD8+ T-cell clones with a stably integrated, yet silent infection of HTLV-I.

3.3.2. HIV-1 Infection of HTLV-I Transformed CD8+ T-Cell Clones

The ability of HIV-1 to infect the CD8+ T-cell clones was assessed. Initial experiments used the HIV_{IIIB} laboratory strain for infection assays due to its preferential ability to infect CD8+ T-cells *in vitro* (Figure 12A). Similar to the analysis of memory CD8+ T-cells (Figure 19C), the activation state of the CD8+ T-cell clones in response to infection was examined. Unlike the memory T-cell population, HIV-1 infection did not alter significantly the expression of CD28, CD38 and the HLA-DR activation markers in the CD8+ T-cell clones (Figure 21). Interestingly, the CD8+ T-cell clones expressed low levels of CD28 and CD38. Conversely, they expressed extremely high levels of HLA-DR. This is in contrast to the results found with memory CD8+ T-cells.

The CD8+ T-cell clones supported higher levels of HIV-1 infection and production than their untransformed primary CD8+ T-cell counterparts and CD4+ T-cells (Figure 22A). The CD8+ T-cell clones: Tri8.D6b.C5, D6b.B3, D6b.H3 and D6b.D7 produced virus at levels 5 - 10 times greater than untransformed CD8+ T-cells and 3 - 5 times greater than CD4+ T-cells, which are considered the primary targets of HIV-1. Of interest are the clones Tri8.A, E and F. These clones supported viral production approximately 20 - 25 times higher than that supported by primary T-cells. HIV-1 production reached a maximum at day 14 in both primary and transformed T-cells. Enhanced productive infection of the CD8+ T-cell clone isolates was further confirmed by intracellular flow cytometry for $HIV\text{-}1_{gag}$ (Figure 22B). Results indicate that the majority of the clones stain intracellularly for HIV-1 at levels comparable to CD4+ T-cells, yet 4 – 6 times greater than primary CD8+ T-cells. However,

Figure 21. Expression of the activation markers CD28, CD38 and HLA-DR in uninfected (□) and infected (■) CD8+ T-cell clones. Results are the mean fluorescence and SEM of three independent experiments as measured by flow cytometry.

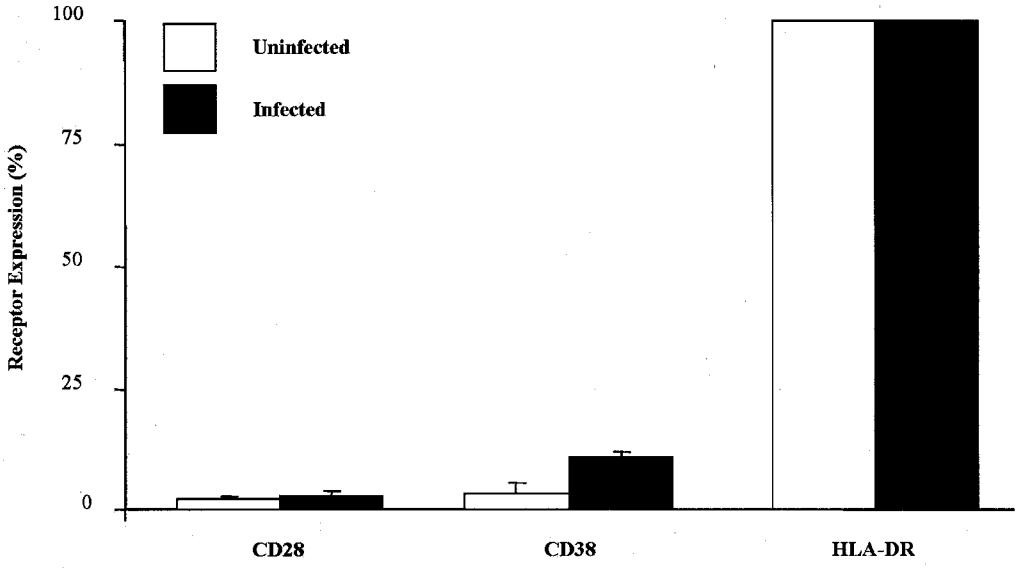
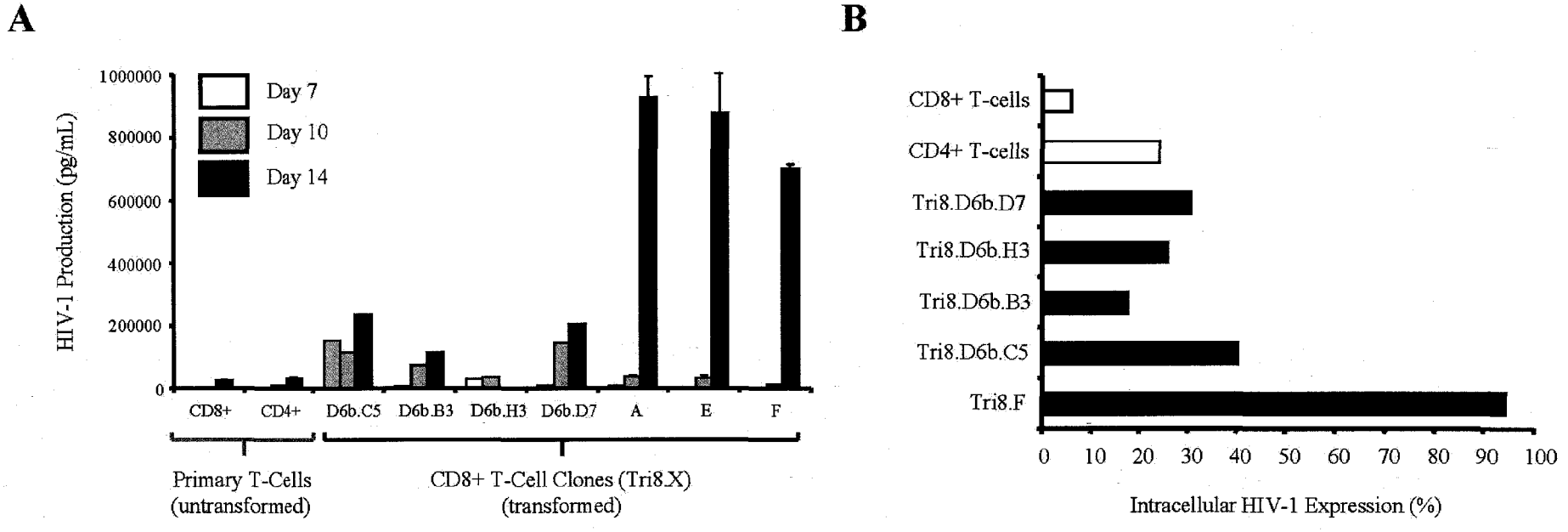

100
75
50
25
0
Receptor Expression (%)
Uninfected
Infected
CD28
CD38
HLA-DR

Figure 22. HIV-1 production in CD8+ T-cell clones. ***A,*** HIV-1 production in primary CD8+ T-cells, CD4+ T-cells and CD8+ T-cell clones. Cells were infected with 300 $TCID_{50}$/mL of HIV_{IIIB} and HIV-1 production was measured from cell free supernatants at days 7, 10 and 14 using an ELISA. Results for primary T-cells and CD8+ T-cell clones Tri8.A, Tri8.E and Tri8.F are expressed as the mean of three independent experiments. ***B,*** Intracellular expression of HIV-1 in primary CD8+ T-cells, CD4+ T-cells and CD8+ T-cell clones. Cells were infected with 300 $TCID_{50}$/mL of HIV_{IIIB} and were stained with HIV-1_{gag}-RD1 conjugated antibody at day 7 post-infection. The number of HIV-1 positive staining cells was assessed by flow cytometry. A minimum of 10, 000 events was assessed for each analysis.


A
HIV-1 Production (pg/mL)
1000000
800000
600000
400000
200000
0
Day 7
Day 10
Day 14
CD8+
CD4+
D6b.C5
D6b.B3
D6b.H3
D6b.D7
A
E
F
Primary T-Cells
(untransformed)
CD8+ T-Cell Clones (Tri8.X)
(transformed)
B
CD8+ T-cells
CD4+ T-cells
Tri8.D6b.D7
Tri8.D6b.H3
Tri8.D6b.B3
Tri8.D6b.C5
Tri8.F
0
10
20
30
40
50
60
70
80
90
100
Intracellular HIV-1 Expression (%)

Tri8.F shows almost 95% intracellular HIV-1 expression compared to only 6.5% infection of primary CD8+ T-cells (Figure 15A). These results suggest that the majority of CD8+ T-cell clones can support viral infection at levels comparable to that of CD4+ T-cells. However, they are stronger mediators of HIV-1 production and replication than primary T-cells.

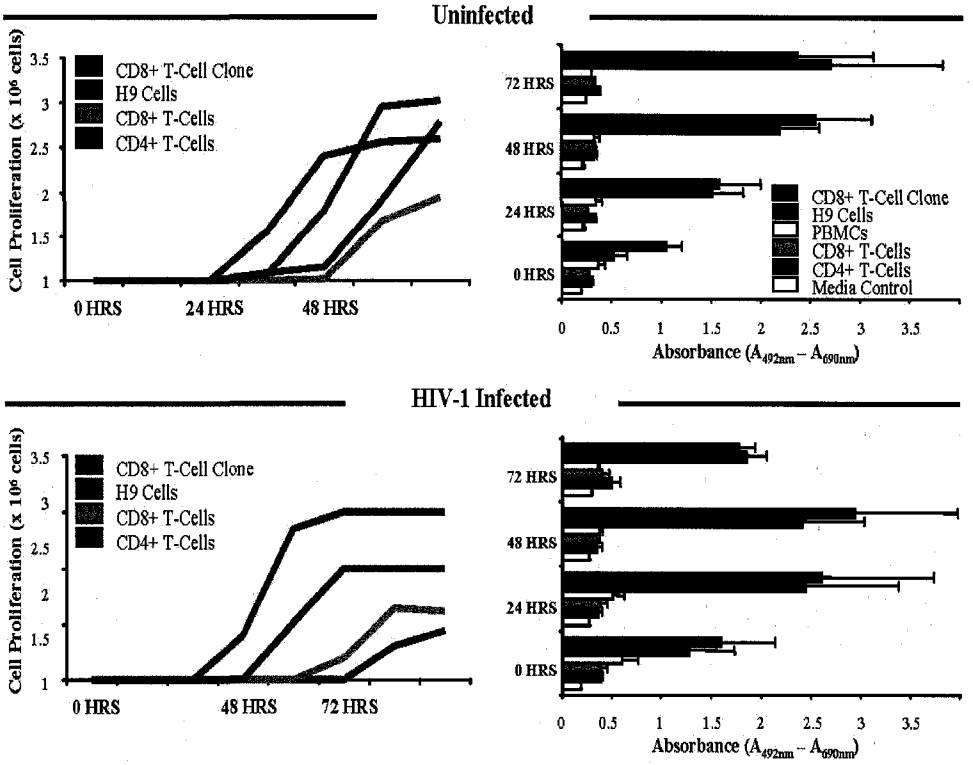
3.3.3. CD8+ T-Cell Clone Proliferation in Response to HIV-1 Infection

In order to determine whether the enhanced production of HIV-1 in the CD8+ T-cell clones was due to an increase in the proliferative capabilities of the clones, their ability to proliferate in response to HIV-1 infection was assessed by trypan blue exclusion (Figure 23A, *left panels*) and a lymphocyte proliferation assay (Figure 23A, right panels). Results indicated that the CD8+ T-cell clones proliferated at a rate higher than lymphocytes but comparable to H9 cells, a CD4+ T-cell line. HIV-1 infection did not seem to alter dramatically the proliferative capabilities of both H9 cells and the CD8+ T-cell clones (Figure 23A, *bottom panels*), suggesting the resistance of these cells to the cytopathic effects of the virus [186, 366-370]. Statistical significance was not observed between the proliferative capabilities of the HTLV-I transformed CD8+ T-cell clones and the H9, CD4+ and CD8+ T-cells.

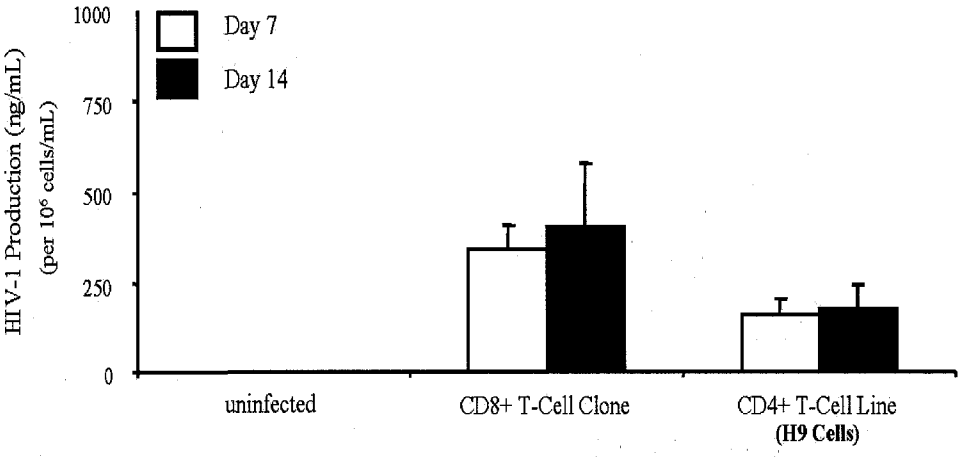
Laboratory adapted T-cell lines have differing capabilities in supporting productive HIV-1 replication [371]. With these studies in mind, a comparison of HIV-1 replication kinetics between the CD8+ T-cell clones and H9 cells was carried out (Figure 23B). Results were normalized to the number of cells in culture at the time of monitoring. Both cell populations were able to support viral production and replication as expected. However, the CD8+ T-cell clones supported approximately 2 times more HIV-1 production than H9 cells. The results illustrated here and in Figure 22A indicate that the HTLV-I transformed CD8+ T-

Figure 23. The effects of HTLV-I transformation on the replication and proliferative capabilities of CD8+ T-cell clones, H9 cells and lymphocytes in response to HIV-1 infection. ***A,*** Cell proliferation of various cell types in response to HIV-1 infection. *Left Panels,* Indicated cells were isolated and cultured in the presence or the absence of HIV-1 infection (300 $TCID_{50}$/mL of HIV_{IIIB}). Cell proliferation was measured by trypan blue exclusion at the indicated time points. *Right Panels,* Proliferative ability of various cell types (legend in upper right panel) in response to HIV-1 infection as measured by the XTT colorimetric assay. Proliferation of infected cultures was measured at days 2-3 post-infection by labeling with an XTT reagent and measuring the absorbance ($A_{492nm} - A_{690nm}$). Results are expressed as the mean and SEM of three independent experiments. ***B,*** Comparison of HIV-1 production between the CD8+ T-cell clones and a CD4+ T-Cell line (H9 cells). Cells were infected with 300 $TCID_{50}$/mL of HIV_{IIIB} and cell-free supernatants were collected on days 7 and 14. HIV-1 production was measured by a p24 ELISA.

A


Uninfected
Cell Proliferation (x 10⁶ cells)
CD8+ T-Cell Clone
H9 Cells
CD8+ T-Cells
CD4+ T-Cells
0 HRS
24 HRS
48 HRS
72 HRS
PBMCs
Media Control
Absorbance (A492nm – A690nm)
HIV-1 Infected

B


HIV-1 Production (ng/mL)
(per 10⁶ cells/mL)
Day 7
Day 14
uninfected
CD8+ T-Cell Clone
CD4+ T-Cell Line
(H9 Cells)

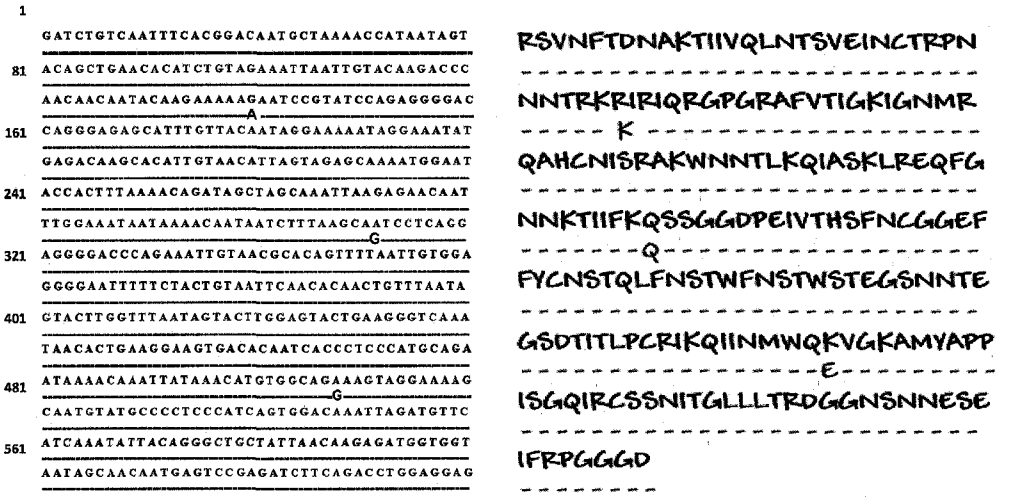
cell clones are enhanced producers of HIV-1 virus. This characteristic attributed to the CD8+ T-cell clones is not dependent upon viral and cellular kinetics (Figure 22A and Figure 23A), nor upon levels of supported virus infection (Figure 22B).

