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Factors Important for the Establishment and Maintenance of HIV-1 Latency in CD4
T Cells

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Molecular Pathology

by

Paula Campos Soto

Committee in charge:

Professor Celsa A. Spina, Chair
Professor Douglas D. Richman, Co-Chair
Professor Lynette B. Corbeil
Professor John C. Guatelli
Professor Stephen M. Hedrick

2008

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Chair

University of California, San Diego

2008

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CURRICULUM VITAE

Education

Ph.D. Molecular Pathology; University of California, San Diego (2008)

B.A. Biology; University of Redlands (1999)

Ph.D. Dissertation: “Identification of Factors Important for the Establishment and Maintenance of HIV-1 Latency in CD4 T cells”, advisor Celsa Spina.

Research conducted involved optimization of a unique *in vitro* model of HIV-1 latency in primary CD4 T cells. This model was used to determine the optimal time of infection during the T cell activation cycle, and activation and proliferation requirements for the development of latently infected cells. It was also used to investigate the presence of viral transcripts in latently infected cells.

Research Experience

University of California San Diego – San Diego, CA

Ph.D. Candidate

September 2001 – February 2008

- Optimized *in vitro* model to investigate HIV-1 latency in primary CD4+ T cells
- Designed and performed experiments to test the optimal time of infection during T cell activation for establishment of a pool of latently infected cells using flow cytometry and real-time PCR
- Determined cell proliferation and activation requirements for the development of latently infected cells using cell staining and cell sorting
- Identified HIV RNA transcripts present in latently infected cells by quantitative RT-PCR

MarDx Diagnostics, Inc. – San Diego, CA

Research Technician

June 2000 – August 2001

- Analyzed and developed new ELISA and Western blot test kits that aid in the diagnosis of Lyme disease, autoimmune disorders, HIV infection and other diseases
- Performed assays to evaluate new products and troubleshoot irregularities
- Ran SDS-PAGE gels, aided in protein purification, and analyzed panels of human sera to aid in the evaluation of products

Quality Control Technician

August 1999 - June 2000

- Tested and evaluated products, using samples from raw materials and finished components
- Ran assays of manufactured ELISA and Western Blot products to verify conformity to specifications

- Recorded test results and created SOPs for new or existing products
- Responsible for reviewing production and training new employees
- Followed GMP and ISO 9000 guidelines

University of Redlands – Redlands, CA
Undergraduate Senior Research
1999

September 1998 - May

- Created mutants of the budding yeast *Kluyveromyces lactis* through EMS mutagenesis
- Identified and isolated temperature sensitive mutants for analysis

Teaching Experience

University of California San Diego – San Diego, CA
Academic Connections Instructor

Summer 2004

- Created the curriculum for a two week Immunology and Disease course for high school students enrolled in the Academic Connections program at UCSD
- Presented lectures and activities, engaging students to learn about immunology and pathology
- Wrote exams and evaluated students

University of Redlands – Redlands, CA
Laboratory Teaching Assistant

September 1995 - May 1999

- Supervised and guided students in their tasks in the laboratory
- Prepared chemical solutions, organized materials, and provided supplies to be used in the lab
- Tutored students that needed help with lab assignments or lab reports

Mathematics Tutor

September 1996 - May 1997

- Assisted students with assignments and questions about material presented in lecture.
- Reviewed and answered questions on topics to be evaluated on final exams.

PUBLICATIONS

Koelsch KK, Liu L, Haubrich R, May S, Havlir D, Gunthard HF, Ignacio CC, Campos Soto P, Little SJ, Shafer R, Robbins GK, D’Aquila RT, Kawano Y, Young K, Dao P, Spina CA, Richman DD, and Wong JK. Dynamics of total, linear non-integrated and integrated HIV-1 DNA *in vivo* and *in vitro*. *J. Infect. Dis.* 197:411-419, 2008.

Campos Soto P, Kawano Y, Koelsch KK, Terry VH, Lewinski MK, Wong JK, Richman DD, and Spina CA. An *in vitro* model of HIV latency in primary CD4 T cells. *In preparation*.

Campos Soto P, Terry VH, and Spina CA. Latent / persistent HIV infection is most likely to develop in minimally activated, non-proliferating CD4 T cells. *In preparation*.

Conference Presentations

Oral Presentations

Soto, PC, Terry V, and Spina CA. Acutely infected CD4 lymphocytes can survive rounds of cell division to become persistently/latently infected. In: The 2006 Palm Springs Symposium on HIV/AIDS – Pathogenesis Versus Host in the Progression to AIDS. Palm Springs, CA. 2006: 34.

Poster Presentations

Soto PC, Terry V, and Spina CA. Non-proliferating HIV-1 Infected CD4 T Cells are More Likely to Become Latently/Persistently Infected. In: 14th Conference on Retroviruses and Opportunistic Infections. Los Angeles, CA. 2007: D-188.

Soto PC, Koelsch KK, Wong JK, and Spina CA. Timing of HIV infection during the T cell activation cycle and the establishment of a latently-infected CD4 cell reservoir. In: Conference program and abstracts book of the Molecular Mechanisms of HIV Pathogenesis Meeting – Keystone Symposia. Whistler, British Columbia. 2004: 419.

Professional Memberships

- American Society for Microbiology (2003 – present)
- Association for Women in Science (2003 – present)

Awards/Grants

- Dissertation Award, Universitywide AIDS Research Program, 2006
- Young Investigator Award, 14th Conference on Retroviruses and Opportunistic Infections, 2007
- Young Investigator Award, 15th Conference on Retroviruses and Opportunistic Infections, 2008

ABSTRACT OF THE DISSERTATION

Factors Important for the Establishment and Maintenance of HIV-1 Latency in CD4

T Cells

by

Paula Campos Soto

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2008

Professor Celsa Spina, Chair

Professor Douglas Richman, Co-Chair

Highly active antiretroviral therapy (HAART) in individuals infected with HIV-1 often lowers plasma viremia to below detection limits. However, cessation of therapy in such individuals results in rebound of virus replication, indicating that HIV-infected cells persist. Resting, memory CD4 T cells in the blood and lymph nodes comprise the major reservoir for persistent HIV infection. To devise new efficient strategies targeted toward the eradication of the latently infected HIV reservoir, a better understanding of the molecular and cellular basis for viral latency

is needed. Dr. Spina's research group has developed a unique *in vitro* T cell model to study HIV latency. In my thesis project, I have used and modified this cell model to investigate: 1) the cellular proliferation and activation requirements for the development of latently infected CD4 cells, and 2) the transcriptional activity of the HIV provirus in a nonproductive, persistent infection.

Results from the first research phase demonstrated that HIV infection immediately prior to T cell stimulation resulted in production of the greatest number of latently infected cells. Infected cells that did not divide, or divided only a few times, following stimulation went on to form the latently infected cell pool. The vast majority of acutely infected, activated CD4 cells were not able to survive multiple rounds of cell division in combination with the cytopathic effects of HIV. Rather, the subset of CD4 cells that exhibited minimal activation, in the presence of fully-activated and productively infected T cells, survived with latent HIV infection.

In the second phase of research, a sensitive qRT-PCR assay was used to examine the transcriptional activity of the HIV provirus in resting, infected CD4 cells. Multiple different species of HIV mRNA were found, with unspliced *gag* transcripts being the most abundant followed by singly-spliced *env*, total multiply-spliced, *nef*, and *tat* species. Detection of viral RNA transcription in latently infected T cells from our *in vitro* model has raised the possibility that HIV latency is not maintained by a simple passive mechanism, but may involve active interactions between viral products and cell processes that influence viral latency and reactivation.

CHAPTER I

INTRODUCTION

HIV AND AIDS

There are an estimated 33 million people worldwide currently infected with the human immunodeficiency virus (HIV), the causative agent of Acquired Immunodeficiency Syndrome (AIDS). In the year 2007 alone, more than 3 million new infections are expected to occur. UNAIDS (<http://data.unaids.org>) estimates that approximately 44% of those infected are women, and 7.1% are children. The most heavily affected area of the world is sub-Saharan Africa, with almost 30 million HIV-infected people. In countries with the highest prevalence, rates of HIV infection may exceed 30% in the general population. The primary method of spread of HIV infection worldwide is through sexual transmission, accounting for approximately 70% of the overall transmission in areas of highest HIV prevalence. In the United States and Europe, acquisition of the virus through homosexual contact remains important, and there is some evidence of a re-emergent increasing incidence of infection among young gay men and ethnic minorities (1).

HIV causes disease by damaging the immune system, leaving the infected person vulnerable to a variety of opportunistic infections. The effect of HIV on the immune system is monitored by quantification of the CD4 lymphocyte count in peripheral blood, in which normal levels are considered to be between 600 and 1,200 cells/ μ L. CD4 cell counts below 350 cells/ μ L indicate impairment of immune function is present, and CD4 cell counts of less than 200 cells/ μ L indicate a high risk of serious opportunistic infections or other complications of HIV disease, and prompt treatment with combined antiretroviral drugs is recommended (2).

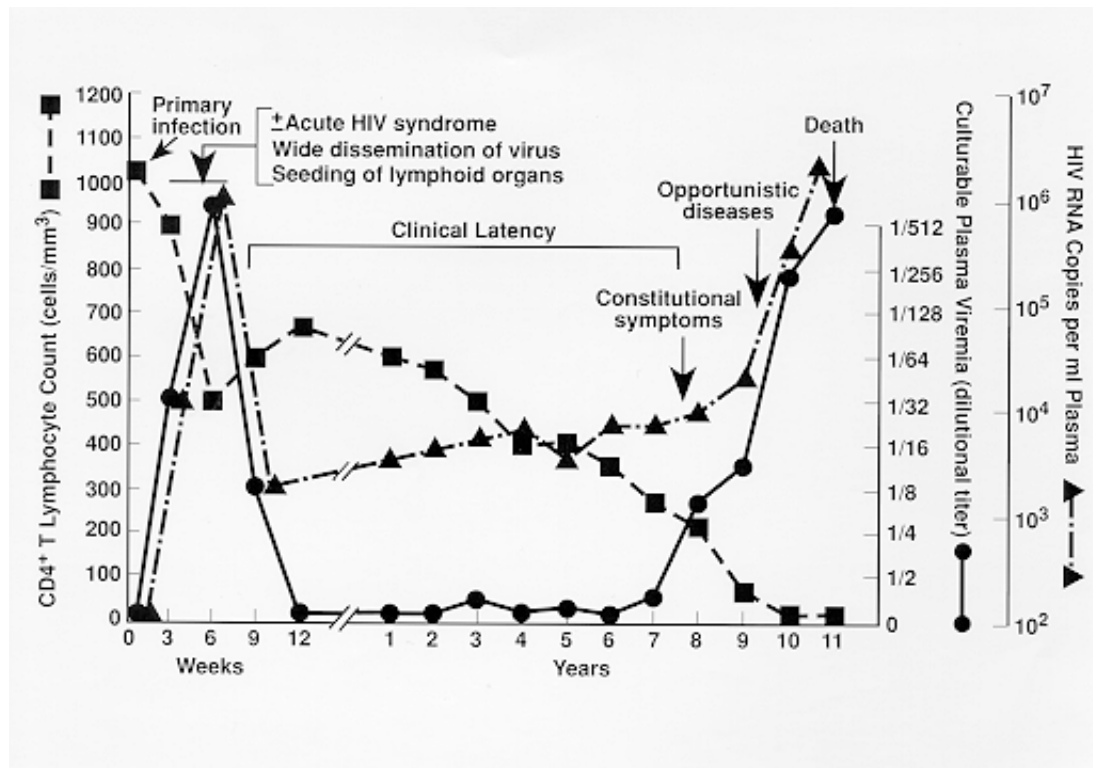


Figure 1.1: Course of untreated HIV infection. From: <http://www.nih.niaid.gov>, adapted from Pantaleo G, Graziosi C, Fauci AS. New concepts in the immunopathogenesis of human immunodeficiency virus infection. *N Engl J Med.* 1993;328:327-35.