3.3.4. CD8+ T-Cells Clones Produce Infectious Virus

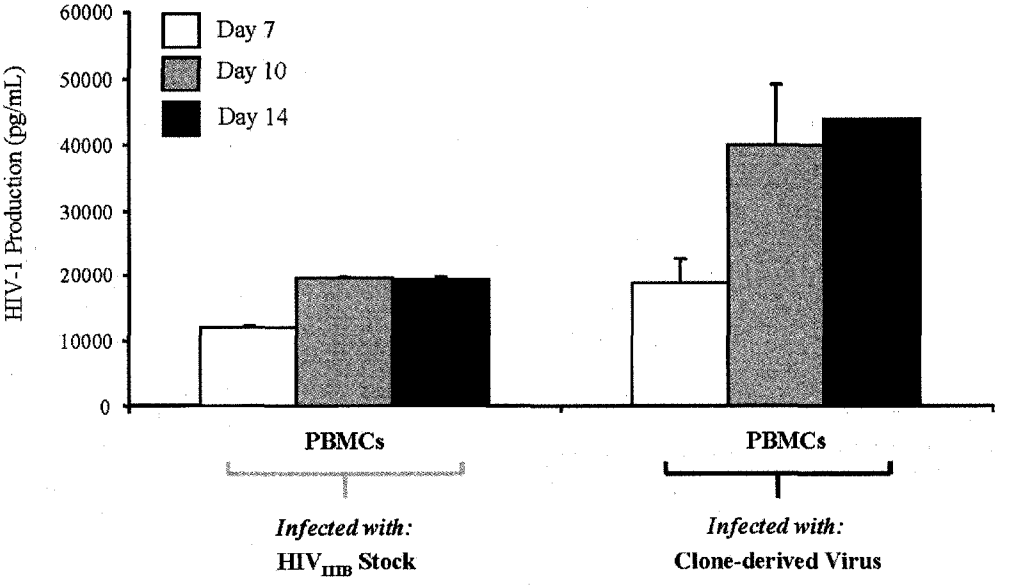
The phenotype of the virus produced by the HIV-1 infected CD8+ T-cell clones was ascertained. One fate of progeny virus exiting from CD8+ T-cell clone is that they may contain numerous mutations or can be considered deleterious and thus non-infectious. A second possibility is that newly-formed virus may be more readily infectious, thus conferring it significant advantages in HIV-1 infection and replication. This second possibility may help explain the high levels of viral production supported by the CD8+ T-cell clones. Virus was isolated from cell-free supernatants of productively HIV-1 infected CD8+ T-cell clones day 7 post-infection. The viral envelope was sequenced and comparison of the nucleotide sequence was done with the published canonical T-cell tropic laboratory strain, HXB2 (HIV_{IIIB}) *(Genbank accession number NC_001802).* Three nucleotide changes were observed at positions 162, 311 and 527 (Figure 24A, *left panel*). These nucleotide changes only resulted in two amino acid changes, $Arg^{306}Lys$ and $Lys^{429}Glu$ (Figure 24A, *right panel*). In order to ascertain the infectivity of the virus, an equal titre of virus derived from the CD8+ T-cell clone infection and the original HIV_{IIIB} viral stock used to initially infect the clones were used to infect PBMCs isolated from a healthy individual. HIV-1 production and replication was measured by an ELISA over a 14-day time-course (Figure 24B). Results demonstrate that the clone-derived virus replicated comparably, if not a little higher (though statistical significance was not found), than that of the HIV_{IIIB} viral stock in PBMCs, indicating that the CD8+ T-cell clones produce infectious HIV-1 particles.

Figure 24. Infectivity of virus derived from the CD8+ T-cell clones. ***A,*** Amino acid sequencing of the HIV-1 envelope region from virus produced by the CD8+ T-cell clones. CD8+ T-cell clone D6b.D7 was infected with 300 $TCID_{50}$/mL of HIV_{IIIB} and at day 7 post-infection, virus produced by the clone was isolated and sequenced against the parental virus (lettered in black). Dashed lines indicate no changes compared to parental virus at that nucleotide/amino acid position. *Left Panel,* Three nucleotide mutations were observed at positions 100, 272 and 468. *Right Panel,* Only two mutations that altered the amino acid sequence were found (denoted in red), Arg306Lys and Lys429Glu. ***B,*** Infectivity of virus derived from the CD8+ T-cell clones. CD8+ T-cell clones were infected with 300 $TCID_{50}$/mL of HIV_{IIIB} for 7 days. Virus produced by the clones was then subsequently isolated and used to infect peripheral blood mononuclear cells (PBMCs). HIV-1 production was measured from cell free supernatants at days 7, 10 and 14 using an ELISA. Similarly, HIV_{IIIB} stock was also used to infect peripheral blood mononuclear cells as a control. Results are expressed as the mean and SEM of three independent experiments.

A


1
GATCTGTCAATTTCACGGACAATGCTAAAACCATAATAGT
RSVNFTDNAKTIIVQLNTSVEINCTRPN
81
ACAGCTGAACACATCTGTAGAAATTAATTGTACAAGACCC
AACAACAATACAAGAAAAAGAATCCGTATCCAGAGGGGAC
NNTRKRIRIQRGPGRAFVTIGKIGNMR
161
CAGGGAGAGCATTTGTTACAATAGGAAAAATAGGAAATAT
GAGACAAGCACATTGTAACATTAGTAGAGCAAAATGGAAT
QAHCNISRAKWNNTLKQIASKLREQFG
241
ACCACTTTAAAACAGATAGCTAGCAAATTAAGAGAACAAT
TTGGAAATAATAAAACAATAATCTTTAAGCAATCCTCACG
NNKTIIFKQSSGGDPEIVTHSFNCGGEF
321
AGGGGACCCAGAAATTGTAACGCACAGTTTTAATTGTGGA
GGGGAATTTTTCTACTGTAATTCAACACAACTGTTTAATA
FYCNSTQLFNSTWFNSTWSTEGSNNTE
401
GTACTTGGTTTAATAGTACTTGGAGTACTGAAGGGTCAAA
TAACACTGAAGGAAGTGACACAATCACCCTCCCATGCAGA
GSDTITLPCRIKQIINMWQKVGKAMYAPP
481
ATAAAACAAATTATAAACATGTGGCAGAAAGTAGGAAAAG
CAATGTATGCCCCTCCCATCAGTGGACAAATTAGATGTTC
ISGQIRCSSNITGLLLTRDGGNSNNESE
561
ATCAAATATTACAGGGCTGCTATTAACAAGAGATGGTGGT
AATAGCAACAATGAGTCCGAGATCTTCAGACCTGGAGGAG
IFRPGGGD

B

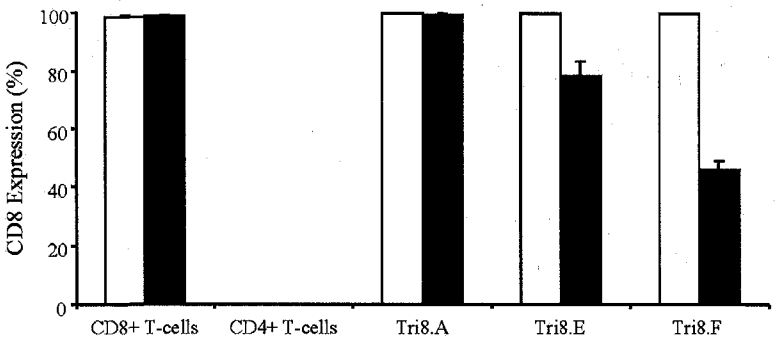

HIV-1 Production (pg/mL)
60000
50000
40000
30000
20000
10000
0
Day 7
Day 10
Day 14
PBMCs
PBMCs
Infected with:
HIV IIIB Stock
Infected with:
Clone-derived Virus

3.3.5. CD8+ T-Cell Clone Cell-Surface Molecule Expression During the Course of *in vitro* HIV-1 Infection

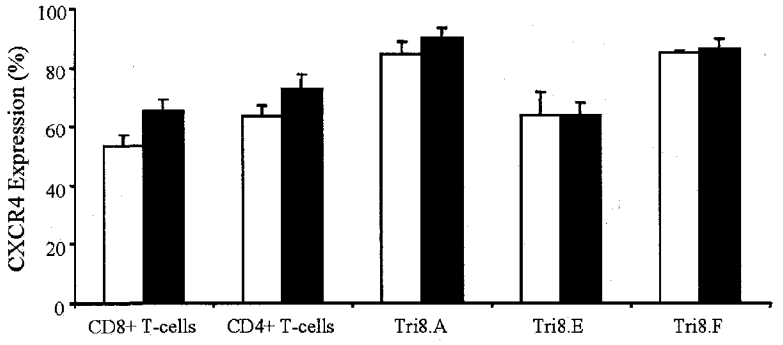
A caveat of successful productive infection of target cells by HIV-1 is the modulation of cell-surface molecules (reviewed in [372]) and the down-regulation of it's receptor (reviewed in [373]). The examination of whether HIV-1 infection of the CD8+ T-cell clones would result in the modulation of extracellular molecules, possibly indicating a potential receptor(s) that may be involved in CD8+ T-cell clone infection was assessed. Initial studies examined the expression of the three most predominantly used receptors/co-receptors used by HIV-1: CD4, CXCR4 and CCR5. Additionally, the levels of the CD8 cell-surface molecule was also assayed as it was the most predominantly expressed molecule on the surface of the CD8+ T-cell clones. Preliminary modulation studies utilized the HIV_{IIIB} laboratory strain. Figure 25A illustrates that HIV-1 infection of the CD8+ T-cell clones Tri8.E and Tri8.F resulted in the down-regulation of the CD8 molecule (Tri8.E: *uninfected*, 99.7% ± 0.1% vs. *infected*, 78.4% ± 4.8%; Tri8.F: *uninfected*, 99.6% ± 0.1% vs. *infected*, 46.0% ± 3.1%) at day 10 post-infection. No modulation of CD8 expression was observed in the CD8+ T-cell clone Tri8.A and primary CD8+ T-cells during the course of infection. This same set of CD8+ T-cell clones did not show any significant alterations in CXCR4 expression in response to HIV-1 infection (Figure 25B). However, there was an approximate 13% - 25% decrease in CXCR4 expression levels of the CD8+ T-cell clones: Tri8.D6b.C5, D6b.B3, D6b.H3 and D6b.D7 (Figure 25C). These clones supported virus replication at levels 5 - 10 times greater than that of CD8+ T-cells, whereas the CD8+ T-cell clones Tri8.A, E and F supported viral replication at levels 20 – 25 times greater than primary T-cells (Figure 22A). These two sets of CD8+ T-cell clones have differing HIV-1 production capabilities and differing patterns of cell-surface molecule modulation, suggesting two

Figure 25. Cell-surface molecule expression on CD8+ T-cell clones during the course of infection. Two subsets of CD8+ T-cell clones (subset no.1: *Tri8.A, Tri8.E, Tri8.F* and subset no.2: *Tri8.D6b.C5, Tri8.D6b.B3, Tri8.D6b.H3, Tri8.D6b.D7*), and primary CD4+ and CD8+ T-cells were examined. Cells were infected with 300 $TCID_{50}$/mL of HIV_{IIIB} and cell surface receptor expression levels were assessed by flow cytometry at day 10 post-infection for ***A,*** CD8; ***B and C,*** CXCR4, and ***D,*** CD4 in uninfected (□) and infected (■) CD8+ T-cell clones. Results are expressed as the mean fluorescence and SEM of three independent experiments. A minimum of 10, 000 events was assessed for each analysis.

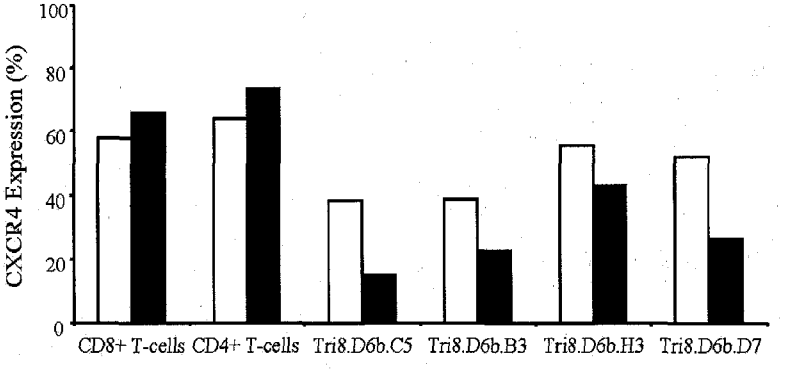
A


CD8 Expression (%)
100
80
60
40
20
0
CD8+ T-cells
CD4+ T-cells
Tri8.A
Tri8.E
Tri8.F

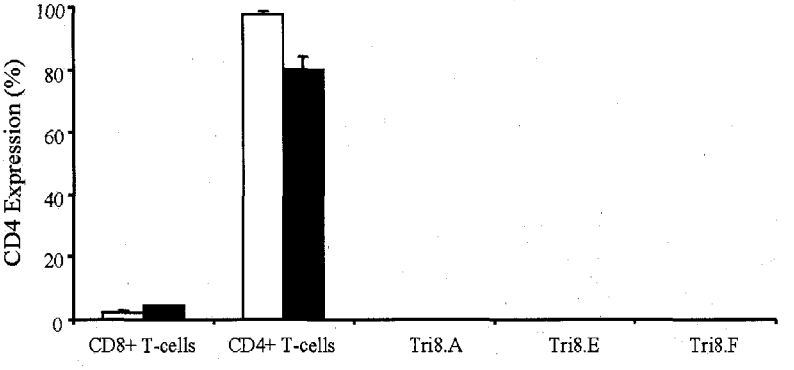
B


CXCR4 Expression (%)
100
80
60
40
20
0
CD8+ T-cells
CD4+ T-cells
Tri8.A
Tri8.E
Tri8.F

C


CXCR4 Expression (%)
100
80
60
40
20
0
CD8+ T-cells
CD4+ T-cells
Tri8.D6b.C5
Tri8.D6b.B3
Tri8.D6b.H3
Tri8.D6b.D7

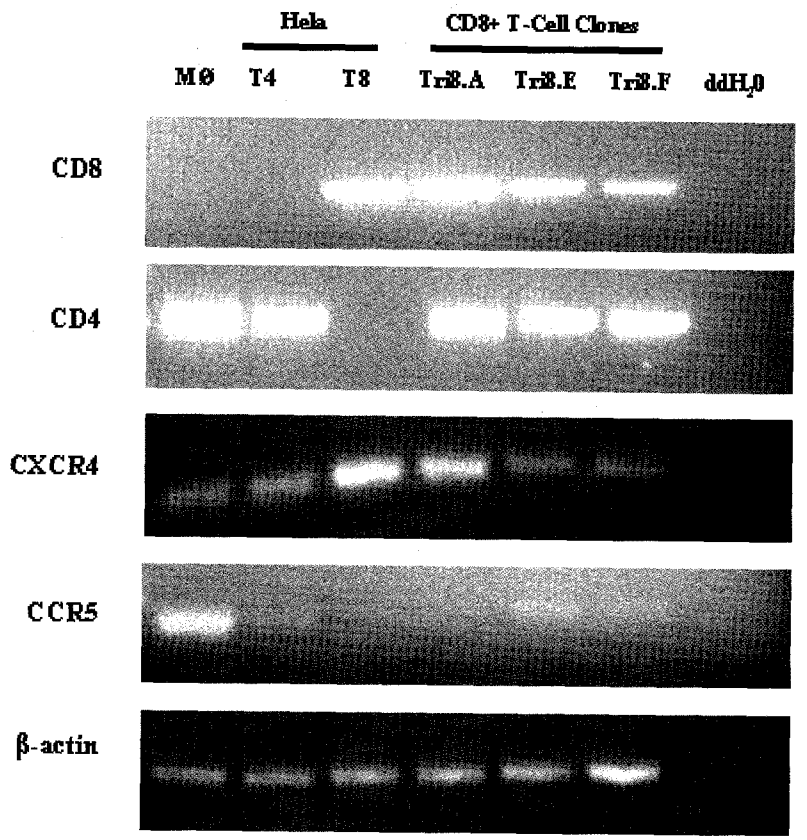
D


CD4 Expression (%)
100
80
60
40
20
0
CD8+ T-cells
CD4+ T-cells
Tri8.A
Tri8.E
Tri8.F

possible routes of receptor-mediated HIV-1 entry into these cells. By flow cytometric analysis, CCR5 expression was found to be < 2% on the CD8+ T-cell clone surface and expression was not altered during the course of infection (data not shown). Lastly, CD4 expression was assessed on these clones. CD4 up-regulation on the surface of CD8+ T-cells has been shown to occur through a variety of mechanisms [118-124]. The emergence of DP cells has also been observed in the *in vitro* infection of PBMCs with HTLV-I [374]. Therefore, it was examined whether the transformation process resulted in the *de novo* expression of the CD4 molecule on the surface of the CD8+ T-cell clones, allowing a route for successful HIV-1 entry into these cells. From our flow cytometric analysis, none of the clones exhibited CD4 expression before or after HIV-1 infection (Figures 25D), indicating that neither the transformation process, or HIV-1 infection could up-regulate the CD4 molecule in the CD8+ T-cell clones. On the other hand, there was a down-regulation of CD4 expression on the primary CD4+ T-cells (*uninfected*, 99.7% ± 1.1% vs. *infected*, 79.9% ± 4.3%), a feature that has been observed in productive HIV-1 infection of T-cells by others [167, 375]. Though no CD4 expression could be detected by flow cytometry, RT-PCR analysis revealed that the CD8+ T-cell clones all expressed high levels of CD4 mRNA transcripts (Figure 26).

Analysis of cell-surface molecule modulation by HIV-1 on CD8+ T-cell clones was broadened to determine whether similar observations would be found with clinical isolates of HIV-1 compared to the laboratory isolate HIV_{IIIB} (Figure 25). Three clinical isolates of HIV-1 were chosen based upon their co-receptor usage: 92HT599 (CXCR4(X4)-mediated), 92HT593 (dual-tropic (R5X4)) and 91US005 (CCR5(R5)-mediated). HIV-1 replication and infection was only supported in the CD8+ T-cell clones infected with the laboratory and

Figure 26. RT-PCR analysis of cell-surface molecule expression on CD8+ T-cell clones. RT-PCR analysis was performed on cDNA samples of the CD8+ T-cell clones and receptor controls (macrophages (MØ), HeLa T4 & HeLa T8). Briefly, 10 uL of samples were loaded and RT-PCR conditions were: 1. 95°C for 2 minutes; 2. 30 cycles of *(a)* 94°C for 1 minute, *(b)* 55°C for 1 minute, *(c)* 72°C for 1 minute; 3. final extension for 7 minutes at 72°C. Samples were normalized with β-actin and visualized on a 2 % w/v agarose gel by ethidium bromide staining.


Hela
CD3+ T-Cell Clones
MØ
T4
T8
Tri3.A
Tri3.E
Tri3.F
ddH2O
CD8
CD4
CXCR4
CCR5
β-actin

clinical X4 and R5X4 isolates as demonstrated by a HIV-1 ELISA (Figure 27A) and RT-PCR analysis (Figure 27B). Failure of the CD8+ T-cell clones to support infection by the R5 clinical strain may be due to the low levels of CCR5 expression found on these cells (Figure 26). Similar to the results found with the HIV_{IIIB} laboratory strain (Figure 25 and Figure 27C, *top middle panel*), productive infection with the clinical X4 and R5X4 isolates resulted in the altered expression of the CD8 and CXCR4 molecules on the CD8+ T-cell clones (Figure 27C). This alteration was most evident at day 14 post-infection. Only the R5 strain, which could not generate a productive infection, failed to down-regulate any of the cell-surface molecules (Figure 27C, *bottom middle panel*). Results for the R5 strain are similar to that of the uninfected control (Figure 27C, *upper left panel*). No expression or the up-regulation of the CD4 molecule was observed in the CD8+ T-cell clones infected with any of the clinical isolates. Thus, productive infection of the CD8+ T-cell clones results in the alteration of the expression levels of the CD8 and CXCR4 molecules.

3.3.6. CD8+ T-Cell Clone Receptor Blocking Studies

In order to elucidate the role of the CD8 and CXCR4 molecules in HIV-1 infection of the CD8+ T-cell clones, blocking studies were performed. The CD8+ T-cell clones were pre-treated with monoclonal antibodies 2 - 3 hours prior to HIV-1 infection. Inhibition of HIV-1 infection and replication was seen in the CD8+ T-cell clones treated with anti-CD8 and anti-CXCR4 antibodies (Figure 28). The inhibition of viral production in the presence of the antibodies was most evident at day 14 post-infection. An equal amount of inhibition was observed between the two antibodies independently of one another. These results were cell-surface molecule specific as treatment of cells with an IgG1 antibody isotype control had no effect on HIV-1 production compared to the untreated HIV-1 infected CD8+ T-cell

Figure 27. The modulation of CD8+ T-cell clone cell-surface molecules in response to productive HIV-1 infection. ***A,*** CD8+ T-cell clones were infected with 300 $TCID_{50}$/mL of laboratory isolates (HIV_{IIIB}) and clinical isolates (92HT599, 92HT593 and 91US005) of HIV-1. Cell-free supernatants were collected on days 7, 10 and 14 and HIV-1 Production was measured by an ELISA. Results are expressed as the mean and SEM of three independent experiments. ***B,*** RT-PCR analysis for HIV-*gag* transcripts of CD8+ T-cell clones infected with various HIV-1 isolates. Briefly, nested RT-PCR analysis was performed on 5uL cDNA samples and PCR conditions were: 1. 95°C for 2 minutes; 2. 30 cycles of *(a)* 94°C for 1 minute, *(b)* 55°C for 1 minute, *(c)* 72°C for 1 minute; 3. final extension for 7 minutes at 72°C. Samples were normalized with GAPDH. ***C,*** Modulation of cell-surface receptors by HIV-1 infection. CD8+ T-cells clones were left uninfected or infected with 300 $TCID_{50}$/mL of the laboratory and clinical isolates of HIV-1. Cells were stained with CD8-PC5, CXCR4-PE and CCR5-PE and receptor expression was assessed by flow cytometry on days 7 and 14. Results are expressed as the mean and SEM of 5 independent experiments.

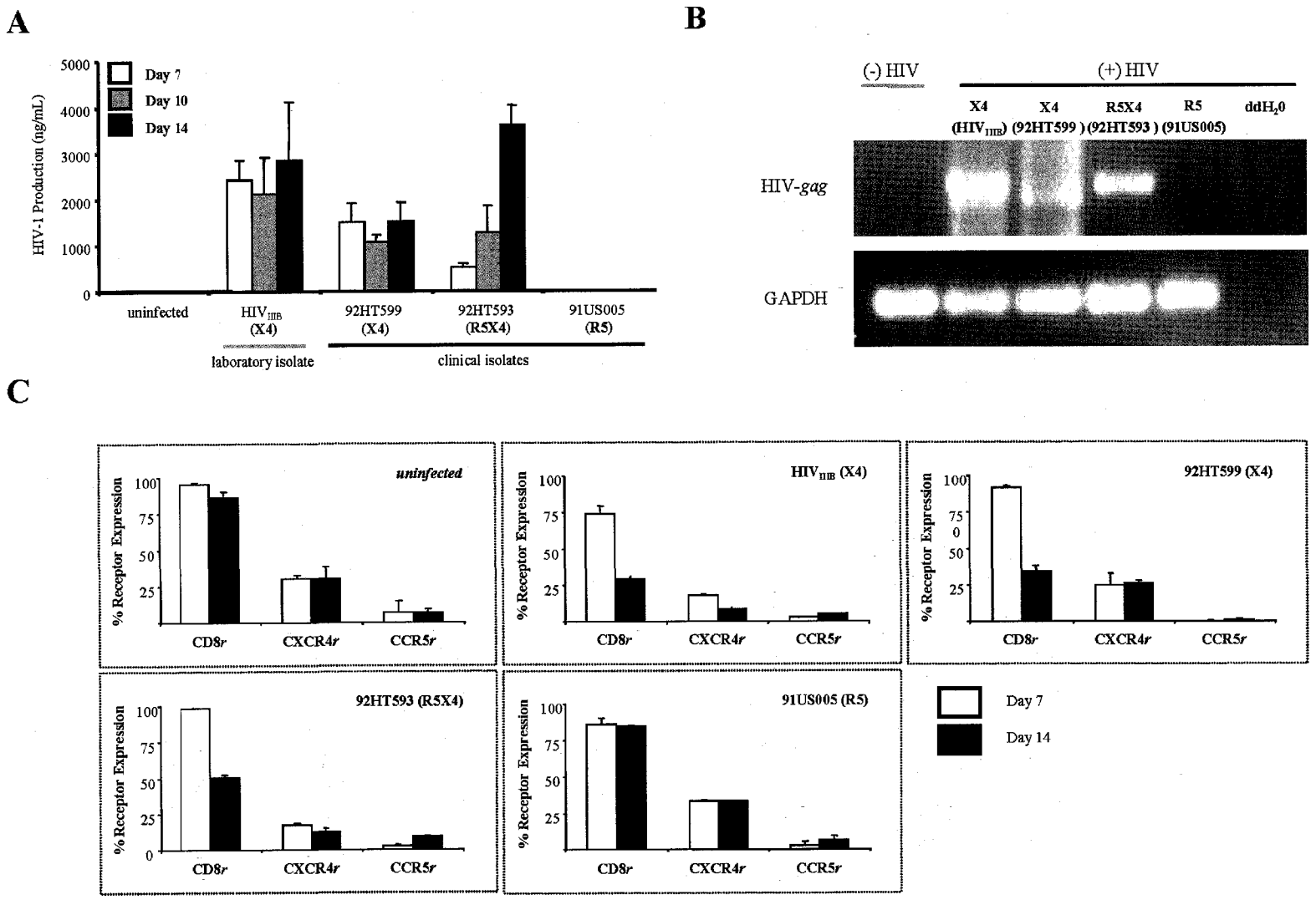
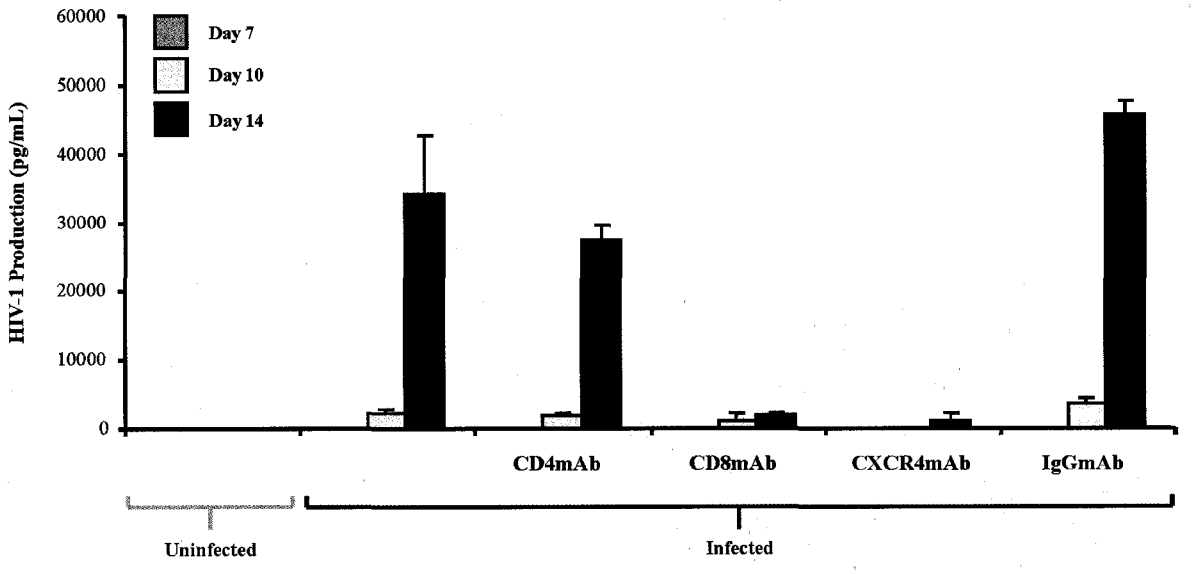

A
HIV-1 Production (ng/mL)
5000
4000
3000
2000
1000
0
Day 7
Day 10
Day 14
uninfected
HIV IIIB (X4)
92HT599 (X4)
92HT593 (R5X4)
91US005 (R5)
laboratory isolate
clinical isolates
B
(-) HIV
(+) HIV
X4 (HIV IIIB)
X4 (92HT599)
R5X4 (92HT593)
R5 (91US005)
ddH2O
HIV-gag
GAPDH
C
uninfected
HIV IIIB (X4)
92HT599 (X4)
92HT593 (R5X4)
91US005 (R5)
% Receptor Expression
100
75
50
25
0
CD8r
CXCR4r
CCR5r
Day 7
Day 14

Figure 28. CD8+ T-cell clone blocking studies. A CD8+ T-cell clone was pre-treated with the appropriate monoclonal antibody and subsequently infected with 300 $TCID_{50}$/mL of HIV_{IIIB}. Cells were assessed for HIV-1 replication at various days post-infection. Viral production was measured from cell-free supernatants using an ELISA. Results are expressed as the mean and SEM of three independent experiments.


60000
50000
40000
30000
20000
10000
0
HIV-1 Production (pg/mL)
Day 7
Day 10
Day 14
CD4mAb
CD8mAb
CXCR4mAb
IgGmAb
Uninfected
Infected

clones. Similarly, anti-CD4 antibody had no statistically significant effect on HIV-1 replication inhibition. The anti-CD4 antibody (clone RPA-T4) used in this study binds to the D1 domain of the CD4 molecule and has been shown to inhibit HIV-1 gp120 binding and syncytium formation. Our results suggest that the CD8 and CXCR4 molecules may act as receptors or part of a larger receptor complex involved in CD8+ T-cell clone mediated HIV-1 adsorption and penetration.

4. DISCUSSION

4.1. *ex vivo* ANALYSES OF HIV-1 INFECTION OF CD8+ T-CELLS: SIGNIFICANCE AND FREQUENCY

CD8+ T-cells are an important component of the immune response and an arbiter of cell-mediated immunity. CD8+ T-cells are involved in the recognition and elimination of foreign pathogens through both their CTL and CNAR abilities. In healthy individuals, the frequency of circulating CD8+ T-cells remains at a very low level until the immune system encounters a foreign pathogen. In the context of HIV-1 infection, during the early stages of disease, the absolute numbers and percentages of leucocytes and lymphocytes (including T-cells and their subsets) remains at a steady state. It is only after seroconversion that takes place in the initial few months, that the frequency of circulating CD8+ T-cells begins to rise dramatically [376, 377]. It has been estimated that the proportion of HIV-1 specific CD8+ T-cells is as high as 18% during established infection [153, 154]. Due to their highly specific CTL ability, it has been speculated that these cells may operate by killing the infected CD4+ T-cell population and thereby, not only partially controlling infection, but also simultaneously contributing to the destruction of the immune system [86, 378, 379], in addition to the anergizing and cytopathic effects of HIV-1 infection that have been established. As AIDS progresses and CD4+ T-cells are lost due to infection, the absolute numbers and functions of CD8+ T-cells begins to decline [76, 380]. In this final stage, the lymphocyte population is almost entirely composed of CD8+ T-cells [381] and it is at this stage that the functions that are normally attributed to CD8+ T-cells become diminished [382]. This directly contributes to the escape of the virus from immune containment as

CD8+ T-cells are involved in the clearance of infected cells and are strong mediators of viral suppression.