If left untreated, HIV disease is chronic and progressive (Figure 1.1). Primary HIV infection, often marked by a mononucleosis-like acute viral syndrome, is followed by a period of clinical latency typically lasting several years, during which time detectable levels of viral replication and CD4 cell turnover slowly progress toward immune dysfunction. Eventually, the number of CD4 cells present is too low to fight off other infections, resulting in the clinical syndrome of AIDS defining illness.

HIV STRUCTURE AND LIFE-CYCLE

HIV virions are spherical, 100 to 120 nanometers in diameter, and consist of a lipid bilayer membrane that surrounds a cone-shaped core. Two identical genomic RNA molecules, 9.2 kb in size, and selected viral proteins such as protease (PR), reverse transcriptase (RT), integrase (IN), Vpr, and Nef reside within the nucleocapsid core (3). The genomic RNA of HIV encodes for a total of 15 different proteins (Figure 1.2). Gag, Pol, and Env are produced as polyproteins that are subsequently proteolyzed into individual protein components, which are common to all retroviruses. The four Gag proteins of matrix (MA), capsid (CA), nucleocapsid (NC), and p6, and the two Env glycoproteins of surface or gp120 (SU) and transmembrane or gp41 (TM), are the structural viral components that comprise the core of the virion and the outer membrane envelope, respectively. The polymerase Pol is cleaved into 3 proteins: protease (PR), reverse transcriptase (RT), and integrase (IN), which provide essential enzymatic functions and are also encapsulated within the virion particle. HIV-1 encodes six additional proteins, called accessory proteins, three of which (Vif, Vpr, and Nef) are found within the viral particle. Two other accessory proteins, Tat and Rev, provide essential gene regulatory functions; and the last protein, Vpu, assists in assembly and budding of the virion (4).

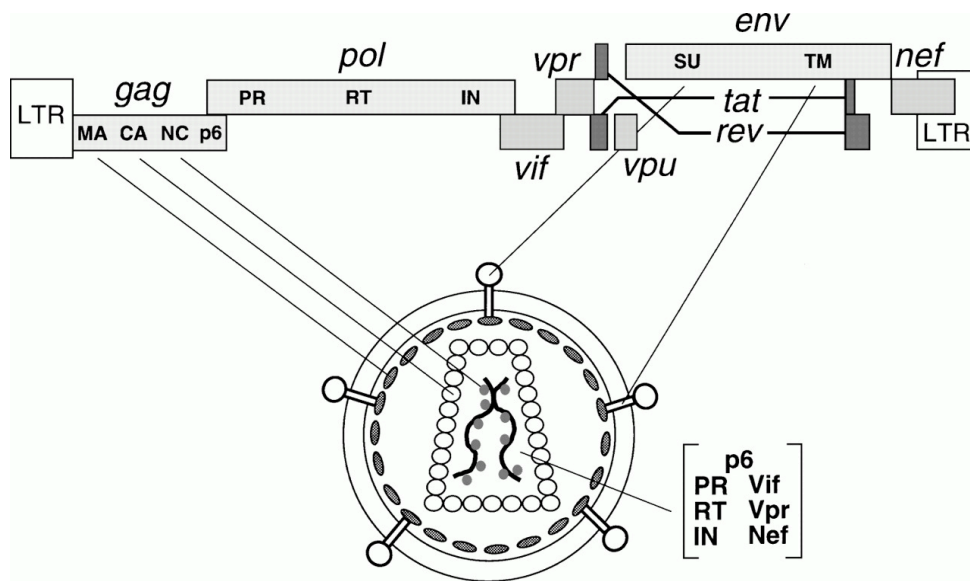


Figure 1.2. The genomic and structural organization of HIV-1. MA, matrix, CA, capsid, NC, nucleocapsid, PR, protease, RT, reverse transcriptase, IN, integrase, env, envelope. From Frankel and Young, Annual Reviews of Biochemistry 1998; 67:1-25.

HIV infection of a host cell begins with the binding of the virion to the plasma membrane of the cell (Figure 1.3). The surface envelope glycoprotein binds to its primary receptor, the CD4 molecule on the outer membrane surface of the target cell, and causes exposure of another portion of the Env trimer. This allows the viral envelope to bind to a cell-specific coreceptor, either the chemokine receptor CXCR4 (in the case of T-cell-tropic) or the chemokine receptor CCR5 (in the case of macrophage-tropic). Coreceptor binding induces a conformational change causing the gp41 trimer portion of the envelope molecule to spring open and then refold to pull the virus and host cell membranes together, allowing fusion to occur. The viral contents, including copies of the viral genetic material and the Pol protein enter the

cytoplasm of the host cell and reverse transcription of the positive strand of genomic RNA begins (5).

The pre-integration complex (PIC), composed of the reverse-transcribed complementary DNA (cDNA) and a number of associated viral and host proteins, enters the cell nucleus. Here, the viral enzyme integrase mediates the insertion of the viral cDNA into the host DNA. Transcription of the integrated viral DNA is under complex control of a number of viral and cellular proteins, including Tat and host transcription factors. Once transcribed, viral RNA may be transported out of the nucleus in its full-length unspliced form to serve as genomic material for new virion production, or it may be singly or multiply spliced into mRNA templates. New viral particles are assembled at the cell plasma membrane and incorporate Gag subunits, Pol, Nef, Env, Vpr, and viral genomic RNA. The HIV viral protease enzyme acts following virion assembly to cleave viral proteins into functional structural and enzymatic components. Gag then functions in the budding of mature virions from the plasma membrane (5).

Current HIV therapies are designed to inhibit the viral replication process at one of several stages: binding and entry (fusion inhibitors), reverse transcription (nucleoside and non-nucleoside reverse transcriptase inhibitors), or Gag protein cleavage (protease inhibitors). Inhibitors of coreceptor binding and maturation have been developed also and are in clinical trials.

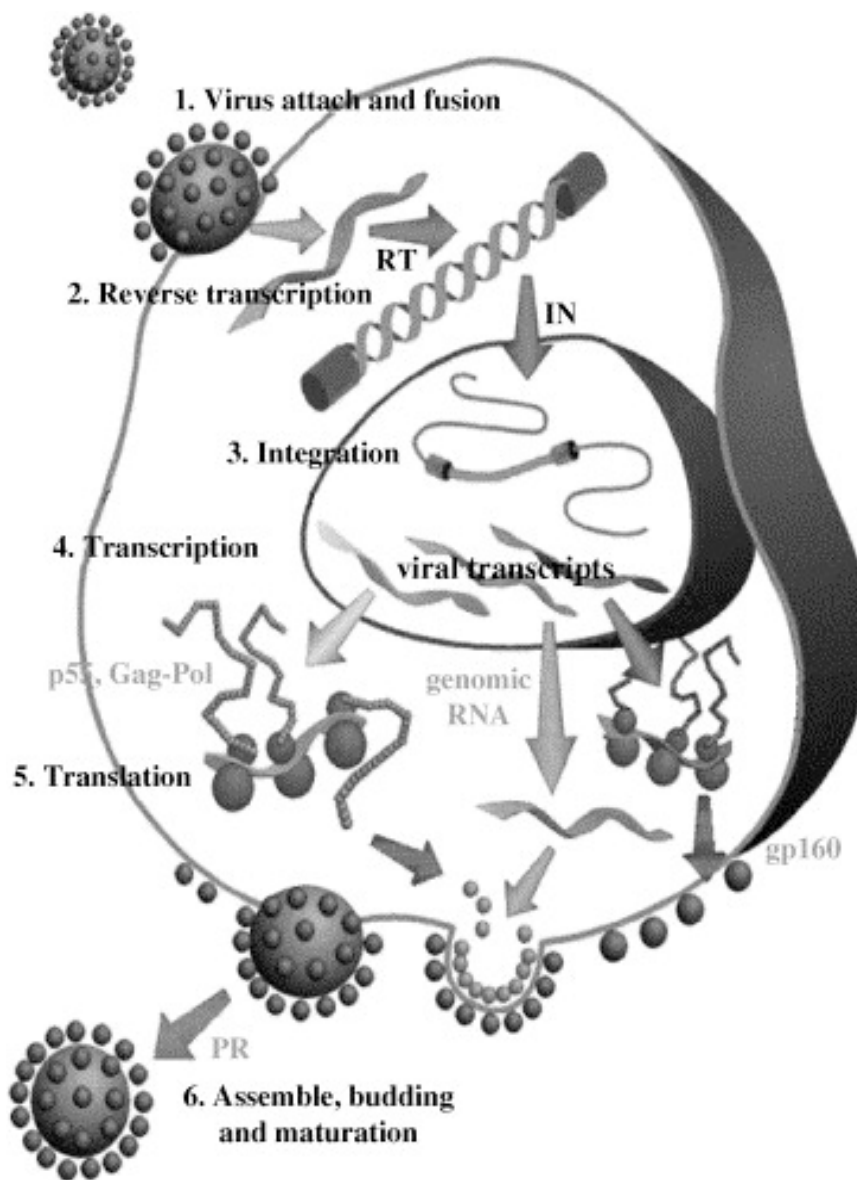


Figure 1.3: Replicative cycle of HIV-1. The main steps in HIV replication are sequentially numbered from 1 to 6. (1) Virus binds to CD4 and the appropriate coreceptor resulting in fusion of the viral envelope and the cellular membrane and the release of viral nucleocapsid into the cytoplasm. (2) Following the uncoating viral RNA is reverse transcribed by the RT. (3) The resulting double-stranded proviral DNA migrates into the cell nucleus and is integrated into the cellular DNA by the IN. (4) The proviral DNA is transcribed by the cellular RNA polymerase II. (5) The mRNAs are translated by the cellular polysomes. (6) Viral proteins and genomic RNA are transported to the cellular membrane and assemble. Immature virions are released. Polypeptide precursors are processed by the viral PR to produce mature viral particles. From: Sierra S, Kupfer B, Kaiser R. Basics of the virology of HIV-1 and its replication. *J Clin Virol.* 2005 Dec;34(4):233-44.

HIV-1 PATHOGENESIS

The major pathologic hallmark of progressive HIV disease is the destruction of CD4 T cells and subsequent loss of immune system competence. Despite considerable efforts to identify the main cause for pathogenicity of HIV infection for CD4 cells, the exact mechanism remains unclear. Two main hypotheses have been proposed. The first suggests that HIV causes depletion of CD4 T cells by infection and direct killing of cells. The second hypothesis proposes that HIV infection indirectly impairs CD4 cell function due to an inappropriate reaction of the host's immune system to the infection (6).

In order to replicate, HIV must integrate into the DNA of the host cell, and therefore its transcriptional activity is directly linked to the metabolic and activation state of the host cell. In activated and proliferating target cells, viral replication is fast and efficient, while truly quiescent cells seem to be refractory to productive viral replication (7, 8). Under optimal conditions of cell activation, where host and viral factors that positively regulate transcription are abundant, infection is cytopathic, resulting in shortened life spans for infected cells (9). The direct cytopathic effects of viral replication have been attributed to a number of viral proteins. The ability of the viral envelope to fuse with the host cell membrane has been shown to correlate with viral cytopathicity *in vitro*. Other viral proteins, such as Vpr may contribute to direct killing of infected cells by promoting apoptosis (10, 11).