4.1.1. High Frequency of HIV-1 Infected CD8+ T-Cells in a Cohort of HIV-1 Seropositive Individuals

Research began with a prospective study of 28 HIV-1 seropositive individuals and 4 HIV-1 seronegative controls. The study subjects were chosen based upon their varying CD4 counts, varying viral loads and their experience with ART. The objectives of the prospective studies undertaken were to examine the frequency and significance of HIV-1 infection of CD8+ T-cells *ex vivo*. The presence of circulating CD8+HIV-1_{gag}+ T-cells was successfully demonstrated in all 28 HIV-1 seropositive individuals. Of great interest was the observation that these HIV-1 infected CD8+ T-cells were found at a significantly higher percentage than HIV-1 infected CD4+ T-cells in the overall majority of the study subjects (36.9% ± 10.0% vs. 26.4% ± 13.1% respectively). Flow cytometric analysis of the HIV-1 infected CD8+ T-cells demonstrated that these cells predominantly exhibited a CD8+CD4-HIV-1_{gag}+ phenotype as less than 1.5% of the infected T-cells analyzed from the PBMCs of the study subjects expressed the $CD4^{dim}CD8^{bright}$ phenotype. HIV-1 infection of CD8+ T-cells was further confirmed by RT-PCR analysis and viral quantitation. It was illustrated that CD8+ T-cells can be found productively infected *ex vivo* and can support HIV-1 viral replication, albeit at very low levels compared to their CD4+ T-cell counterparts isolated from HIV-1 seropositive individuals. Correlation between the frequency of CD8+HIV-1_{gag}+ cells and CD4-, CD8- T-cell counts and treatment was not observed, indicating that the presence of these infected cells is not associated with disease progression. However, the percentage of these HIV-1 infected CD8+ T-cells was consistently higher than the HIV-1 infected CD4+ T-

cell populations regardless of the study subjects CD4 counts, viral load or their treatment options. Statistical significance was shown in the patient groups with low CD4 counts and high viral loads.

4.1.2. CD8+HIV+ T-Cells: *The Role of Circulating CD4+ T-Cells in the Infection of CD8+ T-Cells by HIV-1*

Though the CD4 cell-surface molecule is considered the primary cellular receptor for the HIV-1 envelope glycoprotein, gp120, early studies showed that the requisite expression of the chemokine receptors (CXCR4 and CCR5 being the two most predominantly used by the virus) were required for successful binding and viral entry [16, 96]. In the ensuing years since the discovery of the CD4 receptor and co-receptors, numerous alternate HIV-1 receptors were identified and isolated from target cells [111, 113-115]. These early results and experiments led to the formulation of the hypothesis that CD8+ T-cells may serve as requisite targets of HIV-1 infection. In other words, if HIV-1 has been shown to be detected in and can infect cells of the immune system, such as B-cells, monocytes, macrophages and DCs [34], can the same assumptions hold true for infection of CD8+ T-cells, another cellular subset of the immune system? The results obtained from the patient studies results clearly demonstrated the presence of productively HIV-1 infected CD8+ T-cells *ex vivo*. However, these results were not the first to demonstrate the presence of these CD8+HIV+ cells during the course of HIV-1 infection. Very early studies demonstrated that CD8+CD4- cells from HIV-1 seropositive individuals could harbor the virus [28, 105, 106, 342]. These CD8+CD4-HIV+ cells were isolated from the peripheral blood and lungs of patients, most notably at sites of inflammation. These early reports also hypothesized that transmission of infection to CD8+ T-cells may have taken place due to interactions between productively infected CD4+

T-cells and CD8+ T-cells *in vivo* as a part of the immune response [30, 31, 117]. It is thus conceivable that the repeated contact that occurs between productively infected viral targets and HIV-1 specific CTLs in the immune environment could ultimately lead to the infection of CD8+ T-cells. This may possibly explain the findings obtained in our *ex vivo* patient study. Though the results demonstrated a high percentage of infection of CD8+ T-cells, a significant amount of viral presence was also detected in the CD4+ T-cell population. These CD4+HIV-1_{gag}+ T-cells yielded viral loads 2-3 logs greater than that of their CD8+HIV-1_{gag}+ T-cell counterparts. The viral load observed in the HIV-1 seropositive individuals, based upon the results obtained, was composed predominantly of virus produced by the infected CD4+ T-cell population. From our studies, these productively infected CD4+ T-cells could provide an important source and maintenance of HIV-1 infection in CD8+ T-cells as CD8+ T-cells isolated from lymphocyte populations demonstrated low levels of viral production that decreased over time. Thus, infected CD4+ T-cells may produce readily infectious HIV-1 progeny that can serve to infect CD8+ T-cells and other cells of the immune system. The substantial increase in CD8+ T-cell infection that is observed upon disease progression may thus be related to the phenomenon whereby host immune control is lost and HIV-1 spreads to other cellular subsets and non-lymphoid tissues [105].

Another factor that may contribute to the high levels of CD8+ T-cell infection that was observed during pathogenesis is the loss of the CD4+ T-cell subset from the peripheral blood. During the course of infection, there is an inversion of the CD4:CD8 T-cell ratio due to the loss of CD4+ T-cells. This inversion was also observed in the overall majority of our patients (Table I). Livingstone *et al.* first reported that in individuals with CD4 counts < 200 counts/mm^3, the majority of infected cells were within the DC and CD8+ T-cell populations

[105]. In the studies presented here, a significantly higher frequency of CD8+ T-cell infection in those individuals with CD4 counts < 300 counts/mm^3 was found.

HIV-1 encodes numerous strategies to evade the immune response and to increase viral dissemination. One such strategy utilized by HIV-1 is the direct killing of CD4+ T-cells (for a review of apoptotic strategies used by HIV-1, see [191]). The cytopathic effects of HIV-1 have not been clearly elucidated in the context of CD8+ T-cell infection (for a review, see [383]). By thereby depleting and eliminating a major target of the virus, HIV-1 may adapt and spread to other cell lineages such as CD8+ T-cells. This assumption is most evident in patients p23, p17 and p19 in the study. These patients all had CD4 counts below 100 counts/mm^3 and yet shared some of the highest levels of CD8+ T-cell infection in the study (p17, 44.9%; p19, 58.8%; and p23, 41.8%). Patient p23 also had one of the highest viral loads in the study subjects. This patient's isolated CD4+ T-cells were also high producers of virus as detected by the presence of HIV-1 RNA in their cell-free supernatants. Only patient p26 had a CD4 count less than 100 counts/mm^3 and also had a low level of CD8+ T-cell infection. As explained earlier, CD8+ T-cell infection of these patients may have occurred through CD4+ T-cell contact and viral release. Patient p26's low frequency of CD8+HIV-1_{gag}+ cells may be explained by the lack of readily available CD4+ T-cells that can serve as targets of infection and disseminators of viral release into the cell population. This explanation, however, cannot fully explain the results obtained in the study. High levels of CD8+ T-cell infection in patients p23, p17 and p19 indicate that this phenomenon is not correlated with viral stages of disease as all the patients had varying viral loads and treatment regimens. Furthermore, all the study subjects exhibited the presence of CD8+HIV+ cells, indicating that CD4+ T-cell-mediated viral transfer may occur during any stage of disease and may spread rapidly within a population during pathogenesis.

4.1.3. CD8+HIV+ T-Cells: *CD8-Tropism of HIV-1* in vivo

The tropism and ability of HIV-1 found in the cellular milieu to infect cells of other lineages and subsets may also explain the appearance of infected CD8+ T-cells *in vivo*. Recent studies by Saha and Zerhouni demonstrated the existence of viral strains isolated from the CD8+ T-cells of extremely sick HIV-1 seropositive individuals that could preferentially infect and were acutely cytopathic for CD8+ T-cells [26, 29, 384]. The authors noted that the viral isolates were able to replicate at comparable levels in both CD4+ and CD8+ T-cell populations, indicating that CD8+ T-cells can serve as equally good targets as CD4+ T-cells. Envelope sequencing of these viral isolates demonstrated extensive mutations and changes over the gp120 region. However, the authors could not find any consensus sequences that could explain the CD8-tropism of these viruses. Differences in envelope sequences was also observed from the CD8+ T-cell population in 10 of 13 patients on HAART [104]. The authors noted that the CD8+ T-cells showed lower levels of drug resistance to protease and reverse transcriptase inhibitors, suggesting a lack of viral replication in this population [30]. In accordance, the results from the studies conducted here showed this lack of viral replication in the CD8+ T-cell population of those patients not only on ART, but those that were treatment naïve as well. The existence of CD8-tropic viral strains and the frequency at which these strains may exist in the viral quasispecies may explain the varying levels of CD8+ T-cell infection that we observed in the *ex vivo* patient study undertaken here. Results illustrated that some of the patients had high frequencies of CD8+ T-cell infection (p19, 58.5% CD8+HIV-1_{gag}+) and others had low frequencies (p26, 15.2% CD8+HIV-1_{gag}+), indicating the presence of CD8-tropic strains and lending credence to the possibility that these strains exist at varying levels in HIV-1 seropositive individuals. Thus, there is the possibility that certain individuals may be more susceptible to CD8+ T-cell

infection than other individuals depending on the viral tropism in the cellular milieu. This possibility is echoed in the *in vitro* studies presented here and discussed further on, where differences in HIV-1 replication were observed in CD8+ T-cells from various volunteers. If this is truly the case, then markers and features of these CD8+ T-cells from HIV-1 infected individuals will have to be studied more closely in the future.

4.1.4. CD8+HIV+ T-Cells: *Modulation of CD4 Expression by HIV-1*

Another possible explanation for the presence of CD8+HIV-1_{gag}+ cells in HIV-1 infected individuals was first demonstrated in studies with Severe Combined Immunodeficiency (SCID) mice [338]. It was discovered in these experiments that only a fraction of mature CD8+ T-cells (CD3+CD8+) contained HIV-1 proviral DNA and active viral transcription. The authors found that infection of CD8+ T-cells in the SCID-hu mouse model did not involve cell-to-cell contact, but rather the expression of the CD4 cell-surface molecule, postulating the role of precursor cells in HIV-1 infection in the thymus. On a side note, 1 of the 3 primary isolates used in the studies with SCID-hu mice exhibited preferential tropism to CD8+ T-cells similar to the isolates discovered by Saha and Zerhouni. During thymic maturation of T-lymphocytes, a CD4+CD8+ immature T-cell phenotype exists. Expression of one of these molecules, and the subsequent down-regulation of the other, is indicative of a successful selection of the cell to either a helper or cytotoxic T-lymphocyte lineage. However, evidence of $CD4^{dim}CD8^{bright}$ (in addition to the reciprocal $CD4^{bright}CD8^{dim}$) T-cells have been found outside the thymus [27, 385] in the peripheral blood [340] and lungs [106, 342] of HIV-1 seropositive individuals. It has been estimated that 3% - 5% of PBMCs express both the CD4 and CD8 cell surface molecules [343]. In addition, *in vivo* and *in vitro* studies have shown that antigenic/mitogenic stimulation [119-

121, 124], DC stimulation [27, 121] and viral infection (CMV, Herpes Simplex Virus (HSV), HTLV-I and Human Herpesvirus (HHV)-6) [341, 374] have all shown the ability to induce the *de novo* expression of the CD4 molecule on the surface of CD8+ T-cells. These $CD4^{dim}CD8^{bright}$ T-cells were all found to have a higher activation state [120-124]. Furthermore and more importantly, the expression of CD4 on CD8+ T-cells would allow a route for successful HIV-1 viral binding and entry into these cells [119, 122, 124]. The DP cells also showed greater levels of co-receptor expression than their unstimulated and single-positive counterparts [124]. Zloza *et al.* and Kitchen *et al.* both found a higher degree of expression of the CXCR4 and CCR5 co-receptors on $CD4^{dim}CD8^{bright}$ T-cells, thereby providing a viable mechanism for HIV-1 binding, fusion and entry [122, 124]. The up-regulated CD4 molecule was shown to be functional and associated with p56lck by Kitchen *et al.* and could play a role in trafficking and distribution of these DP cells to sites of inflammation *in vivo* [119, 121]. The $CD4^{dim}CD8^{bright}$ T-cells can then serve to disseminate virus to other potential targets, thereby exacerbating the immune response. HIV-1 infection itself has been associated with hyper-activation of the immune system and could play a role in CD8+ T-cell activation that may result in CD4 up-regulation [123, 386]. In a patient cohort by Imlach *et al.*, the authors found a higher level of activation and CD4 expression on CD8+ T-cells in HIV-1 seropositive individuals regardless of the HIV-1 status of the subject [120]. In another study, the proportion of $CD4^{dim}CD8^{bright}$ T-cells was found to be expressed at low levels (0.8% to 3.3%) in 13 HIV-1 seropositive study subjects and was also found to be correlated with disease [123]. However, the authors discovered that there was an inverse correlation between HIV-1 DNA burden in CD8+ T-cells and CD4 counts, indicating an increase in CD8+ T-cell infection during the latter stages of disease.

In the studies of HIV-1 seropositive individuals presented here, very low levels of $CD4^{dim}CD8^{bright}$ T-cells (0.1% to 1.5%) in the PBMC populations of the study subjects were observed. Conversely, very high frequencies of CD8+ T-cell infection in these same study subjects (23.0% to 58.8%) were found. Thus, the contribution of DP cells to the levels of viral infection seen in the study of HIV-1 seropositive individuals would appear to be extremely minimal. These studies demonstrated that both of the infected T-cell populations were found to be phenotypically distinct. Recent studies by Sullivan *et al.* demonstrated that the induction of cells with the $CD4^{dim}CD8^{bright}$ phenotype was dependent upon the presence of CD4+ T-cells [118]. In our study, no association was observed between CD4 counts and DP cells as patients with high CD4 (p11, 935 counts/mm^3) and low CD4 counts (p19, 10 counts/mm^3) all demonstrated low levels of DP expression that could not account for the high levels of HIV-1 infected CD8+ T-cells found in these individuals. Cochrane *et al.* also did not find an association between $CD4^{dim}CD8^{bright}$ T-cells and the CD4 and CD8 lymphocyte counts [123]. Low levels of DP cells were also found in another study where only 6 (out of 150) HIV-1 seropositive individuals had levels of these cells higher than 5% [122]. The existence of DP cells in HIV-1 seronegative controls (c2 specifically) was also observed, indicating that HIV-1 infection alone in our study cannot account for the presence of these cells. However, we cannot discount the role that these DP cells may potentially play in providing a source of HIV-1 progeny and viral transmission. Furthermore, no correlation was observed between the frequency of CD4+ and CD8+ T-cell infection and the CD4-, CD8 counts and the viral load. The only trend that was observed was between the percentage of $CD4+HIV\text{-}1_{gag}+$ T-cells and the CD4 counts, though no statistical significance was observed. The lack of correlation between the levels of infection of both CD4+ and CD8+ T-cells and

clinical characteristics of HIV-1 seropositive individuals indicates that different factors influence the frequency of infection of these two cell types.

The presence of "contaminating" CD4+CD8- T-cells in our study would also appear to be extremely low and could not account for the levels of viral production and high frequencies of CD8+ T-cell infection observed. Productive infection analysis was performed on CD4+ and CD8+ T-cell subsets isolated through magnetic bead selection. Purity analysis showed that these populations were greater than 99% pure and devoid of any "contaminating" cells. Our isolation system used the Miltenyi MACS system as this system does not require the removal of the magnetic beads after separation for purity analysis or subsequent use in experiments due to their small size (50 nm) [387]. This cell isolation system's efficacy has been demonstrated by numerous studies [106, 120, 122, 123, 388]. A drawback to this method is that the system could not detect the presence (or exclusion) of CD4 cell-surface molecules that are down-regulated during the course of HIV-1 infection [167, 375]. However, moderate levels of CD4+ T-cell infection were still seen in our patient analysis, illustrating that not all CD4 molecules are affected by HIV-1 infection. In addition, as discussed above, only very low levels of $CD4^{dim}CD8^{bright}$ T-cells were detected in our experimental system. Thus, CD4 down-modulation cannot fully explain the levels of CD8+ T-cell infection in HIV-1 seropositive individuals as such a high percentage of observed CD8+ T-cell infection cannot be the result of the possible infection of DP cells which were found at a low level.