The pathology caused by HIV has also been shown to affect T cells indirectly. CD4 and CD8 T cells from infected individuals have been shown to

undergo spontaneous apoptosis *ex vitro* (12). One suggested mechanism for the death of uninfected cells is that up-regulation of Fas ligand on infected cells by the viral protein Nef, and subsequent interaction with Fas on nearby cells triggers apoptosis (13). It has also been proposed that a persistent state of cell activation caused by chronic high viral loads might lead to depletion and exhaustion of immune T cells. This hypothesis is based primarily on studies that show thymic function is impaired in human hosts infected with HIV-1. In contrast, natural hosts of the simian immunodeficiency virus (SIV), such as sooty mangabeys, lack exaggerated immune activation and apoptosis of uninfected bystander cells, and show preserved thymic and bone marrow functions (14, 15).

CD4 T LYMPHOCYTES AND HIV LATENCY

Because HIV preferentially infects CD4 T cells, which can become long lived memory T cells, HIV infection shows lifelong persistence, which can not be eradicated with existing regimens of effective highly active anti-retroviral treatment (16, 17).

Memory CD4 T Cells

The development of memory CD4 T cells involves naive cells exiting the thymus, and entering the peripheral circulation and lymphoid tissues. These newly generated T cells remain in a resting state until they come into contact with their cognate antigen. Following antigen binding and recognition, naive cells become activated, undergo blast transformation, and enter the cell cycle. During this process, metabolic changes take place, including increases in nucleotide pools, up-regulation of expression of transcription factors, cytokines, and surface membrane receptors and adhesion molecules (18). Following peak clonal expansion, most of the responding T cells will die within a few weeks after activation; but a small proportion of these cells will survive, exit the cell cycle, lose surface membrane expression of HLA-DR, CD69, and CD25 (which are considered markers of activation) and revert to a resting quiescent state. The cells will persist in this state as memory T cells, which are able to respond quickly to a second exposure to the same antigen. The survival of resting memory T cells for long periods of time is dependent upon exposure to cytokines such as IL-4, IL-6, and IL-7 (19).

Different expression patterns of cell surface proteins can be used to determine the activation state and history of antigenic exposure for T cells (20, 21). One of the earliest markers of activation is CD69, which quickly and transiently appears on the surface of activated T cells. Expression of the alpha chain of the IL-2 receptor, or CD25, is also up-regulated early and is temporary. HLA-DR (MHC-II), on the other hand, has a slower and more prolonged expression pattern, and it persists while the T cell is in an activated state. Expression of other phenotypic markers is permanently altered following activation, and can be used to identify T cell maturation stages. The major marker in this category is CD45, a membrane tyrosine phosphatase. Naive and memory T cells express different isoforms of this transmembrane enzyme, namely CD45RA and CD45RO, and can therefore be identified based on the differential expression of these isoforms (22). Another marker of naive T cells is the lymphocyte homing receptor CD62L, not usually expressed in memory T cells (23).

To gain cell entry and initiate replication HIV utilizes both the major CD4 receptor and a co-receptor, either CCR5 or CXCR4. CCR5 is the chemokine receptor for RANTES. It is expressed on a fraction of quiescent memory T cells and is up-regulated following cell activation; therefore, R5-tropic viruses can infect and replicate in activated, CCR5+ T cells. CCR5 is also expressed in some naive T cells, albeit at extremely low levels. CXCR4 is the chemokine receptor for SF-1, and it is expressed by naive, memory, resting and activated CD4 T cells at sufficient levels to allow infection of all these cell types.

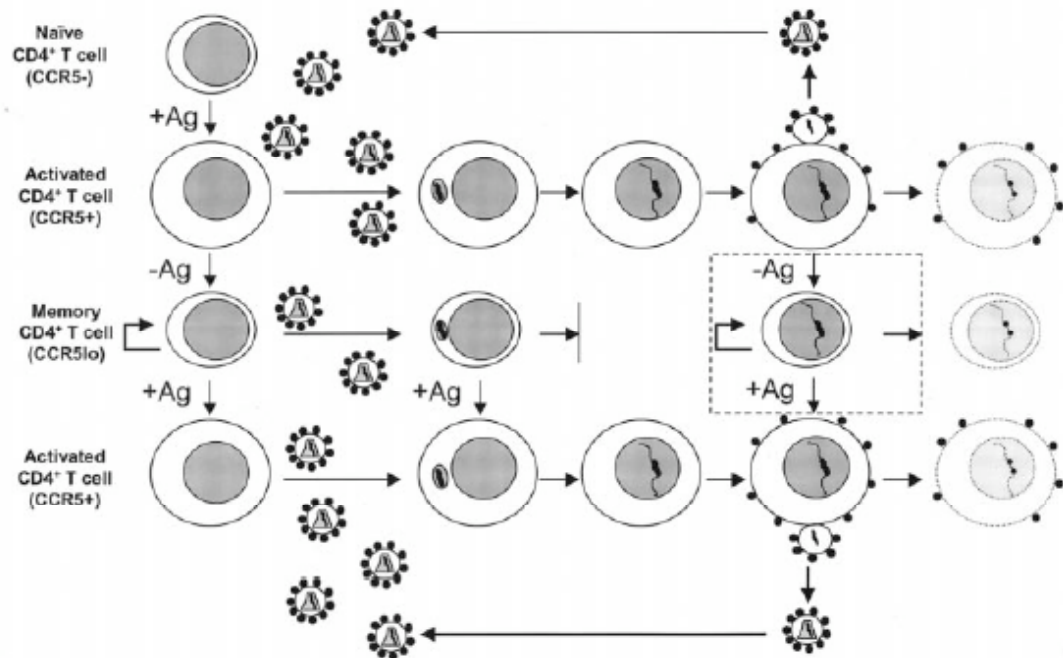


Figure 1.4. Cellular dynamics of HIV-1 infection of CD4 T cells. Transitions between resting (small) and activated (large) CD4 T cells are illustrated by vertical arrows. The normal generation of memory CD4 T cells is illustrated on the left. These cells are derived from antigen (Ag)-activated CD4 T cells that revert back to a resting memory state. Successive steps in the life cycle of the virus are indicated by horizontal arrows. R5 isolates can infect activated CD4 T cells, but may infect only the subset of resting memory CD4 T cells that express sufficient amounts of CCR5. Following infection of resting memory CD4 T cells, there is a block in the virus life cycle, probably at the level of nuclear import of the preintegration complex containing the viral genome. Resting cells with unintegrated HIV-1 DNA are likely to represent a relatively labile reservoir for the virus (preintegration latency). Productive infection requires Ag driven activation of recently infected resting CD4 cells or, more commonly, direct infection of Ag-activated CD4 T cells. Productively infected cells generally die within a few days from cytopathic effects of the infection or host cytolytic effector mechanisms, but some infected lymphoblasts survive long enough to go back to a resting state (boxed), thereby establishing a stable latent reservoir of resting memory CD4 T cells with integrated HIV-1 DNA (postintegration latency). From: RESERVOIRS FOR HIV-1: Mechanisms for Viral Persistence in the Presence of Antiviral Immune Responses and Antiretroviral Therapy. Theodore Pierson, Justin McArthur, and Robert F. Siliciano. *Annu. Rev. Immunol.* 2000. 18:665–708.

Pre-integration Latency

Resting memory CD4 T cells express CD4 and both CXCR4 and CCR5 coreceptors; latent HIV infection is most often found in this phenotypic subset of T cells. Following the infection of resting T cells, the incoming HIV genomic RNA must be reverse transcribed in the pre-integration complex. It has been suggested that the inability of HIV to productively infect resting cells results from the inability to complete reverse transcription (8), or failure to import the pre-integration complex into the nucleus, where it could integrate into the host cell DNA (24). Although short-term *in vitro* infection experiments do not usually result in completed reverse transcription, long-term culture experiments and *in vivo*, studies have demonstrated the presence of complete reverse transcripts in resting CD4 T cells (25, 26). These latter studies indicate that recently infected resting CD4 T cells can serve as an inducible, pre-integration, latent reservoir for HIV. If the infected resting T cell encounters antigen during the period that the pre-integration complex is still functional, resulting cell activation will allow the viral replication cycle to proceed to completion and infectious virion production. Even though pre-integration latency is the most prevalent form seen in untreated patients, it is relatively labile, lasting for only a few days or weeks, and therefore, does not seem to contribute to the latent HIV reservoir found in patients on HAART.

Post-integration Latency

The most predominant form of HIV latency in treated patients is post-integration latency. This more stable form of latency must result from integration of proviral DNA into the DNA of a CD4 T cell. The mechanism responsible for the establishment of HIV latency has not yet been elucidated, but a few possible mechanisms have been proposed (27).

One possible mechanism relies on the fact that nuclear import is dependent upon T cell activation, and therefore suggests that post-integration latency can only result from the return of an activated T cell with integrated provirus back to a resting state. But for an activated, infected T cell to survive, it must first bypass activation-induced cell death, which kills the vast majority of activated cells. The cell must also survive the cytopathic effects of the virus, and the attempts of the immune system to control and destroy infected cells.

Another possible mechanism for how HIV latency is established suggests that a cell becomes infected during a narrow window of time, as the cell is reverting back to a resting state. During this narrow window of time the cell would be activated enough to allow reverse transcription and integration, but not enough to allow initiation of viral replication. Infection during this time would allow the cell to escape the cytopathic effects of productive infection and the cytolytic effector mechanisms of the host's immune defense.

A third hypothesis suggests that cytokine dependent mechanisms allow infection of resting cells. This hypothesis was developed based on studies that

showed that resting CD4 T cells treated with specific cytokines, such as IL-2, IL-7, and IL-15, can become permissive for nuclear import, integration, and virus gene expression (28).

Characteristics of HIV latency *in vivo*

By using inverse PCR to detect the junction between integrated HIV provirus and host chromosomal DNA, studies have shown that integrated virus is found in <0.01% of CD4 T cells in the peripheral blood and lymph nodes of patients in the asymptomatic phase of infection. Frequencies of integrated HIV are not higher in patients with advanced disease, suggesting that a relatively stable steady state is established in which only a minute fraction of the resting CD4 T cell population carries integrated HIV DNA at any given time (17). The frequency of resting CD4 T cells containing replication-competent provirus is even lower than that for integrated HIV-1 DNA. Analysis of the fraction of PBMC, from which virus can be cultured, has shown that cells harboring replication-competent provirus are quite rare (<0.01%) (29). The latent reservoir is established early in acute primary infection, and latently infected cells can be detected in patients who have been started as early as 48 hrs. after presentation with acute retroviral syndrome (30).

Several recent studies have suggested that productively infected T cells can be detected in individuals who are aviremic on HAART. The methods for virus detection include *in situ* hybridization or qRT-PCR assays for the expression of HIV RNA and immunohistochemical detection of cells expressing HIV proteins (31). T

cells in the latently infected reservoir appear to produce little viral mRNA. Some studies have reported approximately 50 copies of unspliced gag RNA/ 10^6 resting CD4 T cells and undetectable levels for multiply-spliced RNA species (32). The viral mRNA that is produced seems to be prematurely terminated (33) or localized into inappropriate compartments (34). Therefore, it is generally assumed that latently infected T cells do not produce viral proteins and are apparently indistinguishable from uninfected cells.

One *in vivo* study of purified resting CD4 T cells from HIV-1-infected individuals on suppressive HAART revealed a strong preference (93%) for proviral integration in protein-encoding genes (35). Most of the identified genes (91%) were transcriptionally active in resting CD4 T cells.

***In vitro* models of HIV latency**

Advancements in the study HIV-1 latency have been hindered severely by the lack of biologically relevant *in vitro* models that can accurately mimic the state of viral latency *in vivo*.

The most clearly defined reservoir of HIV infection consists of latently infected resting memory CD4 T cells, that persist even in the presence of highly active antiretroviral therapy (HAART), and can initiate viral production upon activation (17, 36, 37). This reservoir is extremely stable; clearance has been estimated to be impossible during an infected person's lifetime, thereby rendering HIV infection incurable with current treatment strategies (38, 39). In order to

develop new strategies for the eradication of latently infected cells, a better understanding of how latency is established and maintained, and a better characterization of latently infected cells is needed.

Several cell line models for HIV latent infection have been developed, and despite providing insightful observations, these models were shown not to accurately portray what occurs *in vivo*. The use of chronically infected cell lines, such as the ACH2 T-cell line and the U1 promonocytic cell line, suggested that post-transcriptional mechanisms are involved in maintaining HIV in a latent state (40). The studies also showed that the patterns of HIV RNA expression in these cells consisted of singly and multiply spliced RNA species, with little or no full length viral RNA present. In contrast, studies that examined resting T cells and lymphoid tissue from infected patients, demonstrated that both unspliced and multiply spliced HIV RNA species could be detected, and unspliced transcripts were the most abundant (33, 41).

Conflicting results on preferential sites for HIV integration were also obtained when cell line models were compared to studies with primary T cells. Initial work using a Jurkat cell clone, J-Lat, suggested that integration into heterochromatic regions resulted in latent HIV infection (42). However, later studies that analyzed HIV integration sites in purified resting CD4 T cells from patients on HAART showed a strong preference for viral integration into actively transcribed genes (35).

This past experience with established cell line models highlights the importance of using primary T cells to confirm and advance our understanding of

HIV latency. Ideally, CD4 T cells from infected individuals should be used; but, the extremely low frequency of these cells and the lack of specific markers for their identification and isolation, preclude most *ex vivo* research. Cell models that employ primary T cells to recapitulate latent HIV infection *in vivo* represent a more appealing alternative approach.

Only a few *in vitro* primary cell models have been described to date. Sahu et al. have developed a culture system for post-activation, long-term survival of normal CD4 T cells in a quiescent state. The culture system was then used to generate latently infected, long-lived quiescent CD4 T cells from HIV-infected and activated CD4 lymphocytes from normal donors (43). This model involved co-culturing primary, anti-CD3 stimulated, infected CD4 cells on an adherent tumor-derived brain cell line (H80) to keep the cultured CD4 cells viable indefinitely. Despite yielding long-lived T cells with non-productive viral infection, the validity of this model was unclear. The effects of co-culturing primary cells with an allogeneic tumor-derived brain cell line were not determined, and the activation status of the cultured T cells was not rigorously examined. Another primary cell model of HIV latency was suggested after it was observed that HIV infection of CCL19- or CCL21-treated CD4 T cells resulted in low levels of HIV production, but high concentrations of integrated viral DNA (46). Further, it was demonstrated that re-stimulation of the CCL19-treated, HIV-infected CD4 cells resulted in productive virus replication, which was interpreted as being consistent with the establishment of post-integration latency.

In a recently described T cell model, stable HIV infection was generated in primary CD4⁺ CD8⁺ human thymocytes (44). It was shown that T-cell activation induced >200-fold increase in the activity of a viral reporter gene, which was demonstrated to originate from a fully reverse-transcribed and integrated HIV genome. However, the relevance of a latent infection model established in thymocytes is questionable. In a macaque model of viral latency, thymocytes did not form part of the latently infected cell reservoir (45). Furthermore, latently infected T cells that result from thymocyte infection cannot accurately represent CD4 memory T cells, which are the predominant cell type harboring latent HIV infection in humans.

CHAPTER II

OPTIMAL TIMING OF INFECTION FOR THE SUBSEQUENT DEVELOPMENT OF LATENTLY INFECTED CELLS

INTRODUCTION

The establishment of HIV-1 latency is linked to the normal development of memory T cells. Upon encountering its cognate antigen, a T cell will become activated, enter into the cell cycle and go through multiple rounds of cell division to give rise to a clone of activated memory/effector cells. Most of these effector cells will die, but a few will survive, revert back to resting, and become long-lived central memory cells (18). Because HIV replicates preferentially in activated CD4 T cells, it was initially assumed that latently infected cells developed from infection of activated cells, which would survive both through activation and virus induced cytopathic effects to transition to a latent infection state. (47). However in most circumstances, infection of fully activated cells results in cell death, with cells surviving for only a few days following productive infection (7).

In order to determine the time during the T cell activation cycle when cells are more likely to become infected and survive to form part of the latently infected cell pool, we have used a well-characterized *in vitro* system for HIV replication (25, 37) to derive a model for the study of latency. Our experimental system uses: infection of quiescent, primary CD4 lymphocytes with HIV molecular clones; subsequent induction of the T cell proliferation cascade by T cell receptor (TCR) engagement to activate productive viral replication; long-term culture with the addition of exogenous cytokines; and the recovery of resting T cells that contain latent/persistent infection (Figure 2.1). By day 14 of culture, greater than 99% of the

surviving cells have returned to a resting $G_{0/1}$ state (Figure 2.2). A portion of these cells (5% – 10%) contains integrated HIV DNA, and a subset of which has the capacity to initiate virus production upon a second round of cell stimulation (25).

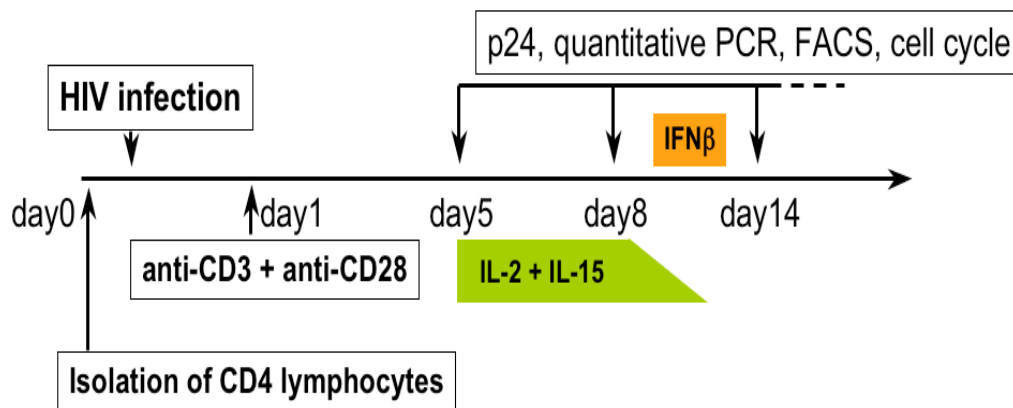


Figure 2.1. Diagram of *in vitro* model of HIV latency. Primary CD4 lymphocytes are isolated from peripheral blood samples of healthy, HIV-seronegative donors, by a negative selection method. Cells are infected with HIV for 6 hrs, and washed to remove excess virus. Cells are then stimulated with immobilized anti-CD3+CD28, returned to culture under optimal cytokine conditions (refer to Materials and Methods) and monitored for productive viral replication.

Resting memory CD4 T cells represent the major reservoir for HIV in the blood and lymph nodes of infected patients. In order to determine whether latently infected cells in our *in vitro* model represent truly resting cells, day 14 cells were analyzed for DNA content and synthesis. As shown in Figure 2.2, on day 14 of culture >95% of cells have returned to a resting state. A very small percentage of cells were in S or G_2 phase of cycle; and of these cells, only 0.1% incorporated BrdU (top-right values). In addition, there was no difference in the number of cells cycling

in infected versus uninfected cell cultures. Therefore, >99% of the CD4 cells recovered at day 14 of culture are truly quiescent.

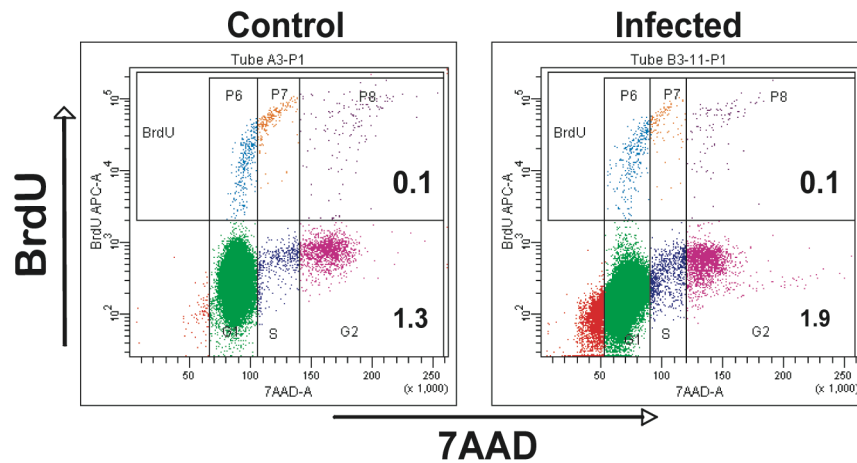


Figure 2.2: BrdU incorporation in CD4 cells at day 14 of culture. Infected and uninfected cells were labeled for BrdU incorporation overnight (BD kit). 7-AAD was then added to stain cellular DNA content.

Using this *in vitro* model, we proceeded to determine that cells exposed to virus early in their activation cycle (e.g. immediately before TCR stimulation), are more likely to become latently infected.

RESULTS

Infection early in the cell cycle results in the establishment of the greatest number of latently infected cells.

In order to determine at what stage of the T cell activation cycle HIV infection is more likely to progress to latency, aliquots of freshly isolated primary CD4 T cells were infected at different time points before (day 0), and after (days 4, 7, and 10) stimulation with immobilized anti-CD3 and anti-CD28 antibodies. Surprisingly, virus production, as measured by soluble p24 production, was greater in the subpopulation of cells that were infected immediately before activation (Figure 2.3-A). Cells infected before stimulation produced approximately four times more virus at the peak of infection than did cells infected 4 days following stimulation. After 14 days in culture, when the cells had returned to a resting state, cells were analyzed for the number of integrated copies of HIV DNA. In agreement with the results of peak virus production, cells infected before stimulation (day 0) contained the highest numbers of integrated HIV DNA copies (Figure 2.3-B).

This data suggested that infection early in the cell activation cycle was more likely to generate a latently infected cell population, raising the possibility that acutely infected, fully activated CD4 T cells were able to survive both infection and the restriction phase of the lymphoproliferative response.