4.1.5. CD8+HIV+ T-Cells: *CD4-Independent Entry by HIV-1*

Though many studies have shown the infection of CD8+ T-cells through CD4-dependent mechanisms, an alternative explanation for widespread infection of these cells is

that HIV-1 infection may be through a non-CD4-dependent route of viral attachment and entry. A more plausible explanation for the high frequencies of CD8+ T-cell infection that was observed in the HIV-1 seropositive study subjects may be through this CD4-independent mechanism, due to the low levels of CD4 expression seen in the study population. Studies have shown that a wide range of cellular subsets and tissues that are CD4 negative are susceptible to HIV-1 infection, such as fibroblasts, epithelial cells and CD4- human cell lines [111, 383]. A recent study by Kaiser *et al.* reported that productive HIV-1 infection in PBMCs took place predominantly in CD4-CD8- double-negative cells [389]. It has been reported that cellular membrane proteins are incorporated into the viral envelope during the process of viral budding and exit from the infected cell [339, 390]. This may allow the possibility of these newly incorporated cellular proteins in the viral envelope to facilitate interactions with receptor and co-receptors independent of the CD4 molecule on target cells. The CD8 molecule has already been shown to be incorporated into the HIV-1 envelope [339, 391]. In addition, one study demonstrated the HIV-1 env-independent infection of CD4-negative cells [111]. The viral strains isolated by Saha and Zerhouni from AIDS patients also showed CD8+ T-cell infection independent of CD4 expression [26, 29, 384]. Two of these viral isolates were able to down-regulate CD8 expression in CD8+ T-cells (and a CD8-expressing T-cell line) and viral infection was abrogated by anti-CD8 antibodies, suggesting that these isolates used CD8 as a receptor. Another viral isolate obtained by this group was able to successfully infect U87 cells independent of CD4, CD8 or co-receptor expression, possibly through an as of yet unidentified receptor. The use of the CD8 molecule as a receptor was also discovered when the gp105 molecule of HIV-2 was studied [109, 392]. The authors found that the HIV-2 gp105, but not HIV-1 gp120, was able to bind to CD8+ T-cells through the CD8 molecule. They also found that the bound CD8 molecule was

functional and associated with p56lck. Thus, CD8+ T-cells could be productively infected by HIV-1 independent of CD4 expression as both resting and activated CD8+ T-cells express the CXCR4 and CCR5 co-receptors required for proper viral attachment and fusion [393, 394]. Both primary CD8+ T-cells and CD8+ T-cell clones expressed the CXCR4 chemokine receptor at significant levels from our *in vitro* studies, thereby providing a mode in CD8+ T-cell tropic HIV-1 entry and infection.

4.1.6. CD8+HIV+ T-Cells: *Supporting Studies*

A similar set of experiments was carried out recently by another group of researchers [339]. The authors, like us, examined the frequency of CD8+ T-cell infection *ex vivo*. They found that CD8+ T-cells were targets of HIV-1 infection and replication and accounted for approximately 15% of the plasma viral load. Concurrently, the authors did not find any association or correlation between the CD4 counts and the CD8-derived plasma virus. Correlation was found with plasma viral load however. Differences observed between the two study groups, ours and Hughes *et al.*, may be explained by methodology and patient sample size. Both studies used sensitive techniques to ascertain the levels of CD8+ T-cell infection that had not been fully explored by previous studies. Flow cytometric analysis, RT-PCR and HIV-1 RNA quantitation techniques were used in our studies. Conversely, Hughes *et al.* assayed *ex vivo* infection through the use of a novel virus immunocapture assay and proviral load quantification. Where the two groups differed was in how the researchers rationalized CD8+ T-cell infection *ex vivo*. Hughes *et al.* examined the phenotype and envelope composition of virus isolated from HIV-1 seropositive individuals, whereas our study examined the tropism and susceptibility of T-cell populations to HIV-1 infection.

However, both studies demonstrated the presence of productively HIV-1 infected CD8+ T-cells *ex vivo*.

4.1.7. Concluding Remarks

The most likely explanation of the high frequency of HIV-1 infected CD8+ T-cells found in HIV-1 seropositive individuals may be a possible combination of all the explanations and theories discussed above. There is an ongoing battle between the immune control of the virus and viral fitness in the host immune system. Numerous alterations and mutations occur in the viral quasispecies on a daily basis in order for the virus to survive and to better adapt and replicate in susceptible cells. Thus, the viral quasispecies is composed of a number of viral strains that each have their own phenotypic characteristics. Therefore, CD8+ T-cells may possibly be infected through viral mutation, cell-to-cell contact, $CD4^{dim}CD8^{bright}$ T-cells, CD4-independent mechanisms or through some combination of the above.

Our research clearly established the presence of HIV-1 infected CD8+ T-cells *ex vivo* and suggested that CD8+HIV+ T-cells serve as a viral reservoir during the early and late stages of disease. Isolated CD8+ T-cells from study subjects all demonstrated viral production regardless of their CD4 counts and viral loads (no correlation was observed however). Though there was a higher frequency of HIV-1 infection in CD8+ T-cells of seropositive individuals, these cells contributed minimally to the viral load. The establishment of a latent reservoir for HIV-1 has already been noted in CD4+ T-cells from patients undergoing HAART [395] and the same phenomenon may also be evident in CD8+ T-cells. The contribution of CD8+ T-cells as a possible viral reservoir in the host immune system has been demonstrated before. However, these studies were limited to the analysis of

HIV-1 DNA and integrated/unintegrated forms of the virus. These early studies demonstrated that CD8+ T-cells from the peripheral blood and lungs of HIV-1 infected individuals could harbor detectable amounts of integrated HIV-1 proviral DNA and linear and circular forms of unintegrated viral DNA [30, 106]. The detection of proviral sequences were also found in CD4-CD8+ cells from HIV-1 patients, though these same sequences were also observed in $CD4^{dim}CD8^{bright}$ cells [120, 123]. McBreen *et al.* found proviral copies of HIV-1 more frequently in the naïve subset than the memory subset of CD8+ T-cells [386]. Our research clearly illustrated active replication of HIV-1 in CD8+ T-cells by RT-PCR analysis and HIV-1 RNA quantitation in the cell-free supernatants of these cultured cells. The majority of our study subjects all had higher CD8 counts than CD4, thus potentially establishing a large pool of cells capable of being viral targets and a potential source of HIV-1 dissemination. From our results, the high degree of infection found in CD8+ T-cells and yet their low levels of viral production make this population of infected cells an ideal candidate for a viral reservoir that may serve to disseminate virus during the various stages of disease.

4.2. *in vitro* HIV-1 INFECTION OF CD8+ T-CELLS: SUSCEPTIBILITY AND EFFECTS ON CD8+ T-CELL FUNCTION

Despite the accumulating research (and controversy) over the nature of HIV-1 infection of CD8+ T-cells, there is still a lack of information with regard to the effects of viral infection on CD8+ T-cells and their functions. Though previous studies have examined the effects of HIV-1 infection on CD8+ T-cells and their functions, these studies were limited predominantly to *in vivo* observations and did not have the availability of sensitive techniques used currently. Many of these studies also focused on the specific and secondary cytolytic abilities of CD8+ T-cells in response to infection and failed to examine or acknowledge the role that CD8+ T-cell subsets such as memory cells play in controlling not only HIV-1 infection, but other viral infections as well. Therefore, we set out to examine the susceptibility of CD8+ T-cells and their subsets to *in vitro* HIV-1 infection and to examine the effects of infection on CD8+ T-cell function.

4.2.1. CD8+ T-Cells are Susceptible to *in vitro* HIV-1 Infection

Before answering the question of whether or not HIV-1 has an effect on the abilities and functions of CD8+ T-cells and their subsets, the experimental system designed in our research had to be tested for its ability to support productive HIV-1 infection. CD8+ T-cells were obtained through isolation from PBMCs of healthy volunteers. We observed that CD8+ T-cells were able to support viral infection and replication. To determine whether or not the infection was productive, we detected HIV-1 presence by RT-PCR analysis and treated the CD8+ T-cells with AZT, a viral replication inhibitor. We found infection to be productive as the presence of the reverse transcriptase inhibitor abrogated HIV-1 production. Furthermore, *in situ* RT-PCR analysis, like RT-PCR detection, demonstrated the presence of

in vitro virally infected CD8+ T-cells in the PBMC population. The nature of HIV-1 infection required the use of this technique as the HIV-1 provirus frequently integrates into the host cell genome and establishes a persistent viral infection. Furthermore, these cells are characterized, amongst other things, by a transcriptionally silent state that allows these HIV-1 infected cells to escape immune surveillance. These latently infected cells play an important role in the future as providing a reservoir for the virus. Thus, *in situ* RT-PCR was used to detect the presence of HIV-1 transcripts that may be found in low-copy numbers, in CD8+ T-cells.

Of interest was the observation that CD8+ T-cells supported higher levels of replication with T-cell tropic isolates and strains of HIV-1. Similarly, CD4+ T-cells also showed a preferential susceptibility to infection with T-cell tropic strains *in vitro* [95]. An observation we found throughout our experiments were the differences in viral production and kinetics when CD8+ T-cells were isolated from numerous volunteers. This phenomenon is not restricted solely to CD8+ T-cells as it is also apparent when CD4+ T-cells and macrophages of different individuals are infected *in vitro* [396, 397]. Our results with T-cell tropic strains and isolates were expected as both CD4+ and CD8+ T-cells express high levels of the chemokine receptor CXCR4 that is predominantly used by the T-cell tropic HIV-1 strains for binding and entry [398]. Specifically, the T-cell tropic laboratory strain HIV_{IIIB} resulted in the greatest replication and production in the isolated CD8+ T-cell population. Thus, this strain was used subsequently throughout the remainder of our experiments as it would be the most useful strain to examine the effects of productive HIV-1 infection on CD8+ T-cells. Interestingly, the M-tropic laboratory strain, and to a significantly lesser extent the M-tropic clinical isolate, were also able to successfully infect CD8+ T-cells. This may be explained by the presence of moderate CCR5 expression on the surface of the CD8+

T-cells. Recently, it was demonstrated that the CD4 receptor was associated with the chemokine receptor CCR5 on the cell surface [98]. This association was not dependent on the presence of gp120 or any other receptor-specific ligand. Though it has not been observed with the CD8 molecule to date, this suggests a possible route of M-tropic HIV-1 binding and entry into these cells if the CD8 molecule would also be found to co-localize with the CCR5 receptor or another receptor/co-receptor. Interestingly, the CD8+ T-cell clones were unable to support productive infection of M-tropic (R5) clinical isolates even though very low levels of CCR5 expression were observed on the surface of these cells. The failure of these cells to support M-tropic infection may be due to the restricted entry of M-tropic strains of HIV-1 into HTLV-I transformed cells [399]. Conversely, suitable levels of CCR5 may not have been present at the extracellular surface to allow proper viral binding and fusion. The envelope of SIV has been discovered to bind and infect target cells through CCR5 and independent of CD4-usage [400-402]. HIV-1 clinical isolates have also been shown to productively infect cells independently of CD4 and through the use of chemokine receptors [403-405]. Some viral strains were found to infect CD4- cell lines independent of HIV-1 envelope expression [111].

A consistent feature of infection with the T-cell tropic and macrophage tropic laboratory strains and the T-cell tropic clinical isolate was the increase in viral production over time. Infection with the macrophage tropic clinical isolate resulted in a time-dependent decrease in viral production. This may be due to the inability of the clinical isolate to sufficiently utilize CCR5 of the positively selected CD8+ T-cells. In examining HIV/AIDS patients, it is known that M-tropic viruses persist throughout the infection, whereas viruses that exploit CXCR4 emerge late in the course of disease. It can be postulated in an *in vivo* setting that CD8+ T-cells may be infected at low levels with M-tropic virus and this low

level of infection may persist throughout viral pathogenesis. However, near the end stages of disease, there may be a shift in the preferential infection of CD8+ T-cells by CXCR4-utilizing viruses.

4.2.2. CD8+ T-Cell Infection Independent of CD4 Expression

Our experimental results have shown that CD8+ T-cells were able to support productive HIV-1 infection. Since the CXCR4 co-receptor was highly expressed on the surface of the CD8+ T-cells and the affinity of gp120 for the CD4 molecule had already been established, we examined the possibility of CD4 up-regulation on the surface of CD8+ T-cells during infection, similar to our patient studies. The up-regulation of CD4 has already been observed in other viral infections [406, 407]. Based upon mean fluorescence values of gp120 co-expressing CD8+ T-cells, one report has suggested that CD8+ T-cells were low producers of HIV-1 [30]. These results were further supported by our *ex vivo* studies. In addition, our results indicate that CD8+ T-cells supported approximately 10-fold lower levels of *in vitro* HIV-1 replication than what was routinely observed in CD4+ T-cells. A possible explanation for this observation may be due to the low expression of the CD4 molecule on the surface of CD8+ T-cells and subsequent down-regulation of this receptor following infection. However, in our experiments, increases in CD4 expression by CD8+ T-cells over time in culture did not show a dependence upon infection. In fact, up-regulation and detection of the CD4 molecule was not observed in the *in vitro* HIV-1 infected CD8+ T-cell population. Of note was the decrease in the mean fluorescence of the CD8 cell-surface molecule in the infected population. This was most likely due to the cytopathic and anergizing effects of the culturing of CD8+ T-cells in the presence of HIV-1. These results were further substantiated in our later studies with CD8+ T-cell clones (discussed further

on). In addition, the contribution of the CD8+CD4+ T-cells in the isolated CD8+ T-cell population during HIV-1 infection was minimal (approximately 1.5%). Similar results were found in our study of HIV-1 seropositive individuals. Modulation of CD4 expression was found to be the same both *ex vivo* isolated and *in vitro* infected CD8+ T-cells, indicating that the effects of HIV-1 infection on CD8+ T-cells occurs independently of culture conditions or HIV-1 status of an individual.

Brenchley *et al.* observed that the number of HIV-1_{gag} DNA copies in $CD4^{dim}CD8^{bright}$ lymphocytes were 5 to 100 fold more than in the CD8+CD4- lymphocytes [408]. However, the majority of cells that were infected with HIV-1 in our *in vitro* experiments were of the CD8+CD4- phenotype. These findings suggest the possibility of an alternate portal of entry that may be used by HIV-1 to enter CD8+CD4- T-cells. The role of the CD8 cell-surface molecule in HIV-1 infection cannot be discounted as recent evidence has shown that CD4-independent HIV-1 variants exist in AIDS patients and these viruses preferentially infect CD8+ T-cells using CD8 as a receptor [26, 29] (discussed in Section 4.1.3 and Section 4.1.5). Another factor limiting the replication of HIV-1 in CD8+ T-cells may be the differences in the strength of signal transduction pathways between CD4+ and CD8+ T-cells [233, 409].

4.2.3. HIV-1 Infection Induces Cell Death but Does Not Impair CTL Function in CD8+ T-Cells

HIV-1 replication persists in target cells and lymphoid tissues despite the vigorous immune response. HIV-1 specific CD8+ T-cells, which can identify and eliminate virally infected cells through their CTL and CNAR functions, exist at high frequencies in the majority of HIV-1 seropositive individuals. It has been reported that

CD8+ T-cells produce IFN-γ, a strong mediator of the antiviral state, from as low as 0.8% induction to as high as 18% in response to various HIV-1 proteins [154]. The question is then addressed as to why antiviral CD8+ CTLs fail to prevent or control productive HIV-1 infection. Numerous studies have shown the various defects in CD8+ CTL-mediated control of HIV-1, from the impairment of the lytic ability of CD8+ T-cells, to anergy, dysfunction in antigen presentation, maturation defects, reduced cytokine/chemokine production to defects in T-cell signaling (for a comprehensive review, see [349]). Recently, it has been hypothesized by Petrovas *et al.* that the impairment of the maturation and effector function of HIV-1 specific CD8+ CTLs may be explained by apoptosis [410]. Though the cytopathic effects of HIV-1 infection on CD8+ T-cells has been known for over 10 years, apoptosis induction of HIV-1 specific CD8+ CTLs was not known until recently. HIV-1 specific CTLs were shown to be highly susceptible to CD95/Fas-mediated apoptosis [192] and exhibited reduced levels of Bcl-2 and Bcl_{XL} [197], two anti-apoptotic proteins. The studies by Mueller *et al.* demonstrated that HIV-1 specific CD8+ T-cells were 3-fold more prone to apoptosis than CMV-specific CD8+ T-cells [192]. Our results showed that a general *in vitro* HIV-1 infection of CD8+ T-cells resulted in the appearance of DNA fragmentation, thus indicating the cytopathic effects of HIV-1 on these cells. However, these results did not translate into a defect in CD8+ T-cell function as observed in a heterologous MLR. The HIV-1 infected CD8+ T-cells were found to have greater cytolytic abilities than their uninfected counterparts. A drawback to this assay was that it only measured the non-specific ability of CD8+ T-cells to lyse target cells. It was not an accurate measurement of the function of HIV-1 specific CD8+ CTLs. Overall, impairment of CD8+ T-cell function and viability may be enhanced in the HIV-1 specific CD8+ T-cell compartment, leaving functions of non-HIV-1 specific CD8+ T-cells unaffected.