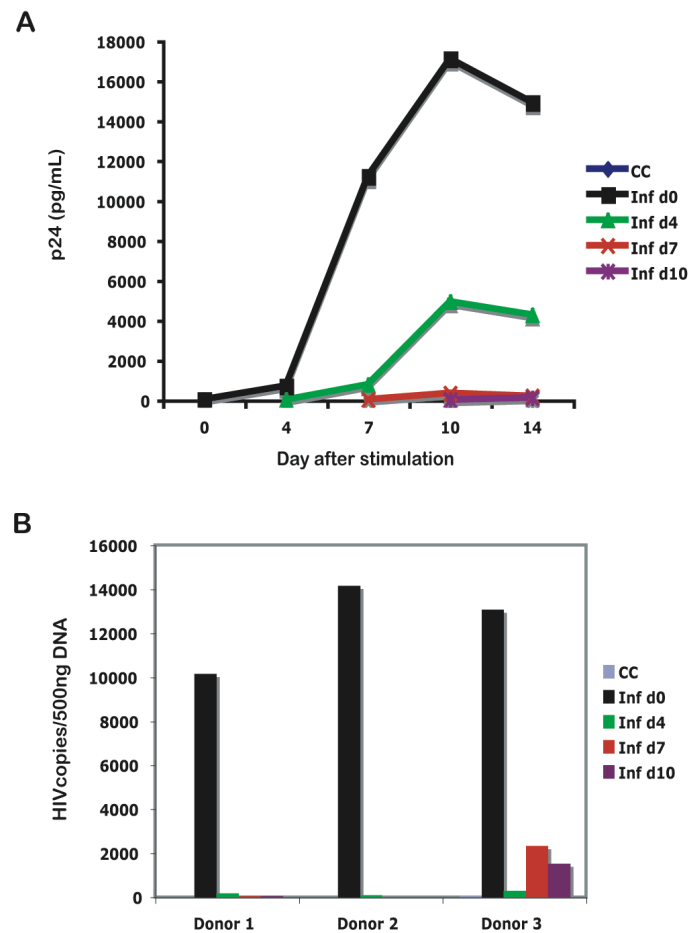


Figure 2.3. Infection early in the cell activation cycle results in the greatest number of latently infected cells. **A)** Aliquots of 10×10^6 cells were infected at different time points before (day 0) and after (days 4, 7, and 10) TCR stimulation. Each infected or uninfected (CC) subpopulation was then cultured separately out to 14 days, and soluble p24 production was monitored over time. Data shown are representative of three experiments. **B)** At day 14, each subpopulation was analyzed by real-time PCR for number of integrated copies of HIV DNA. Data from all three donors tested are shown.

It has previously been shown that the state of CD4 lymphocyte maturation influences significantly the cell's ability to support productive HIV replication. In this published study (78), primary CD4 lymphocytes were sorted into two major phenotypic subsets representative of "memory" ($CD45RA^-$) and "naïve" ($CD45RA^+$)

cells; and viral replication was compared between the two subsets following infection with the NL4-3 clone of HIV-1. Interestingly, naive cells (RA⁺) exhibited significantly greater response to stimulation with anti-CD3/CD28 antibodies than did memory cells (RA⁻); however, the majority of virus replication occurred in the memory cell subset.

To determine whether the timing of HIV infection also affected the establishment of latency differentially in these major T cell maturation subsets, freshly isolated CD4 cells were sorted into “memory” and “naïve” phenotypic subpopulations, and infected at day 0 (before stimulation) or day 4 (after 4 days of stimulation). As expected, HIV replication occurred preferentially in RA⁻ memory cells, with these cells producing approximately two fold more virus than the RA⁺ cells (Figure 2.4-A). In addition, the RA⁻ cells also contained higher numbers of integrated HIV DNA copies, as shown in figure 2.4-B. These results were in agreement with the results obtained using unfractionated CD4 T cells; infection immediately prior to cell stimulation yielded higher production of virus throughout culture, and resulted in the greatest number of latently infected cells at day 14 (Figure 2.3). HIV infection close to the activation event seems to have a greater impact on the development of a latent/persistent reservoir in memory CD4 cells than in naive CD4 cells.

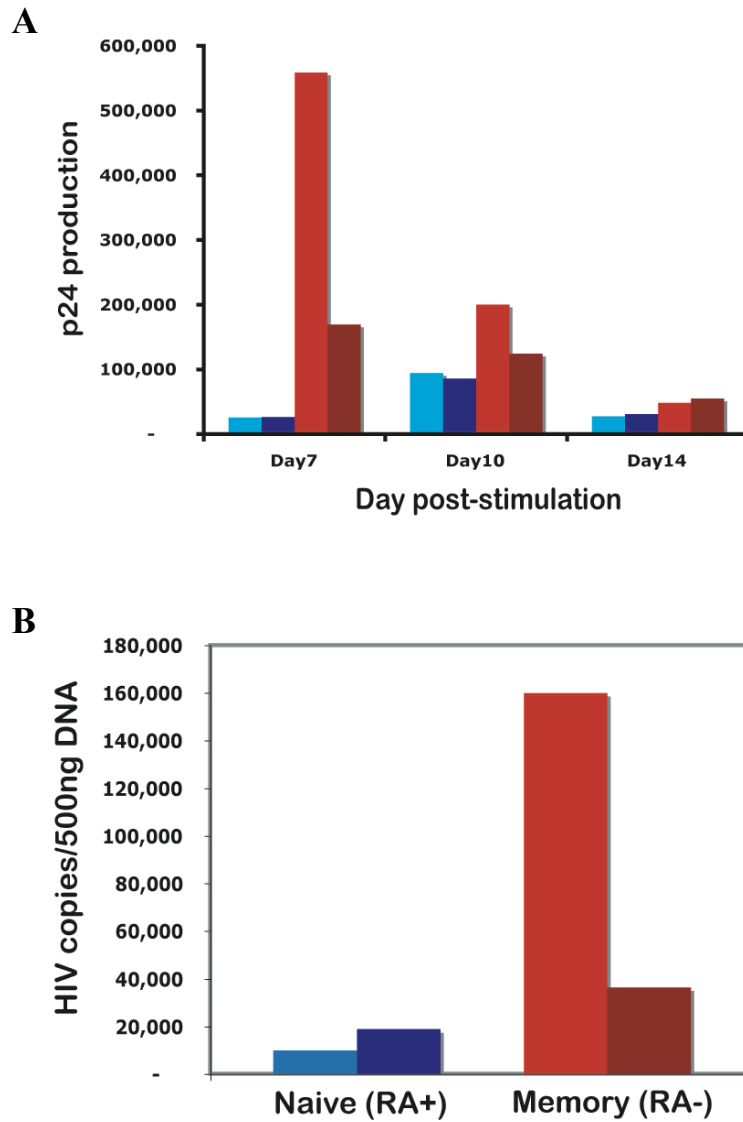


Figure 2.4. Memory phenotype cells (RA-) yield a more productive infection and result in higher numbers of cells with integrated HIV DNA. Freshly isolated CD4 lymphocytes were sorted into RA+ (blue) or RA- (red) subsets, and infected either at day 0 (light color) or 4 days after (dark color) stimulation with anti-CD3/CD28 antibodies. p24 production in the supernatant was measured by ELISA at days 4, 7, and 10 (A) and the number of integrated copies of HIV present at day 14 of culture was determined by real-time qPCR (B).

DISCUSSION

The mechanism responsible for the establishment of HIV latency in CD4 T cells is still unknown. It is generally thought that HIV latency arises as a consequence of the tropism of HIV for long-lived memory CD4 T cells. One proposed hypothesis suggests that infection of an activated CD4 T cell, which is able to survive through the contraction phase of the lymphoproliferative response, and the cytopathic effects of a productive viral infection, results in the establishment of latency in that cell (47). However, until now, this hypothesis has not been tested.

In order to further characterize the activation status of latently infected cells in our model, we analyzed the cell cycle status of day 14 cells by BrdU and 7AAD labeling. After overnight labeling, the great majority of CD4 cells (>95%) are in the G₀ phase of the cell cycle, and a small percentage of the cells are in S or G₂ (1.2% to 1.9%). Interestingly, cells in the S or G₂ phase of the cycle were not actively synthesizing DNA, as shown by the fact that none of these cells stained positive for BrdU. The existence of this population of cells, which appears to be in cycle but not synthesizing DNA, is puzzling. They could represent cells that have arrested in S or G₂, or they could represent an artifact of the staining procedure. However, this population was seen in all donors tested, and has been seen using other methods of DNA staining.

After characterizing the metabolic state of latently infected cells obtained using this model, we showed that CD4 T cells exposed to virus before activation are more likely to become part of the latently infected cell pool. This result was

confirmed both in unfractionated preparations of CD4 cells, and in sorted subpopulations of naïve (RA^+) and memory (RA^-) phenotype subsets. Such findings were surprising, because most studies of HIV infection have used exposure of pre-stimulated cells to virions to establish infection and obtain quick, abundant virus production. Infection of pre-stimulated cells, however, is more likely to result in cell death, while unstimulated cells seem to survive infection more readily (48). In our studies, infection of resting cells followed by immediate activation resulted in the greatest number of latently infected cells surviving out to 14 days in culture.

Based on our observations, such latently infected cells in our model may result from two potential scenarios: 1) these cells become infected early in the cell activation cycle (days 0 to 4) and survive to day 14; or 2) these cells become infected later during culture, as the cells are reverting back to a quiescent state (days 8 to 12). Based on experiments described here, cells that are reverting back to resting are not likely to become infected and establish viral latency (Figure 2.3, cells infected at day 10). And although our data demonstrate that some cells infected early are able to survive to day 14 with latent infection, these results do not provide information on the actual activation status of such cells at the time of acute infection. This question is addressed in the next chapter.

In summary, the results obtained with our *in vitro* cell model of HIV infection show that viral latency is established very early in a population of CD4 T cells that are undergoing activation in the presence of infectious virus. Several publications have reported that *in vivo*, a significant portion of the CD4 cell pool

with latent infection is established throughout various tissue compartments in patients very early after the detection of acute HIV infection (30). Therefore, given the restrictions inherent in any cell culture model to mimic biologic events occurring in the whole organism, our primary CD4 cell model seems to reflect accurately at least one mechanistic component that is involved in the establishment of HIV latency.

MATERIALS AND METHODS

Primary CD4 lymphocyte cultures. Peripheral blood mononuclear cells (PBMCs) were isolated from HIV-seronegative donors by density gradient centrifugation of heparin sodium, anti-coagulated whole blood using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). CD4 T cells were isolated by negative selection using the RosetteSep cell separation procedure (StemCell Technologies, Vancouver, B.C., Canada). The resulting cell preparations were routinely >95% CD4⁺ by flow cytometry. The isolated CD4 cells were cultured in RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA) supplemented with 1mM L-glutamine, 50 U/mL penicillin, 50ug/mL streptomycin, and human AB serum (5%, vol/vol). For stimulation, cells were distributed at concentrations ranging from 2 to 4 x 10⁶ cells/2 mL/well into flat-bottom wells of 6-well culture plates that were coated with anti-CD3 (0.03ug/mL) plus anti-CD28 (0.2ug/mL) antibodies. Cultures were maintained up to 14 days post-stimulation using a mixture of cytokines to optimize cell proliferation and survival: IL-2 (5 U/mL final, NIH AIDS Research & Reference Reagent Program) was added on day 4, IL-15 (10 ng/mL final, R&D Systems, Minneapolis, MN) was added on days 4 and 7, and IFN-beta (10 ng/mL final, PBL InterferonSource, New Jersey, NJ) was added on days 10 and 12.

Cell staining and flow cytometry analysis. The purity of CD4 lymphocyte preparations was monitored by direct staining with dual antibody combinations: anti-

CD4 (FITC) and anti-CD8 (PE), and anti-CD19 (FITC) and anti-CD16 (PE), (BD Biosciences, San Jose, CA). Anti-CD25 and anti-HLA-DR antibodies conjugated to APC and PE, respectively (BD Biosciences) were used to measure levels of CDR cell activation. Cell surface staining was performed for 30 min. at 4°C, per the manufacturer's instructions (73, 74) with cell aliquots containing 0.5×10^6 to 1×10^6 cells. Flow cytometry analysis was performed on a FACS Canto instrument using DiVa software 2.1 (BD Flow Cytometry Systems).

Virus infection. Infectious virus stocks of the NL4-3 clone of HIV-1 (75) were prepared by transfecting plasmid DNA into the CEM T lymphoblastoid cell line with FuGene Transfection Reagent (Roche Molecular Diagnostics, Alameda, CA) and harvesting supernate at the time of peak viral replication and spread, as described previously (64). Aliquots of $4-6 \times 10^6$ CD4 lymphocytes were incubated with 0.5 mL of the NL4-3 virus stock for 5-6 hrs at 37°C at multiplicities of infection (MOI) of 0.1 to 0.5 TCID₅₀ per cell. After infection excess virus was removed by extensive washing with calcium-free and magnesium-free Dulbecco's phosphate buffered saline (PBS; Mediatech, Herndon, VA) plus 2% human AB serum (Omega Scientific, Tarzana, CA). Cells were stimulated as described above and virus infection was performed at different time points during culture (see results section). After three days, CD4 lymphocytes were removed from the CD3/CD28 antibody coated plates and maintained in 6-well tissue-culture treated plates with RPMI medium supplemented with 5% human AB serum, at $3-4 \times 10^6$ cells per well for the

remainder of the culture period (13-14 days). Productive HIV replication was assessed by quantifying the amount of soluble p24 antigen released into culture supernatants using an ELISA method (Abbott, Abbott Park, IL).

Detection of integrated HIV DNA. Infected cells and uninfected cell controls were collected at different time-points and stored frozen in dry pellet aliquots of $1-2 \times 10^6$ cells. For batched analysis, pellets were thawed and resuspended in 200 μ L Dulbecco's PBS. Total DNA was extracted using the Qiagen DNA extraction kit (Qiagen, Chatsworth, USA) following the manufacturer's protocol. High and low molecular weight fractions of genomic DNA were recovered, using a published protocol (65). Replicates of 500ng total DNA were loaded on a 0.5% SeaKem Agarose gel (Cambrex, Rockland, USA); fractionation of DNA was achieved with electrophoresis for 3 hours at 60V. The ~20 kb high molecular weight (HMW) band was excised from the gel with a gene capsule cutter (Geno Technology, St. Louis, USA) to achieve uniform gel slice-sizes. DNA was extracted from the gel using the Qiaex II gel extraction kit (Qiagen, Chatsworth, CA) following the manufacturer's protocol with minor variations. The incubation time in QS buffer was extended to 20 minutes after adding silica. Following incubation, the silica was washed 3 times in buffer QXI and 3 times in buffer PE. DNA was quantified using a real time quantitative PCR assay (Taqman qPCR). Beta-actin specific primers and probe were provided with the ABI TaqMan[®] beta-actin detection reagents (ABI, Foster City, USA). Real-time qPCR was performed in 25 μ l containing 5 μ l DNA target, 12.5 μ l

TaqMan® Universal Master Mix (ABI) and 7.5µl primers and probe (400nM forward and reverse primers, 200nM probe) using these cycling conditions: 50°C/2min, 95°C/10min, then 95°C/15sec, 60°C/1min (45 cycles). A standard curve was generated ranging from 30 - 0.1ng genomic DNA using a reference human DNA (TaqMan® Control Genomic DNA, ABI). DNA content was calculated using the sample cycle threshold (ct) signal values correlated to the standard curve ct values. HIV-1 DNA copy numbers were adjusted to the amount of input DNA and expressed as copies/µg DNA. Primers and probe were designed against p24 gag, and their sequences were as follows:

Forward: 5'-AAAAGAGACCATCAATGAGGAAGC-3'

Reverse: 5'-TGGTGCAATAGGCCCTGC-3'

Probe: 5'-FAM/CAGAATGGGATAGATTGCATCCAG-3'

CHAPTER III

CD4 T CELLS THAT PROLIFERATE THE LEAST DURING HIV INFECTION ARE MORE LIKELY TO BECOME LATENTLY INFECTED

INTRODUCTION

To initiate its replicative cycle, HIV must reverse transcribe its genomic RNA into DNA, and the viral DNA must integrate into the host cell DNA. In an activated cell, this process is rapid and efficient, due to the abundance of cellular factors that are necessary for integration and transcription of viral genes. However, in resting cells that appear to be refractory to HIV infection *in vitro*, there are blocks at and immediately after reverse transcription (6). Consequently, the ability of the virus to establish infection is greatly influenced by the metabolic and activation state of the host cell.

The specific, minimal requirements for the establishment of HIV infection in CD4 T cells have not been defined. The level of cell activation that is needed for viral DNA integration is still unknown. Some studies have shown that HIV vector constructs can infect and integrate into the DNA of non-dividing T cells, following treatment with cytokine mixtures in culture supernate derived from mitogen-stimulated T cells (28). This prior study did not determine the precise identity and concentrations of the active cytokines, nor whether T cell division was an absolute requirement for the persistent establishment of HIV provirus during infection.

In experiments described herein using our *in vitro* model, we demonstrate that T cell proliferation is not a requirement for the establishment of persistent HIV infection. Rather, minimally activated T cells that do not enter mitosis become stably infected and survive with replication competent virus. Based on these observations, we hypothesize that cells comprising the latently infected CD4 cell reservoir *in vivo*

are most likely descended from minimally activated T cells that are “bystanders” to both antigen-specific immune responses and productive HIV replication occurring in immune tissue microenvironments.

RESULTS

Cells that proliferate the least are more likely to become latently infected.

To determine whether an acutely infected, fully activated CD4 T cell can survive to a state of latent infection, we used our CD4 cell model, optimized for the development of HIV-1 latency in culture, to track infected cell proliferation using CFSE viable cell staining. Fourteen days following infection and stimulation, when more than 99% of cells have returned to resting, cells were sorted based on their cell division profile (see figure 3.1-A for cell sorting diagram). The recovered cell subpopulations were analyzed for the amount of integrated HIV DNA present, as well as virus production following a second round of stimulation. A reverse transcriptase inhibitor was added on day 5 of culture to block the subsequent spread of infection during later stages of the cell activation cycle.

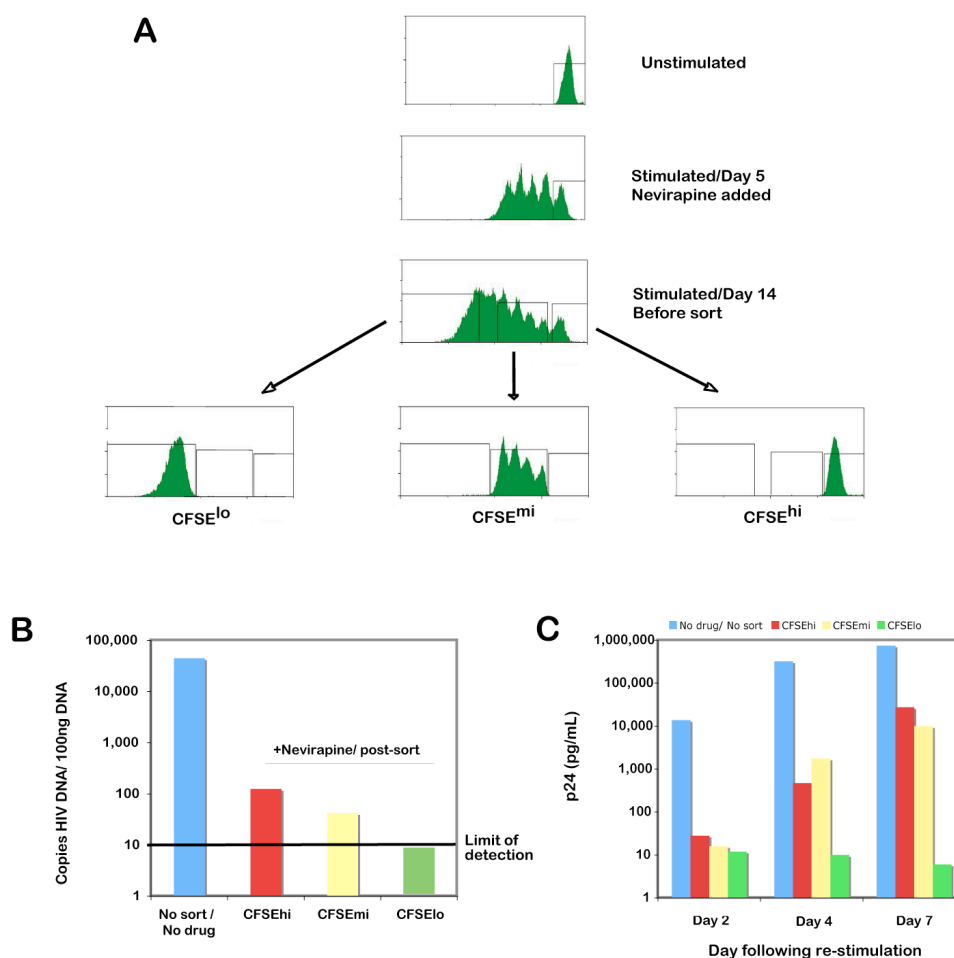


Figure 3.1. Cells that proliferate the least, following HIV infection, are more likely to become latently infected. **A)** Cell sorting procedure. CD4 lymphocytes were isolated by negative selection, stained with CFSE, infected with NL4-3 (MOI=0.1), and then induced to proliferate using immobilized anti-CD3 + anti-CD28. On day 5 after stimulation, Nevirapine was added to prevent further cycles of infection. At day 14, cells were FACS-sorted based on CFSE content into subsets that had: i) not divided (CFSE^{hi}); ii) divided a few times after day 5 (CFSE^{mi}); or iii) divided many times after day 5 (CFSE^{lo}). The recovered subsets were analyzed for integrated HIV DNA, and tested for induction of HIV replication. **B)** High molecular weight, cellular DNA was extracted from total CD4 cells and from each of the sorted CFSE subsets. The copy number of HIV provirus in each cell sample was determined by qPCR analysis (limit of detection of the assay is 10 copies/100ng DNA). Representative data, one of four experiments performed with different cell donors. **C)** Viable cell samples from each sorted subpopulation were taken at day 14, washed and restimulated with anti-CD3/anti-CD28 to induce productive virus replication. The levels of soluble p24 production were determined by ELISA following secondary stimulation. Representative data, one of four experiments.

Surprisingly, cells that did not divide (CFSE^{hi}) or cells that divided only a few times (CFSE^{mi}) during the 14 day culture contained the greatest number of integrated HIV DNA copies: 125 and 42 copies respectively, per 100 ng of genomic DNA (Figure 3.1-B). Cells that divided multiple times after the addition of the antiretroviral drug usually contained 2 to 4 fold fewer copies than cells that did not divide (Table I). We also tested for the presence of replication competent HIV in each sorted subpopulation by analyzing soluble p24 production in supernatant, and by staining for intracellular p24 expression at 2 and 7 days following a second round of cell stimulation. In agreement with the integrated HIV DNA data, cells that did not divide or divided only a few times produced higher amounts of p24 and showed greater percentages of cells expressing intracellular p24 (Figure 3.1-C and Figure 3.2).