4.2.4. HIV-1 Replication in CD8+ T-Cells Occurs Predominantly in the Memory T-Cell Subset

Levels of HIV-1 replication were examined in CD8+ T-cells co-expressing CD28, CD38 or HLA-DR. These subsets were selected for study due to their significance in asymptomatic infection and disease progression. From our results, we did not see an increase in susceptibility to HIV-1 infection in the CD28+, CD38+ or HLA-DR+ CD8+ T-cell subsets. It may be argued that the observation that the positively selected populations were unable to support full HIV-1 replication was due to an inherent artifact present in the selection process with conjugated beads. However, when the memory and naïve subsets were examined later on, the positively selected memory population resulted in the higher level of HIV-1 production, thereby discounting the notion that positive selection hindered susceptibility to infection. Interestingly, these positively selected subsets showed a decrease in viral kinetics over time when compared to the negative fractions. The decrease in replication kinetics may be due to the inability of these cells to support more than a minimal level of replication. Thus, without any suitable cellular target, the virus would eventually be unable to propagate. Though our results did not show any significant HIV-1 replication in the CD8+CD38+ subset, a recent study revealed that HIV-1 specific CD8+ T-cells were skewed toward a CD8+CD38+ phenotype and these cells were more sensitive to Fas-induced apoptosis [411]. A strong correlation between HIV-1 specific CTLs and CD8+CD38+ T-cells was also found in that study.

Of note are the levels of HIV-1 replication in the CD8+CD28- subset. The number of these cells increase early on and throughout the course of infection, [412, 413] suggesting that the CD8+CD28- T-cells may be involved in the control of virus replication. If the CD8+CD28- cells are indeed involved in the suppression of HIV-1 replication and, based

upon our observations, show an increase in susceptibility to HIV-1 infection, then a key subset of CD8+ T-cells involved in fighting disease may be hampered or eliminated early on in the course of disease. This would aid the virus in escaping immune containment and further aiding AIDS pathogenesis.

As mentioned earlier, high levels of replication of HIV-1 were found in the CD8+CD45RO+ memory T-cell subset when compared to the CD8+CD45RA+ naïve T-cell subset. Both the CD8+CD45RO+ and CD8+CD45RA- memory T-cell populations showed significantly higher levels of replication than the CD8+CD45RO- and CD8+CD45RA+ naïve T-cell populations. However, previous research demonstrated that the CD8+CD45RA+ naïve subset was found to have a higher frequency of infection than the memory subset *in vivo* [386]. In addition, the naïve CD8+ T-cell population has also been shown to be the most susceptible to HIV-1 infection following activation-induced up-regulation of the CD4 cell-surface molecule by antibodies to CD3 and CD28 [124]. While susceptibility to productive infection was observed in the CD8+CD45RA+ naïve T-cell subset *in vitro*, our results indicate that HIV-1 replicated more efficiently and productively in the memory phenotype of CD8+ T-cells in the absence of activation-induced up-regulation of CD4 surface expression. In CD4+ T-cells, it has been well documented that the CD45RO+ memory population serves as the main contributor to the pool of infected T-cells *in vivo* [414, 415]. It was also found that activation or antigenic stimulation of CD4+CD45RO+ T-cells rendered them susceptible to the cytopathic effects of HIV-1 [416]. Based upon our *in vitro* results, infection of the CD8+CD45RO+ memory T-cells resulted in the up-regulation of activation markers, most notably CD28. The increase in the activation state of susceptible memory cells during infection may result in bystander T-cell activation [417, 418]. Bystander activation of CD8+ T-cells has been shown to not only increase cytokine

production [419] and proliferation [420], but has also been shown to sensitize cells to the anergizing effects of the virus [421, 422]. This may explain the failure of memory T-cells to respond to recall antigens during acute and persistent viral infections. Conversely, the down-regulation of CD3 and CD28 on HIV-1 specific CTLs has been shown to result in a defect in TcR activation, thereby contributing to the state of anergy observed in HIV-1 pathogenesis [247, 423]. The low level of HIV-1 replication in CD8+CD45RA+ naïve T-cells may be explained by the observation in CD4+ T-cell studies that infection of naïve CD4+ T-cells by laboratory isolates of HIV-1 results in insufficient T-cell activation that is required for the reverse transcription and integration of the viral genome [393, 416, 424-426]. In addition, CD4+ T-cells express low levels of CCR5 that is required for the entry of NSI variants of HIV-1 [393]. These same observations may hold true for the infection of memory and naïve CD8+ T-cells *in vitro* that is reported here. Work done by Wills *et al.* may partly explain the high frequency of infection of CD8+CD45RA+ naïve T-cells *in vivo*. The researchers found that the CD8+CD45RA+ T-cells are not comprised solely of naïve T-cells, but also contain a significant proportion of memory T-cells that can change from a $CD45RO^{high}$ to $CD45RA^{high}$ phenotype *in vivo* [427]. Thus, during the course of HIV-1 infection *in vivo*, the memory T-cell subset may play a role as the initial proviral reservoir, but may subsequently revert to the naïve T-cell phenotype that has been observed in response to various stimuli [427, 428].

4.2.5. HIV-1 Infection Impairs the Memory CD8+ T-Cell Proliferative Capacity in the Presence of PHA

In order to assess the effects of HIV-1 infection on CD8+CD45RO+ memory T-cells, we examined the ability of the infected memory cells to proliferate in response to *in vitro* HIV-1 infection. It has been postulated that T-cell dysfunction could be attributed to the

altered sensitivity of CD8+ (and CD4+) T-cells to various co-stimulatory signals in HIV-1 infected patients. *In vivo* results have already demonstrated the significant increase in memory T-cell numbers in acute viral infections [429, 430]. These experiments were performed in the absence of any CD4+ T-cell help. A long-standing paradigm in immunology has been the requirement for CD4+ T-cell help in the expansion and maintenance of memory CD8+ T-cells *in vivo* [431, 432]. However, more recent evidence has demonstrated that CD4+ T-cell help was not required for the proliferation and persistence of fully functional antigen-specific memory CD8+ T-cells in the primary immune response [433]. Our findings demonstrated that HIV-1 infection of the CD8+CD45RO+ memory T-cell subset did not impair the proliferative response of these cells to the majority of the antigens used for stimulation. In the presence of PHA, the infected memory T-cell population showed a nearly 30% decrease in proliferation when compared to the uninfected memory cell population. PHA is a mitogen that has been show to induce the activation of T-cells. Similarly, HIV-1 infection may also result in the activation of T-cells. However, improper activation of T-cells may give rise to the triggering of the apoptotic pathway in the cell. Recently, Holm *et al* demonstrated that HIV-1 virions induced CD8+ T-cells to up-regulate CD25 and HLA-DR and preferentially induced apoptosis in the CD8+CD25+HLA-DR+ T-cell subset [354]. Our isolated CD8+CD45RO+ T-cells also showed an up-regulation of HLA-DR during *in vitro* infection. However, our results also demonstrated that HIV-1 infection only minimally decreased proliferation in response to antigenic stimulation when compared to the uninfected population. The lack of proliferation in the CD8+ memory T-cell population may be partly explained by the observations of Pantaleo *et al* [434]. The researchers found that the CD8+HLA-DR+ subset was severely defective in their proliferative response to anti-CD28, anti-CD3, anti-CD2, PHA and PMA. From our *in vitro*

results, HIV-1 infection only resulted in a noticeable decrease in proliferation in the cells stimulated with PHA. Therefore, strong mitogenic stimulation with PHA in the presence of HIV-1 resulted in the decrease in proliferation. This may have led to the triggering of an apoptotic pathway or anergy in the infected CD8+CD45RO+ memory T-cell population by an aberrant form of T-cell activation induced by PHA stimulation [435].

In addition, there was also a 3-fold increase in proliferation of the CD8+CD45RO+ populations, both uninfected and infected, when stimulated with antibodies to CD3 and CD28. *In vivo* studies have demonstrated that CD8+ T-cells from HIV-1 infected patients demonstrated a decrease in proliferation in response to PMA and antibodies to CD28 [436]. These CD8+ T-cells were thus found to be hypo-responsive at the level of proliferation and cytokine production. Similarly, the response to T-cell activation through super-antigen stimulation was found to be defective in both CD4+ and CD8+ T-cell subsets [437]. This observation was found to occur early during disease progression in the CD8+ T-cells and occurred in conjunction with both CD3 and CD28 co-stimulation and was also correlated with increased levels of apoptosis. Limited proliferative responses that were observed in our study were also observed by other researchers who found that gp120 stimulation led to only marginal proliferation of PBMCs in HIV-1 infected individuals [438]. In the same study, responses to the p24 antigen of HIV-1 led to a more robust response than to the envelope proteins. This may be due to the greater variability in the *env* proteins as compared to the *gag* proteins [263]. The observed increase in CD8+ T-cell proliferation after viral infection has been proposed to be due to the contribution of antigen-specific and bystander proliferation [420]. Recently, it was demonstrated that a generalized immune activation, rather than a homeostatic response, was responsible for the increased proliferation of naïve and memory CD4+ and CD8+ T-cells in HIV-1 infected individuals on HAART [319].

4.2.6. Concluding Remarks

In our *in vitro* system, CD8+CD45RO+ memory T-cells only showed an impairment of their proliferative capacity in response to HIV-1 infection in the presence of PHA stimulation. The observation that memory T-cells serve as one of the main reservoirs for the virus has profound implications as a high proportion of the expanded CD8+ T-cell population in HIV-1 infected individuals is comprised of CD45RO+ cells [439]. These memory cells have an enhanced susceptibility to cell death [421] and die upon bystander activation [418]. In addition, the CD45RO+ phenotype contributes significantly to the HIV-specific CTL response and these CD45RO+ CTLs are preferentially lost upon disease progression [302]. The effect of infection of CD8+ T-cells on CTL function, chemokine and cytokine production, and the susceptibility to apoptosis could have a significant impact upon disease progression.

4.3. MODULATION OF CELL-SURFACE MOLECULES ON CD8+ T-CELL CLONES IN RESPONSE TO *in vitro* HIV-1 INFECTION

After determining the significance and frequency of HIV-1 infection of CD8+ T-cells *ex vivo* in HIV-1 seropositive individuals (Section 4.1) and the susceptibility of CD8+ T-cells to *in vitro* infection (Section 4.2), attention was turned to identifying cellular receptor(s) on the surface of the CD8+ T-cells involved in viral adsorption and penetration. The discovery of a cellular receptor on the surface of these cells that would impart a route for viral entry has profound implications in understanding HIV-1 pathogenesis both *in vivo* and *in vitro* and also in identifying novel therapeutic strategies that would aid in treating the infection.

4.3.1. HTLV-I Transformation of CD8+ T-Cells

Before attempting to discover and identify extracellular receptors on CD8+ T-cells that would confer susceptibility to productive HIV-1 infection, we wished to normalize our experimental system. From the analysis of the 28 HIV-1 seropositive individuals, it was observed that the levels of CD8+ T-cell infection varied from as low as 15.2% to as high as 58.8%. Furthermore, no correlate was found to explain these variances as CD8+ T-cell infection in HIV-1 infected individuals was not associated with CD4- and CD8- cell counts, viral loads or treatment regimens. The ability of CD8+ T-cells from different individuals to support varying levels of HIV-1 production and replication was further extended to our *in vitro* studies where it was observed that CD8+ T-cells isolated from numerous individuals all supported viral infection at different frequencies. These differences may be attributed to the state of immune activation of the given individual at the time of CD8+ T-cell isolation or even to endogenous host cellular factors that may promote (or inhibit) the degree of HIV-1

infection. The variances in the ability of CD8+ T-cells to support productive HIV-1 infection was not solely restricted to these studies and has been previously observed by others [396, 397]. Thus, in order to normalize our experimental results, a CD8+ T-cell line was generated by the transformation of primary CD8+ T-cells from the PBMC population of a healthy volunteer.

Over the past 10 - 15 years, numerous strategies and techniques have evolved in order to establish long-term cultures of T-cells from the ectopic expression of the human telomerase reverse transcriptase gene [306, 307] to the use of viruses that have inherent transforming properties such as Herpesvirus saimiri [440-446] and HTLV-I [447-450]. A caveat of cellular transformation and immortalization by these viruses is the ability of the transformed cell to grow independently of stimulation with APCs or antigens/mitogens such as IL-2. HTLV-I was chosen due to its ease of use and availability. An advantage of HTLV-I transformation is its greater ability to establish a longer lifespan of human T-cell clones, maintenance of the cellular phenotype (i.e. cytotoxic or helper) and constitutive expression of IFN-γ [448, 451]. Thus, HTLV-I transformed T-cell lines are more indicative and serve as a suitable model of activated T-cells. However, disadvantages are also found with the use of this virus. One disadvantage of HTLV-I transformation is that infected T-cells may lose their CD3-TcR complex or cytotoxic potential [452-455]. The second disadvantage is that infectious virus may be produced by the HTLV-I transformed T-cell [456-458], thus complicating the interpretation of results. However, in our studies, as will be discussed further on, productive HTLV-I infection was not observed. Of note is that careful interpretation of experimental results must be taken as the HTLV-I transformed CD8+ T-cells clones may differ from "normal" activated CD8+ T-cells.

Primary blood-derived CD8+ T-cells from a healthy volunteer were immortalized by co-culture with UV-irradiated MT2 cells, an established HTLV-I transformed CD4+ T-cell line that is capable of producing readily infectious HTLV-I. CD8+ T-cells were transformed by co-culture as opposed to infection with cell-free virus because HTLV-I transmission *in vitro* requires cell-to-cell contact [457]. HTLV-I was the first characterized human retrovirus and it is the etiologic agent responsible for adult T-cell leukemia, a CD4+ T-cell malignancy in a small percentage of infected individuals [459, 460]. The virus, similar to HIV-1, is also associated with a variety of immunoregulatory disorders, including the neurodegenerative disease HTLV-I myelopathy/tropical spastic parapesis [461]. HTLV-I has been thought to primarily infect CD4+ T-cells [462], though infected CD8+ T-cells have been found at low frequencies in infected individuals [463]. Additionally, a reservoir of HTLV-I in symptomatic HTLV-I infected individuals was found in the CD8+ T-cell compartment [464]. In contrast, HTLV-II tropism has been less clearly studied but seems to favor CD8+ T-cells [465, 466].

Isolated CD8+ T-cells were successfully transformed by HTLV-I co-culture and CD8+ T-cell clones were established by limiting dilution. Before examining the modulation of cell-surface molecules by HIV-1, and hence identifying candidate host cell-receptors, the CD8+ T-cell clones were first characterized. The clones were predominantly of the CD8+CD4- phenotype and devoid of any DP cells. Previous studies by Macchi *et al.* illustrated that HTLV-I infection resulted in the appearance of a DP cell population and these cells were the preferential targets of immortalization in both isolated CD8+ and PBMC populations [374]. However, these same studies showed a time-dependent decrease in DP expression in culture. A possibility may therefore exist that our immortalized CD8+ T-cells may have exhibited a DP phenotype at a certain point during the transformation process, that

may have diminished through the subsequent weeks of culture. In fact, all the CD8+ clones illustrated the positive presence of CD4 transcripts, whereas CD4 protein expression on the cell surface was not found by flow cytometric analysis. This suggests that these cells may have expressed CD4 at one point or another. Modulation of cell-surface molecules by HTLV-I infection has been observed previously [467] and thus the lack of CD4 extracellular expression may be due in part to receptor shuttling dynamics or the down-regulation of a potential target receptor that has been well characterized in HIV-1 infection [167, 169-171, 375, 468, 469]. Another likely scenario is that CD4 expression on the CD8+ T-cell clone surface was at a level below the detection limits of flow cytometry.

The CD8+ T-cell clones also had an increased activation state characteristic of HTLV-I transformation. It has been noted that following HTLV-I infection and in the early stages of the transformation process, the IL-2 receptor and HLA-DR are up-regulated [454, 470], whereas CD3 is down-regulated and the transformed cells lose their antigen responsiveness and IL-2 dependence [453, 454]. The CD8+ T-cell clones in our studies all exhibited increased HLA-DR expression and IL-2 independence. Furthermore, a low level of CD28 expression was observed in the CD8+ T-cell clones. The CD28 co-stimulatory pathway is involved in IL-2 transcription/stabilization and promotion of T-cell survival [471]. Studies by Sholz *et al.* demonstrated that HTLV-I infected cells did not require the presence of CD80 or CD86 (the CD28 ligands) co-stimulation for proliferation and IL-15 and IFN-γ secretion [472], thereby suggesting that HTLV-I induced T-cell activation may substitute for CD28 co-stimulation.