Table 3.1. Quantification of integrated HIV DNA and induction of replication competent HIV (p24 production) after second round of cell stimulation. Cumulative data for all donors tested using the procedure described in Figure 1. n/a, not assayed; bd, below limit of detection.

	Integrated HIV DNA (copies/100 ng DNA)				p24 Production after 2 ^o Stimulation (pg/mL)			
	No Drug Unsorted	CFSE ^{hi}	CFSE ^{mi}	CFSE ^{lo}	No Drug Unsorted	CFSE ^{hi}	CFSE ^{mi}	CFSE ^{lo}
Donor 1	206,104	216	n/a	51	263,763	142,118	n/a	308
Donor 2	177,307	226	225	89	385,701	n/a	7,983	60
Donor 3	257,149	1,338	893	313	n/a	33,784	144,730	9,512
Donor 4	44,860	125	42	bd	746,047	27,428	10,020	26
Mean	171,355	476	387	151	465,170	67,777	54,244	2,477

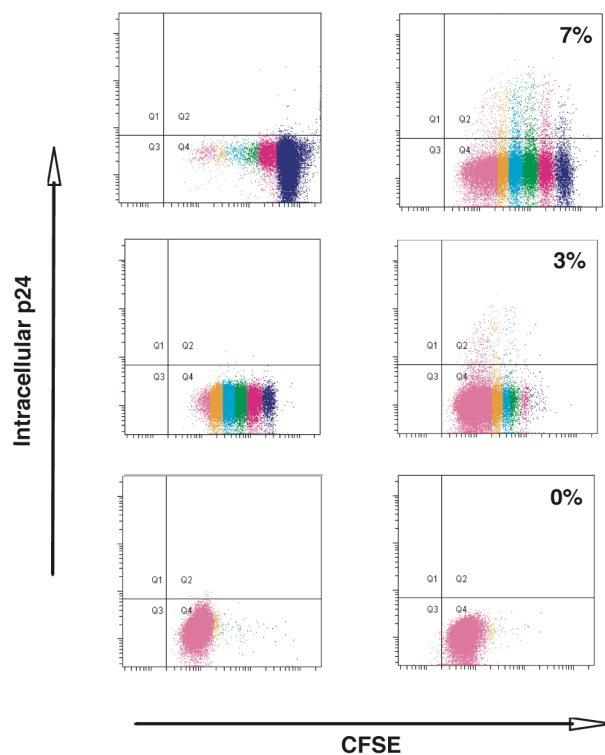


Figure 3.2. Cells that proliferate the least following HIV infection produce larger amounts of p24 following reactivation. After 14 days following initial HIV infection, and after sort based on CFSE profile, each subpopulation was washed and restimulated with anti-CD3 + anti-CD28 to induce productive virus replication. Cell subsets were analyzed by flow cytometry for levels of intracellular p24 expression, after 2 and 7 days of secondary stimulation. Representative data, one of four experiments.

These data suggest that even though a few cells seem able to survive acute infection and full activation to become latently infected, the majority of cells that survive to become part of a latently infected cell pool are cells that do not divide or divide only a few times in the presence of active HIV replication.

To verify that the differences observed in virus production following reactivation were not due to differences in the ability of highly-proliferating (CFSE-) cells to respond optimally to a second round of stimulation, cell cycle analysis was performed during the induced re-stimulation, using aliquots of cells taken from each sorted subpopulation. As shown in Figure 3.3, all three subpopulations responded equivalently well to TCR re-stimulation, with 25% to 50% of the cells exhibiting active cycling (BrdU incorporation).

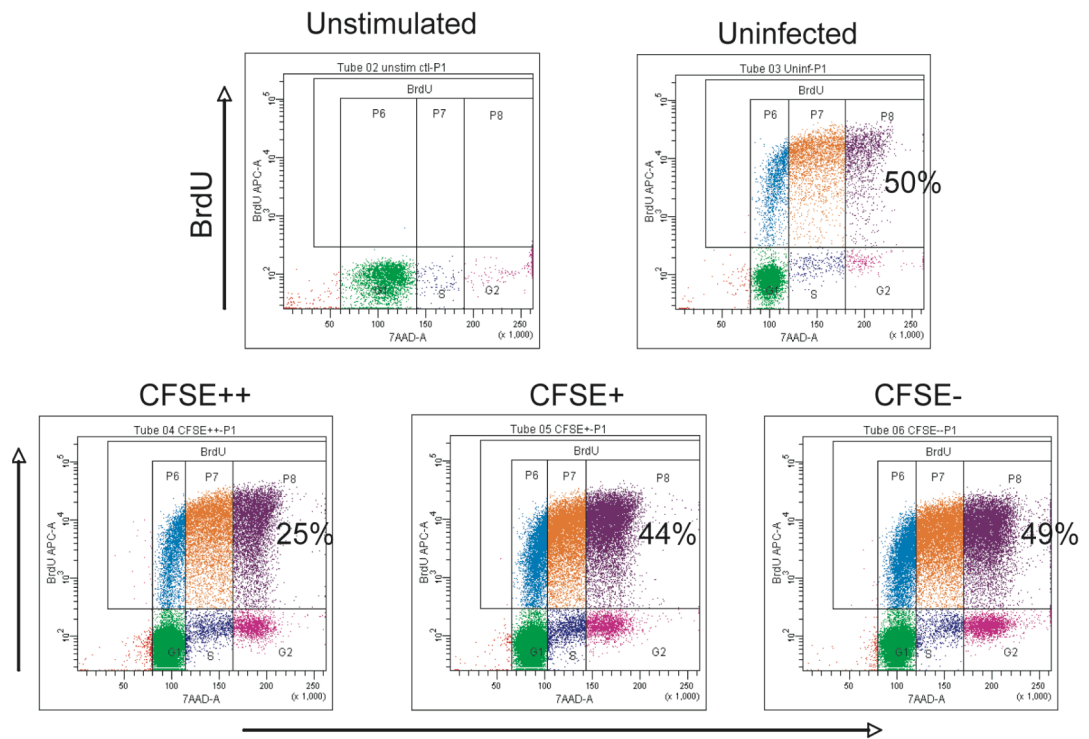


Figure 3.3. Sorted cell subpopulations respond similarly to a second round of stimulation. Following 4 days of re-stimulation, small aliquots from each sorted cell subpopulation and from unstimulated and uninfected cells were labeled with BrdU for 2 hours and then permeabilized, fixed, and stained for BrdU content.

Cells that divide the least remain IL-2R positive but do not express HLA-DR after 14 days in culture

To characterize the activation status of infected cells with different proliferation profiles, we stained CFSE labeled cells that had been in culture for 14 days, with antibodies against CD25 (alpha chain of the IL-2 receptor and a marker of early activation) and HLA-DR (MHC-II molecule and a marker of late activation). As shown in Figure 3.4-A and 3.4-B, most cells remained CD25 positive 14 days after infection and stimulation, regardless of the number of cell divisions. In contrast, only the cells that divided the most during culture (CFSE low or negative) expressed high levels of HLA-DR.

These results, along with the prior results, indicated that the majority of latently infected cells derived from our *in vitro* culture system, were those cells that had become only minimally activated while being in close contact with fully activated and productively infected cells. We hypothesized that minimal activation of such “bystander” cells would be sufficient to allow HIV to integrate into the host cell DNA, but not enough to activate virus production, allowing these cells to survive with persistent or latent infection.

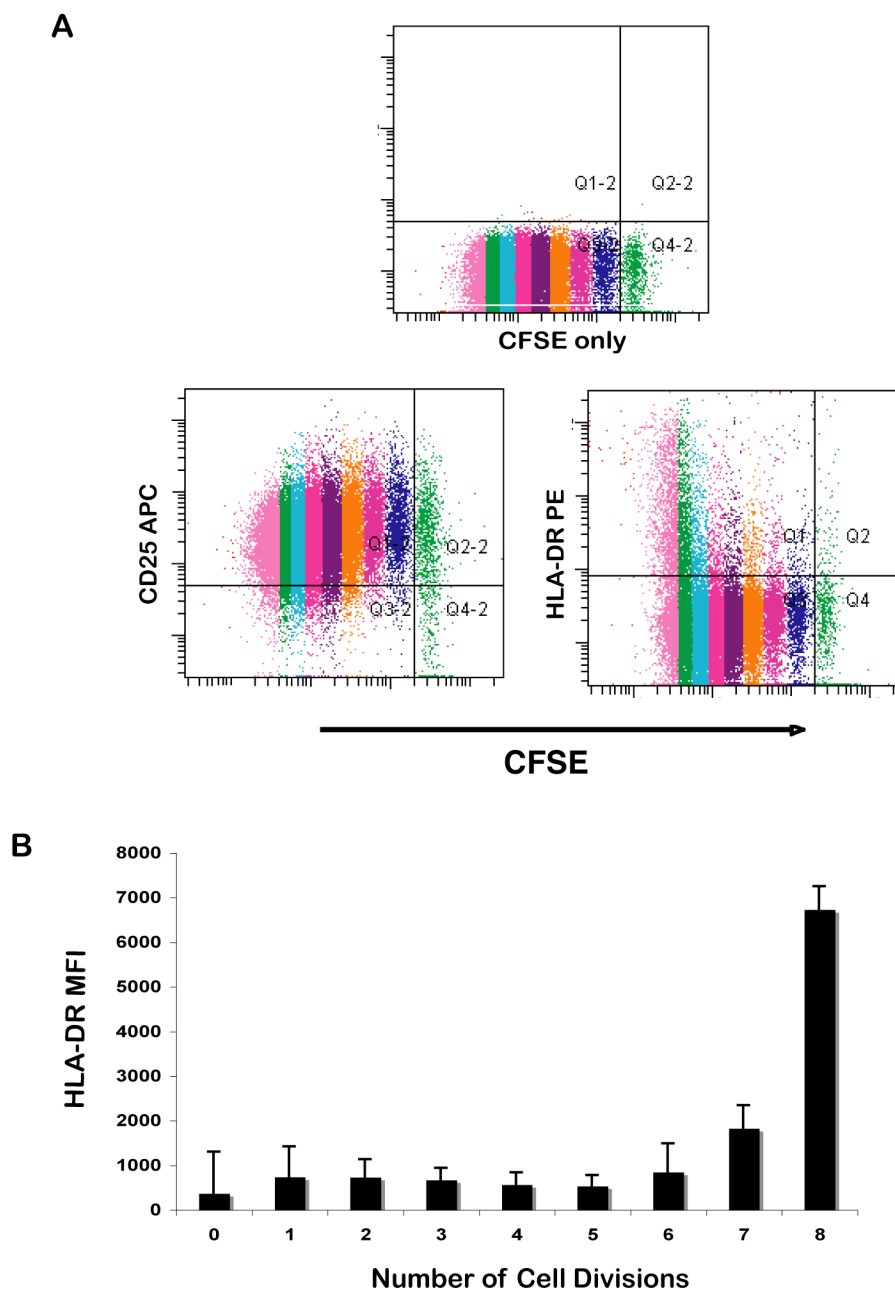


Figure 3.4. After 14 days in culture, the majority of cells remain CD25 positive; HLA-DR expression is seen primarily on highly proliferating cells. **A)** Cell samples were taken after 14 days in culture, following initial HIV infection and CFSE labeling. Recovered cells were stained for expression of IL-2R (CD25) and MHC-II (HLA-DR), and analyzed by flow cytometry. **B)** Graphic representation of HLA-DR expression on cells with different proliferation profiles. Mean Fluorescence Intensity (MFI) values represent the mean of three experiments. Error bars indicate standard deviation.