4.3.2. Enhanced HIV-1 Production is Observed in the CD8+ T-Cell Clones

Infection of the CD8+ T-cell clones by the HIV_{IIIB} laboratory strain resulted in markedly different results in comparison with untransformed primary T-cells. All the CD8+ T-cell clones supported much higher levels of HIV-1 production and replication. Differences between the CD8+ T-cell clones themselves were also observed. These variances in the ability of transformed T-cells and T-cell lines to support HIV-1 production is not solely restricted to HTLV-I and has been observed in T-cells immortalized with various oncogenic viruses [371, 443]. Studies by Moriuchi *et al.* demonstrated that the Tax protein of HTLV-I was able to increase the expression and secretion of the β-chemokines MIP-1α, MIP-1β and RANTES [473, 474], thereby inhibiting M-tropic HIV-1 disease while enhancing T-cell tropic HIV-1 infection. The authors also found that Tax was able to enhance HIV-1 fusion and entry. However, our results demonstrated that the majority of the CD8+ T-cell clones (with Tri8.F being the exception), all supported similar levels of HIV-1 infection in comparison with untransformed T-cells shown by intracellular HIV-1 staining by flow cytometry. This is correlated with the lack of expression of HTLV-I Tax in our CD8+ T-cell clones.

Mitogenic stimulation by non-infectious HTLV-I virions was also shown to dramatically induce the production of large quantities of HIV-1 for *in vitro* HIV-1 infected PBMCs [475]. Similarly, numerous studies have suggested that co-infection with HTLV-I was found to exacerbate and accelerate disease progression in HIV-1 infected individuals [476-479]. HTLV-I is known to induce the expression of the IL-2 receptor and IL-2, thus providing one source of cellular activation [480] and the virions themselves have mitogenic properties [481]. The CD58 and CD54 co-stimulatory molecules were also found to be over-expressed in transformed T-cells [482, 483]. All these studies suggest that the increased

activation state and phenotype of the CD8+ T-cell clones may thus favor HIV-1 infection and replication.

Minimal to moderate levels of HIV-1 replication were observed in our CD8+ T-cell clones at the early time points of infection. At the later stages of HIV-1 infection of the clones, viral production and replication reached a maximum. It may be hypothesized that HIV-1 may have to overcome endogenous host factors expressed by the CD8+ T-cell clones first before maximal viral replication can be attained. Two components of the innate immune response have been identified as mediators of HIV-1 infection inhibition. The factors were identified as Trim5α and Apolipoproten B mRNA-Editing Catalytic (APOBEC) protein and the activities of these proteins were found to be involved in HIV-1 replication abrogation [484]. In the case of APOBEC, several groups have found that viral reverse transcripts made in the presence of this restriction factor were littered with mutations [485-487]. The APOBEC family of proteins possess "editing-like" functions whereby catalysis results in the hydrolytic deamination of cytidine and thereby converting it to uridine. These "U"-containing viral DNA transcripts may have two non-exclusive fates: *a.* they may be maintained as proviral sequences that are genetically compromised to such an extent that they would not yield infectious progeny; and *b.* the U residues may be recognized by cellular DNA repair enzymes and the viral DNA may be cleaved and degraded. Both APOBEC3G and 3F are expressed in primary T-cells [488-490] and exist in a primarily active state [491]. In order to overcome these restriction factors, the HIV-1 encoded protein, Vif, acts as an adaptor protein connecting APOBEC to the E3 ubiquitin ligase and thereby promoting polyubiquitination and proteasomal degradation of the protein [492-495]. Thus, a complex interplay exists between host cellular factors and the virus in an infected cell. In the case of the CD8+ T-cell clones, the lag in viral production observed may be attributed to the

amount of time it takes HIV-1 to overcome these restriction factors for successful productive infection to proceed. The presence of these endogenous restriction factors may also help to explain the low levels of HIV-1 production seen in our *ex vivo* and *in vitro* studies with CD8+ T-cells.

The transforming ability of HTLV-I has been attributed to the property of one of the encoded proteins of the virus, Tax [496-498]. Though a conflicting report has attributed the transforming properties to the HTLV-I envelope protein [499], initial studies found that Tax was both necessary and sufficient for *in vitro* immortalization of T-cells [500, 501]. HTLV-I Tax is able to enhance viral transcription in T-cells [463] and is involved in the induction and modulation of numerous cellular genes [496, 502, 503]. Early studies demonstrated an association between Tax and the HIV-1 LTR [502]. However, studies have suggested that Tax is a relatively weak enhancer of HIV-1 transcription [504]. It has the ability, however, to synergize with other inducers of HIV-1 infection to increase viral transcription and replication. Numerous viral transactivators have been illustrated to up-regulate expression of the HIV-1 LTR [505-508]. With these studies, it was postulated that enhanced Tax-mediated transcription of the HIV-1 LTR would be responsible for the dramatic increase in HIV-1 infection and replication in the CD8+ T-cell clones (Figure 20D). Though the HTLV-I genome was stably integrated into the CD8+ T-cell clones, no expression of the HTLV-I *tax* gene was found through RT-PCR analysis. These results indicated that Tax is not responsible for the enhanced HIV-1 production observed in the HTLV-I transformed CD8+ T-cell clones.

Another phenomenon observed during HTLV-I infection and transformation is the enhanced proliferation of HTLV-I infected CD8+ T-cells independent of IL-2 or any other mitogens both *in vivo* and *in vitro* [509-511]. Suppression of apoptosis has been proposed as

the mechanism involved in the transforming action of HTLV-I and of the lymphocyte proliferation observed [370]. A recent study demonstrated that resistance to apoptosis in HTLV-I infected cells was due to anti-apoptotic signaling that was attributed to the over-expression of chemokines [512]. Furthermore, HTLV-I T-cell lines showed reduced susceptibility to Fas [513] and to TNF-α stimulation [514]. This resistance may be due to the activation of NF-κB via the Tax protein [515] or the expression of anti-apoptotic genes [516, 517]. Proteins encoded by EBV [518], Adenovirus [519] and Sindbis Virus [520] all have shown to inhibit apoptosis. HTLV-I itself has a dichotomous effect on CD4+ and CD8+ T-cells as a recent study by Sibon *et al.* demonstrated that HTLV-I infection resulted in the recruitment of CD4+ T-cells into the cell cycle, while protecting the latter from cell death [369]. In fact, early studies found that transformed CD8+ T-cells were resistant to the cytopathic effects of HIV-1 [368]. The CD8+ T-cell clones used in this study proliferated at a rate higher than untransformed lymphocytes, yet consistent with that of an established CD4+ T-cell line, H9 cells. HIV-1 infection did not seem to alter the rate of proliferation significantly in the clones. However, the CD8+ T-cell clones produced roughly 2-fold more HIV-1 virus compared to the CD4+ T-cell line, indicating that the rate of proliferation and suppression of apoptosis induced by the HTLV-I transformation process is not solely responsible for the greater susceptibility of the CD8+ T-cell clones to HIV-1 infection and replication.

4.3.3. Down-Regulation of the CD8 and CXCR4 Molecules from the Surface of the CD8+ T-Cell Clones During Productive HIV-1 Infection

Focus was then turned toward identifying CD8+ T-cell clone extracellular molecules involved in mediating and enhancing HIV-1 binding and entry. Analysis of the three most

predominantly expressed cell-surface molecules and those associated with HIV-1 binding and entry (CD4, CXCR4 and CCR5) [521, 522] were studied in addition to the most predominantly expressed molecule on the surface of the CD8+ T-cell clones (CD8). Down-regulation of the CD8 and CXCR4 cell surface molecules was observed on the CD8+ T-cell clones during productive HIV-1 infection using both laboratory and clinical isolates of HIV-1. R5-tropic viruses failed to replicate in the T-cell clones and thus no modulation of extracellular molecules was found when cells were infected with this viral strain. Surface expression of CCR5 on the CD8+ T-cell clones was minimal and therefore concentration of this chemokine receptor may not have been sufficient to allow permissive HIV-1 infection, especially with the lack of CD4 expression found in these cells as well. Platt *et al.* demonstrated that in HeLa cells with low levels of CD4 expression, a much larger quantity of CCR5 expression is required for maximal HIV-1 infection by R5-tropic strains [523]. These studies, along with other studies suggest that cell-surface concentrations of extracellular receptors may play a role in altering tropism of HIV-1 infection [524-526]. Furthermore, increased production of the β-chemokines by the HTLV-I transformed CD8+ T-cell clones may have inhibited R5-tropic infection [473, 474]. The down-regulation of CD4 was observed in primary untransformed CD4+ T-cells during the course of *in vitro* infection, a consistent feature observed during productive HIV-1 infection [167, 171, 375, 527]. HIV-1 encodes numerous proteins and functions that are involved in down-regulating its primary receptor, CD4. HIV-1 infection itself has been shown to inhibit CD4 transcription [528]. Furthermore, CD4-gp160 complexes have been illustrated to be both sufficient and necessary to impair CD4 expression on the cell surface and the extent of this impairment was demonstrated to correlate with the levels of activation and production of gp160 [529-531]. Additionally, HIV-1 Vpu induces the intracellular degradation of ER-resident CD4,

decreasing its half-life from 6 hours to 12 minutes [532, 533]. Lastly, Nef has shown to interfere with numerous cell-surface molecules in order to aid viral pathogenesis [167, 534]. All these virally encoded proteins may play a role in the down-regulation, and hence lack of cellular expression, of the CD4 molecule (and other cell-surface molecules) in the HIV-1 infected HTLV-I transformed CD8+ T-cell clones. Likewise, HTLV-I may encode some as-of-yet unidentified protein(s) that has similar functions to these HIV-1 encoded proteins. These studies may be further extended to the CD8+ T-cell clone experiments in attempting to explain the down-regulation of the CD8 and CXCR4 molecules observed. Interestingly, a recent study by Stove *et al.* demonstrated that Nef down-regulated the surface expression of the β-chain of the CD8 molecule independently of CD4 down-regulation by accelerated endocytosis [535]. The authors also found that CD8 modulation was localized to the same motifs and residues in Nef involved in CD4 down-regulation, suggesting common molecular interactions.

The regulation of expression of a host cellular receptor for HIV-1 implies that this phenomenon is important for the fitness and survival of the virus. For example, decreased expression of a receptor may enhance viral particle release or prevent receptor incorporation into newly-formed progeny virus, thereby preventing aggregation of virions or the inactivation of the viral envelope. This has been observed during influenza virus infections where neuraminidase-associated inactivation of the viral receptor is critical for viral release [536]. Modulation of receptor expression may also alter the activation state of the infected cell through cell signaling pathways, thereby improving the cellular environment for infection and replication. Lastly, receptor down-regulation may protect an infected cell from receptor mediated apoptosis [90, 353] or from the effects of superinfection toxicity [537].

Overall, internalization of receptors may contribute to the subversion of the host immune system and disease progression.

The microenvironment on the surface of CD8+ T-cell clones may facilitate HIV-1 binding and entry. Our results demonstrated that treatment of the CD8+ T-cell clones with monoclonal antibodies to CD8 and CXCR4 independently, had an equal effect on the inhibition of HIV-1 infection, suggesting a possible close association between the two molecules. Recent studies have shown the co-localization and association between CD4 and CCR5 on the surface of the cellular membrane [98, 538]. These studies were extended to show further co-localization of CCR5, CXCR4 and CD4 in macrophages and T-cells [539]. Surprisingly, the authors found that these clusters of receptors were found frequently to be separated by less than a diameter of one HIV-1 virion, suggesting that such distributions are involved in facilitating co-operative interactions with HIV-1 during virus binding and penetration. In addition to the interaction between CD4 and CXCR4, it was revealed that CXCR4 interacts with the cytoplasmic domain of the α-subunit of CD8 and this may serve to amplify "cross-talk" between both signaling molecules [540]. This evidence may also raise the possibility of CD8 recruitment into HIV-1 gp120 adsorption/penetration complexes during infection. In our studies, antibodies to both CD8 and CXCR4 abrogated HIV-1 replication in CD8+ T-cell clones equally, indicating an association or close contact between the two molecules in these cells. The aforementioned examples of co-clustering and close association between cell-surface molecules all point to the existence of receptor (or protein) rich domains that may facilitate HIV-1 infection. These micro-rich environments were identified as lipid rafts. These domains play an important role in membrane trafficking, cell morphogenesis, signal transduction and are involved in the recruitment of the TcR complex [541-543]. A report by Popik *et al.* demonstrated that lipid raft integrity was required for

productive HIV-1 entry and viral penetration was mediated by CD4, CXCR4 and CCR5 co-localized in these rafts [99]. However, a conflicting report showed that the presence of lipid rafts is dispensable for HIV-1 infection [544]. Nevertheless, the CD8 molecule has been associated with lipid rafts [545] and was shown to be activated and involved in signaling in the presence of these rafts [546, 547]. Thus, CD8 association with lipid rafts may confer it opportunities to interact with not only other membrane receptors, but with the gp120-binding complex as well.

Early studies by Lusso *et al.* demonstrated that HTLV-I and HIV-1 co-infection broadened the spectrum of HIV-1 tropism to include CD8+ T-cells, B-cells, epithelial cells and skeletal muscle cells [548]. Both HIV-1 and HTLV-I have been know to integrate host cellular proteins into their membranes, thereby increasing the possibility of these viruses to adsorb to the cell surface of non-traditional targets [202, 339, 390]. The recent discovery of the cellular receptor for HTLV-I may also identify an alternative receptor for HIV-1 binding in the CD8+ T-cell clones. The receptor for HTLV-I was found to be Heparan Sulfate Proteoglycan (HSPG) and more specifically syndecans [366, 549]. Though HSPG is predominantly expressed on CD4+ T-cells, minimal expression was observed on the surface of CD8+ T-cells. Previous work indicated that the requirement for syndecans and HSPG in HIV-1 infection was accentuated when target cells express low levels of entry receptors on cells such as macrophages [550]. HSPGs themselves have been known to migrate into lipid rafts and these HSPGs have been implicated in HIV-1 binding and infection of T-cell lines, primary lymphocytes and macrophages [549-552]. Sapphire *et al.* found that CD4 expression also was not sufficient to support the initial adsorption of HIV-1 on macrophages and rather the abundantly expressed syndecans served as the main class of attachment receptor [550]. The association between HIV-1 and the HTLV-I receptors was shown by de

Parseval *et al.* where a highly conserved arginine residue at position 298 in the V3 loop of gp120 bound to the syndecan receptor [553]. Interestingly, this same residue was also shown to recognize and bind CCR5. One can thus envision that syndecans can substitute for CCR5 in the initial binding of HIV-1. These results demonstrate that HIV-1 can exploit this highly conserved residue in gp120 to utilize two entirely different receptors. Thus, syndecans and HSPGs may facilitate HIV-1 entry by concentrating CCR5 molecules poorly expressed on target cells. Additionally, syndecans and the chemokine receptors have been shown to exist as preformed complexes [554-556], thereby providing an enriched extracellular environment for HIV-1 adsorption and also providing a feasible route for HIV-1 infection in cells lacking CD4 expression such as the CD8+ T-cell clones. An interesting feature of syndecans is that HIV-1 bound to these molecules remains infectious for a week, whereas cell-free virus loses its infectivity after a single day [549]. These findings suggest that if a syndecan-rich environment exists in the CD8+ T-cell clones, this can provide a microenvironment that may amplify HIV-1 replication in surrounding cells.

4.3.4. Concluding Remarks

Caution must be applied in the interpretation of the results in order to not generalize the findings obtained from the infection of CD8+ T-cell clones to that of primary CD8+ T-cells. The CD8+ T-cell clones may differ markedly from primary T-cells due to the HTLV-I transformation process. However, these cells do provide a very useful tool and system in examining *in vitro* HIV-1 infection. The CD8+ T-cell clones may be considered as a "hyper"-model of CD8+ T-cell infection due to the high levels of HIV-1 replication and resistance to the cytopathic effects of the virus observed in these cells. The CD8+ T-cell clones can also provide a suitable model for the isolation of candidate viral receptors in the

hope of identifying novel therapeutic targets on primary CD8+ T-cells that can aid in preventing and treating disease.

4.4. LIMITATION OF DETECTION ASSAYS

Careful interpretation of the data and the results must be taken as each detection assay has its advantages and disadvantages. A striking observation from our analyses of HIV-1 seropositive individuals was that there was a higher proportion of CD8+HIV-1gag+ than CD4+HIV-1gag+ T-cells as detected by flow cytometry. Due to the controversy surrounding this area of research, additional studies are required to lend more credence to the novel findings. A limitation of flow cytometric analysis, though a powerful tool, is the sensitivity of the instrumentation. This was most evident in our analysis of cell-surface molecules as conflicting results were obtained with the flow cytometric data and the more sensitive RT-PCR analysis of the same molecules. Another limitation, in regards to the intracellular flow cytometry experiments, is the non-specific uptake of antibodies. The use of multiple anti-HIV antibodies would be required to test this limitation. However, we were not able to address this potential problem as the antibody used was the only one available commercially.

Throughout our studies, cells were isolated from the peripheral blood of HIV-1 infected individuals and healthy volunteers and manipulated. Ex vivo and in vitro handling of the cells may result in alterations of cellular functions and phenotype in comparison to in vivo analysis of cells inside their natural cellular environment. Therefore, diligence and careful analysis of the results and the conclusions made therein is essential.

4.5. FUTURE DIRECTIONS

Follow-up experiments to identify the interactions between HIV-1 viral proteins and candidate receptors (CD8 and CXCR4) on the surface of the CD8+ T-cell clones will have to be performed. These studies will require immunoprecipitation and short-interfering RNA (siRNA) knock-down analyses in order to further elucidate the role that these extracellular molecules may play in conferring susceptibility to HIV-1 infection. Of greater importance is the need to translate these studies in primary CD8+ T-cells.

The finding that HIV-1 seropositive individuals have a high frequency of circulating HIV-1 infected CD8+ T-cells, irrespective of their disease status, has profound implications in our knowledge of HIV-1 pathogenesis. Of interest will be the sequencing of the viral envelope of HIV-1 particles derived from the CD8+HIV+ T-cell compartment in order to determine if there is a consensus sequence for CD4-independent HIV-1 entry in CD8+ T-cells.

The studies presented here only touch upon the surface in the examination of HIV-1 infection of CD8+ T-cells. With recent studies by Hughes *et al.* [339] and Kaiser *et al.* [389] supporting our observations, hopefully new avenues and developments will open up in characterizing this novel cellular target of HIV-1 and identifying therapeutic strategies to prevent HIV-1 infection.

4.6. CONCLUSIONS

The events that mediate infection of CD8+ T cells have not been thoroughly studied. CD8+ T cells are important mediators of virus inhibition *in vivo* and their function is compromised in HIV-1 infection. Infection of these cells *in vivo* may contribute to their functional dysregulation. If CTL function could be restored, better control of viral replication would be attained. We presented here studies that examined the susceptibility and the effects of HIV-1 infection in CD8+ T-cells both *ex vivo* and *in vitro*. The identification of the mechanism of entry of HIV-1 into these cells may allow future studies to determine how entry may be blocked, thus improving CD8+ T cell responses to HIV-1 infection *in vivo*. Moreover, the identification of an alternate receptor for HIV-1 entry has the potential to lead to the development of new treatments for HIV-1 infection. Understanding the mechanism of HIV-1 entry into CD8+ T cells would contribute to our knowledge of HIV pathogenesis *in vivo*.

Over the past 25 years, the identification and characterization of HIV-1 and its effects has evolved and progressed rapidly. It is said that this virus is the most extensively studied and best-understood agent that causes human disease [557]. Many recent discoveries have provided new roads for improvement in prevention, vaccine development, treatment and care. With sufficient emphasis by researchers on not only recognizing approaches that target the virus, but also the host immune response, one can hope to see immune control of HIV-1 to a level of that seen by LTNPs and the eventual eradication of the viral disease from all parts of the world.

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CONTRIBUTION OF COLLABORATORS

Detection of HIV-1 *pol* transcripts (Section 3.1.5), quantitation of HIV-1 production in cell-free supernatants of HIV-1 seropositive individuals (Section 3.1.6) and detection of HTLV-I integration in CD8+ T-cell clones (Section 3.3.1) was done in collaboration with Dr. John E. Kim (Public Health Safety Agency of Canada, Ottawa, Canada).

AZT treatment (Section 3.2.1) and measurement of HIV-1 production and replication in CD8+ T-cell subsets (Section 3.2.4) were performed by Sowmya Balasubramanian.

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EDUCATION

Ph. D. Microbiology and Immunology
Thesis Title: Frequency and Significance of HIV-1 Infection of CD8+ T-Cells: Implications for Viral Pathogenesis
University of Ottawa, Ottawa, Ontario
September 2001 – December 2007

B.Sc. Hons. Biochemistry (*cum laude*)
Thesis Title: Determination of the Complement Control Repeat Regions of Decay Accelerating Factor Required for Enterovirus 70 Binding and Infection
University of Ottawa, Ottawa, Ontario
September1997 - May 2001

WORK EXPERIENCE

Research Associate, October 2007 – Present
Public Health Agency of Canada, Ottawa, Ontario
Involved in the implementation and formulation of research objectives and experimental assays. Supervised the day-to-day laboratory operations.

Teaching Assistant, November 2007 – September 2008
University of Ottawa, Faculty of Medicine, Ottawa, Ontario
Taught and supervised 1st year medical students in Problem Based Learning courses dealing with infection and immunity topics

Graduate Student, September 2001 – September 2007
University of Ottawa, Department of Biochemistry, Microbiology and Immunology
Ottawa Health Research Institute, Ottawa, Ontario
Examined the significance and susceptibility of CD8+ T-cells to HIV infection.

Laboratory Technologist, June 2001 – June 2003
GJS Mass Measurement, Fisher Scientific Ltd. Canada, Ottawa, Ontario
Involved in the calibration of weights and balances.
Responsibilities included the handling and processing of various customer accounts.

Research Assistant, September 2000 - May 2001
University of Ottawa, Ottawa, Ontario
Research in the characterization and properties of Enterovirus 70.

Emergency Room Assistant, Summer 1999
Ottawa Hospital General Campus, Ottawa, Ontario
Aided doctors and nurses in attending to the needs of the patients. Supervised waiting room and guided patients and visitors to proper locations. Adapted successfully to a high stress environment.

AWARDS/ ACHIEVEMENTS

Scholarships

Ontario Graduate Scholarship
Value: $15, 000 per year
Duration: May 2006 – April 2007

University of Ottawa Excellence Scholarship
Value: Tuition Fees
Duration: September 2006 – August 2007

Canadian Institutes of Health Research Doctoral Award
Research Proposal: The Effect of HIV Infection on CD8+ T-cell Function
Value: $22, 000 per year (3 years)
Duration: June 2003 – May 2006

University of Ottawa Excellence Scholarship
Value: Tuition Fees
Duration: September 2005 – August 2006

University of Ottawa Excellence Scholarship
Value: Tuition Fees
Duration: September 2004 – August 2005

University of Ottawa Excellence Scholarship
Value: Tuition Fees

Duration: September 2003 – August 2004

University of Ottawa Admission Scholarship
Value: $1, 200
Duration: September 1997 – April 1998

Awards

Keystone Symposia Scholarship
Keystone Symposia: Molecular and Cellular Determinants of HIV Pathogenesis (X8)
Awarded March 25th, 2007

Young Investigator Award
14th Conference on Retroviruses and Opportunistic Infections (CROI 2007).
Awarded February 25th, 2007.

Ottawa Health Research Institute Trainee Award
Awarded November 2006

Canadian Scholarship
XVI International AIDS Conference, Toronto, Canada
Awarded August 13th, 2006

University of Ottawa - Biochemistry, Microbiology and Immunology Graduate Poster Competition
Top 3 Finalist Award
Awarded May 18th, 2006

OHTN Scholarship
Ontario HIV Treatment Network, 8th Annual Meeting, Toronto, Canada
Awarded November 24th, 2005

OHTN Scholarship
Ontario HIV Treatment Network, 7th Annual Meeting, Toronto, Canada
Awarded November 25th, 2004

University of Ottawa - Biochemistry, Microbiology and Immunology Graduate Poster Competition
Top 3 Finalist Award
Awarded April 1st, 2004

OHTN Scholarship
Ontario HIV Treatment Network, 6th Annual Meeting, Toronto, Canada
Awarded November 3rd, 2003

Ottawa Health Research Institute Trainee Award
Awarded November 2003

University of Ottawa - Biochemistry, Microbiology and Immunology Graduate Poster Competition
Top 3 Finalist Award
Awarded April 11th, 2002

PUBLICATIONS AND CONFERENCE PROCEEDINGS

Publications in Refereed Journals

Gulzar, N., and Copeland, K. F. CD8+ T-Cells: Function and Response to HIV Infection. Current HIV Research. (2004). 2(1): 23-37.

Haddad, A., Nokhbeh, M. R., Alexander, D. A., Dawe, S. J., Grisé, C., **Gulzar, N.**, and Dimock, K. Binding to Decay-Accelerating Factor Is Not Required for Infection of Human Leukocyte Cell Lines by Enterovirus 70. J. Virology. (2004). 78: 2674-2681.

Gulzar, N., Diker, B., Mihowich J., Deschatelets, J., Arsenault M. E., Lamoureux, N., Cameron, W. D., Kim, J. K., Copeland, K. F. T. Proportion of HIV-1 Infected CD8+CD4- T Lymphocytes *in vivo*. Manuscript *in prep.*

Gulzar, N., Balasubramanian, S., Jiang, J., Beaudoin, G., Sanchez-Dardon, J., Munger, J., and Copeland, K. F. Preferential Infection of CD8+CD45RO+ Memory T-cells by HIV-1 and Their Proliferative Response to Antigens. Manuscript *in prep.*

Gulzar, N., Balasubramanian, S., Diker, B., Jiang, J., and Copeland, K. F. The Effect of HIV-1 Infection of CD8+ T-Cells on Cytokine Responses. Manuscript *in prep.*

Presentations in Refereed Conference Proceedings (Oral)

Gulzar, N., Diker, B., Shroff, A., Buberoglu, B., Klonowska, D., Kim, J. E., Cameron, D. W., and Copeland, K. F. Frequent Infection of Primary CD8+ T-Cells by HIV-1 *in vivo*: Significance in Viral

Pathogenesis. The 6th Annual Ottawa Health Research Institute Research Day. Ottawa, Canada. November 30th, 2006.

Gulzar, N., Shroff, A., Klonowska, D., Buberoglu, B., Kim, J. E., and Copeland, K. F. Productive HIV-1 Infection of CD8+ T-Cells is Associated with the Down-Regulation of the CD8 and the CXCR4 Cell-Surface Molecules: Viral Targets for Receptor-Mediated Entry?. Ontario HIV Treatment Network, 9th Annual Meeting. Toronto, Canada. November 27-28, 2006.

Gulzar, N., Shroff, A., Buberoglu, B., Klonowska, D., Kim, J. E., and Copeland, K. F. High Levels of HIV-1 Infection and Replication in CD8+ T-Cell Clones Results in the Down-Regulation of the CD8 and CXCR4 Receptors. XVI International AIDS Conference. Toronto, Canada. August 13-18, 2006.

Gulzar, N., Shroff, A., Klonowska, D., Kim, J. E., and Copeland, K. F. The Role of Extracellular Receptors in Human Immunodeficiency Virus-1 Infection of CD8+ T-cell Clones. Canadian Association for AIDS/HIV Research, 14th Annual Meeting. Vancouver, Canada. May 12-15, 2005.

Gulzar, N., and Copeland, K. F. Differential Replication Kinetics and Effects of HIV-1 Infection. Canadian Association for AIDS/HIV Research, 13th Annual Meeting. Montreal, Canada. May 13-16, 2004.

Gulzar, N., and Copeland, K. F. HIV-1 Infection of CD8+ T-cells. The 3rd Annual Ottawa Health Research Institute Research Day. Ottawa, Canada. November 2003.

Gulzar, N., Bernard, N. F., and Copeland, K. F. The Impact of HIV Infection on CD8+ T-cell Function. Canadian Association for AIDS/HIV Research, 12th Annual Meeting. Halifax, Canada. April 10-13, 2003.

Presentations in Refereed Conference Proceedings (Poster)

Gulzar, N., Shroff, A., Buberoglu, B., Klonowska, D., Kim, J. E., and Copeland, K. F. Host-Viral Interactions Induce the Down-Regulation of the CD8 and CXCR4 Receptors in CD8+ T-Cell Clones. Keystone Symposia: Molecular and Cellular Determinants of HIV Pathogenesis (X8). Whistler, Canada. March 25-30, 2007.

Gulzar, N., Shroff, A., Buberoglu, B., Klonowska, D., Kim, J. E., and Copeland, K. F. CD8 and CXCR4 Receptor Mediated Infection of CD8+ T-cells by HIV-1. 14th Conference on Retroviruses and

Opportunistic Infections (CROI 2007). Los Angeles, USA. February 25-28, 2007.

Gulzar, N., Shroff, A., Buberoglu, B., Klonowska, D., Kim, J. E., and Copeland, K. F. HIV-1 Infection of CD8+ T-Cells is Associated with the Down-Regulation of the CD8 and CXCR4 Receptors. Fourth Northern Lights Conference (CFBS 50th Scientific Conference). Ottawa, Canada. October 10-13, 2006.

Gulzar, N., Shroff, A., Buberoglu, B., Klonowska, D., Kim, J. E., and Copeland, K. F. Down-Regulation of the CD8 and CXCR4 Receptors Following HIV-1 Entry into CD8+ T-Cell Clones. Canadian Association for AIDS/HIV Research, 15th Annual Meeting. Quebec City, Canada. May 25-28, 2006.

Gulzar, N., and Copeland, K. F. High Frequency of HIV-1 Infection of CD8+ T-Cells *in vitro* is Associated with Receptor Modulation. University of Ottawa, Biochemistry, Microbiology and Immunology Graduate Poster Presentations. Ottawa, Canada. May 18th, 2006.

Gulzar, N., Shroff, A., Klonowska, D., Buberoglu, B., Kim, J. E., and Copeland, K. F. Interactions between Host CD8+ T-Cell Clone Receptors and the HIV-1 Glycoprotein Confer Susceptibility to Infection. Ontario HIV Treatment Network, 8th Annual Meeting. Toronto, Canada. November 24-25, 2005.

Gulzar, N., Balasubramanian, S., Beaudoin, G., and Copeland, K. F. Infection of CD8+ T-cell Subsets by HIV-1. Ontario HIV Treatment Network, 8th Annual Meeting. Toronto, Canada. November 24-25, 2005.

Gulzar, N., Shroff, A., Kim, John E., and Copeland, K. F. Virus-Receptor Interactions During HIV-1 Infection of CD8+ T-Cell Clones. The 5th Annual Ottawa Health Research Institute Research Day. Ottawa, Canada. October 24th, 2005.

Gulzar, N., and Copeland, K. F. Human Immunodeficiency Virus-1 Modulation of CD8+ T-cell Clone Gene and CXCR4 Expression. Ontario HIV Treatment Network, 7th Annual Meeting. Toronto, Canada. November 25-26, 2004.

Gulzar, N., and Copeland, K. F. Modulation of Chemokine Receptor and Gene Expression in CD8+ T-cell Clones by HIV-1. The 4th Annual Ottawa Health Research Institute Research Day. Ottawa, Canada. November 19th, 2004.

Gulzar, N., and Copeland, K. F. Differential Replication Kinetics and Effects of HIV-1 in CD8+ T-cell Clones. University of Ottawa, Biochemistry, Microbiology and Immunology Graduate Poster Presentations. Ottawa, Canada. April 1st, 2004.

Gulzar, N., and Copeland, K. F. The Impact of HIV Infection on CD8+ T-cell Function. The 2nd Annual Ottawa Health Research Institute Research Day. Ottawa, Canada. January 24th, 2003.

Balasubramanian, S., **Gulzar, N.**, Smaill, F., and Copeland, K. F. HIV Infection of CD8+ T-cells is Associated with Altered Cellular Function. International Society for Sexually Transmitted Disease Research. Ottawa. July 27-30, 2003.

Gulzar, N., and Copeland, K. F. Differential Replication Kinetics and Effects of HIV-1 in CD8+ T-cell Clones. Ontario HIV Treatment Network, 6th Annual Meeting. Toronto, Canada. November 3-4, 2003.

Gulzar, N., and Copeland, K. F. HIV Infection of CD8+ T-Cells. University of Ottawa, Biochemistry, Microbiology and Immunology Graduate Poster Presentations. Ottawa, Canada. April 11th, 2002.