Unstimulated “bystander” CD4 cells, cultured in the presence of fully activated and infected cells, become the basis for a latently infected cell pool.

It has been shown previously that HIV can establish infection in resting CD4 T cells that have been cultured in the presence of cytokines, such as IL-2, IL-7, and IL-15 (28). To investigate whether latently infected cells produced in our model were derived through a similar scenario during culture with cytokines (namely IL-2 and IL-15) and productively infected proliferating T cells, we designed an experimental model for “bystander” cell infection. The model involved staining a portion of freshly isolated CD4 T cells with CFSE, and maintaining these stained cells without stimulation for 4 days (labeled #2 in Figure 3.5-A). Another portion of the CD4 cells were not stained, but were infected and stimulated according to our standard *in vitro* protocol (#3, Figure 3.5-A). After 4 days of culture, the unstimulated, uninfected, CFSE stained cells were mixed with the fully activated, infected cells at a ratio of 1:1 (2+3, Figure 3.5-B). As a positive experimental control for infection of CFSE stained cells, we infected and stimulated another portion of freshly isolated CFSE stained CD4 cells (#1, Figure 3.5-B) and cultured them in parallel for 14 days. Infection of each cell subpopulation was monitored by flow cytometry for expression of intracellular p24 staining, throughout the 14 day culture. As shown in Figure 3.5-B, 20% – 30% of fully activated, infected cells were expressing intracellular p24 at the peak of the infection. Interestingly, resting cells that were CFSE stained and cultured in the presence of fully activated infected cells, also exhibited intracellular p24 expression at day 7, demonstrating the infection of such non-proliferating cells.

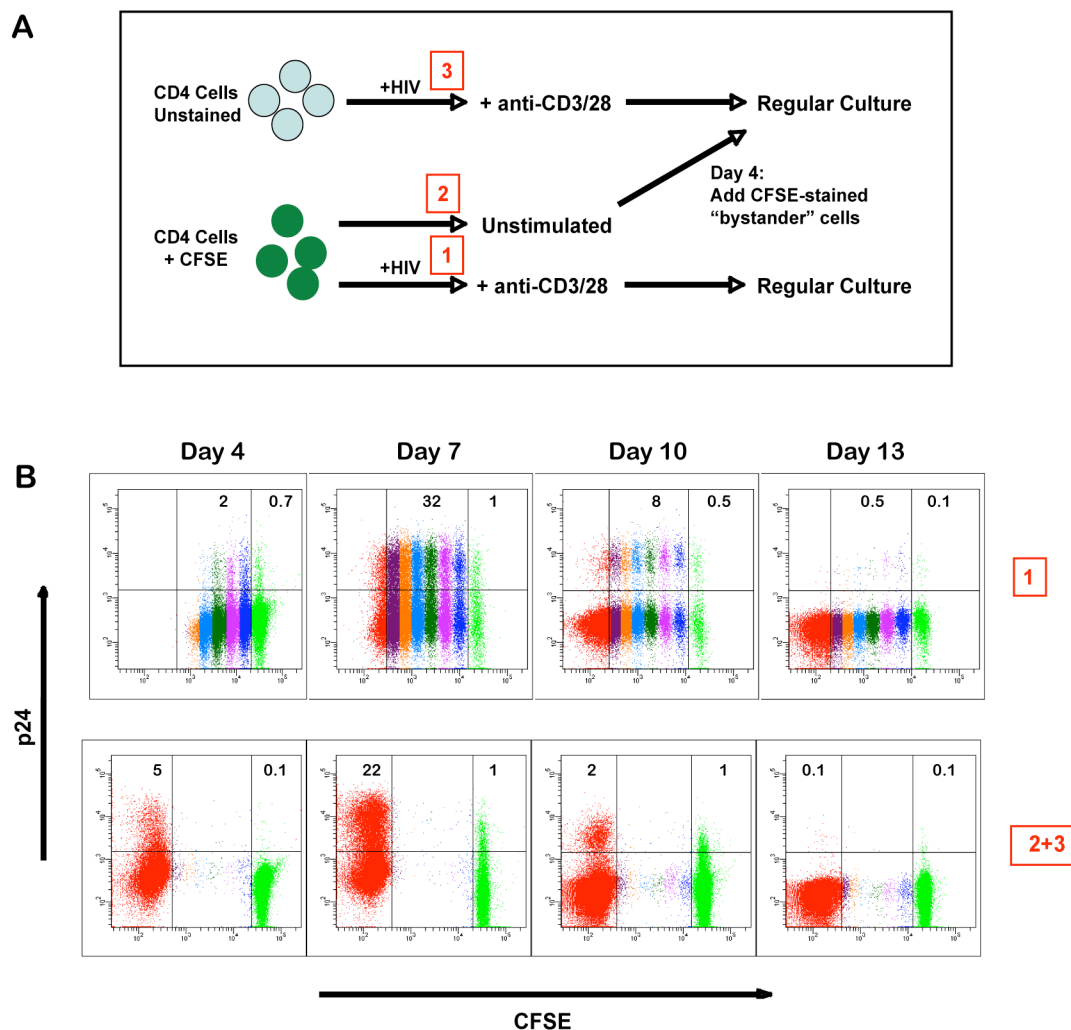


Figure 3.5. Unstimulated, non-proliferating cells become latently infected after culture with stimulated, infected cells. **A)** Diagram depicting experimental design for testing the infection of unstimulated, non-proliferating cells. After isolation of CD4 cells from healthy donors, half the cells were stained with CFSE, and the other half was left unstained. Unstained cells were infected, stimulated and cultured according to our standard *in vitro* model protocol (#3 in diagram). After 4 days in culture, unstimulated uninfected, CFSE stained CD4 cells (#2 in diagram) were added to the infected stimulated cells. The cell mixtures were cultured for an additional 10 days in the presence of cytokines. Indinavir (1 μ M) was added at day 7. An aliquot of CFSE stained cells was infected, stimulated, and cultured in parallel for comparison (#1 in diagram). **B)** Cells were monitored throughout the 14 day culture for intracellular p24 expression. Top row: CFSE stained, infected, and stimulated cells. Bottom row: CFSE stained, unstimulated cells mixed at day 4 with infected, stimulated, unstained cells. Representative data, one of three experiments.

After 14 days of culture, the cells were sorted into CFSE-bright (unstimulated, bystander cells) and CFSE-negative (fully activated, proliferating cells) subsets, and each cell subset was then analyzed for the amount of integrated HIV DNA and the frequency of replication competent virus. Even though the percentage of productively infected cells in the fully activated cell population (CFSE-negative) was much greater than the unstimulated cell population (CFSE-bright) at the peak of culture on day 7, the number of integrated HIV copies and the number of infectious units per million cells (IUPM) were similar in both subpopulations at day 14 (Table 3.2). In fact, the amount of latent HIV infection appeared to be greater in the unstimulated cell population.

Our data indicate that the majority of the latently infected CD4 cells result from infection of “bystander” cells, which become minimally activated by being cultured in the presence of fully activated, infected cells. The results suggest that the direct infection of resting (non-proliferating) CD4 memory T cells may be the major contributor to development of the latently infected T cell reservoir *in vivo*.

Table 3.2. Non-proliferating, minimally-activated CD4 cells form the major portion of latently infected cells. After 14 days in culture, cells were sorted into CFSE-bright and CFSE-negative populations. Each subpopulation was analyzed for number of copies of integrated HIV DNA, and number of infectious units per 10^6 cells.

	D14 Cell Subset	HIV _{intg} / 10^6 Cells	Infectious Units/ 10^6 Cells
Donor 1	CFSE +	464,563	25,030
	CFSE -	192,000	25,030
Donor 2	CFSE +	93,938	817
	CFSE -	80,500	162
Donor 3	CFSE +	51,250	1,429
	CFSE -	78,813	817
In vivo*		100	1

*Hermankova et al. J Virol. 2003.

DISCUSSION

The mechanisms involved in the establishment of HIV-1 latency in CD4 T cells have not been clearly defined. Studies into possible mechanisms of HIV latency have been hindered by the lack of a biologically relevant *in vitro* model. Our lab has developed a unique *in vitro* cell model, which has enabled us to identify activation and proliferation requirements for the establishment of HIV-1 latency in primary CD4 T cells.

Three main hypotheses have been suggested for how HIV latency is established in CD4 T cells: 1) an activated, proliferating cell becomes infected and reverts back to resting; 2) a cell becomes infected during a narrow window of time as it is reverting back to a resting state; or 3) a resting memory cell becomes infected without full activation (27). Using our optimized *in vitro* model for development of HIV latency, we show that resting CD4 cells cultured in the presence of fully activated, infected cells become a major part of the latently infected cell pool. Our data suggest strongly that direct infection of resting, or non-proliferating, cells is an important mechanism for the establishment of HIV-1 latency in CD4 T cells.

Other *in vitro* studies examining direct infection of resting T cells have produced conflicting results. Some early studies have shown that reverse transcription is inefficient in resting T cells (8), and nuclear import and integration are not detected unless cells are activated to enter the cell cycle (49). More recent studies, however, have demonstrated infection of resting primary T cells following

virus spinoculation (50), and that virus-producing endothelial cells can promote HIV replication in non-dividing memory T cells (51).

Most importantly, HIV infection of resting CD4 cells has been detected *in vivo* in both animal models and infected humans. The population of resting T cells that exhibited infection had a longer half life than that of infected cells, which were activated (52). These investigators hypothesized that cytokines present in the immune tissue milieu could provide a permissive environment for viral replication in resting T cells. Other investigators (53) have proposed that unintegrated HIV, which has been shown to affect the response of resting T cells to activating stimuli, may alter the resting state of the cells, making them more permissive to infection. More studies on a molecular level are needed to determine which of these, or other, mechanisms are involved in direct infection of resting T cells.

Understanding the mechanism(s) responsible for the establishment of HIV latency should help to identify possible unique targets for the development of new treatment strategies. If specific markers of resting T cell infection can be defined in the future, it may be possible to use such markers to identify and purge the latent HIV reservoir to eradicate infection.

MATERIALS AND METHODS

Primary CD4 cell cultures. See Material and Methods Section, Chapter II.

Virus infection. See Materials and Methods Section, Chapter II.

Quantification of integrated HIV DNA. Aliquots of infected cells and uninfected cell controls were collected from culture at different time-points, and kept frozen in dry pellets of $1-2 \times 10^6$ cells. For batch analysis, pellets were thawed and resuspended in 200 μ L calcium-free and magnesium-free Dulbecco's PBS (Mediatech). Genomic DNA was extracted using the Qiagen Blood and Cell Culture DNA Mini Kit (Qiagen, Valencia, CA) and eluted in 100 μ L of 10 mmol/L Tris, pH 8.0. After determination of DNA concentration by spectrophotometry, real-time qPCR was performed with 100-200 ng of input genomic DNA, using ABI Prism 7700 Sequence Detection System with software version SDS 1.9.1 (Applied Biosystems, Foster City, CA). Sample DNA content was calculated using the sample cycle threshold signal values (ct sample) correlated to the standard curve ct values (ct standard). Real-time qPCR was performed in 25 μ L containing 5 μ L DNA target, 12.5 μ L TaqMan® Universal Master Mix (Applied Biosystems) and 7.5 μ L primers and probe (900nM forward and reverse primers, 200nM probe) using the following cycling conditions: 50°C 2min, 95°C 10min, then 95°C 15sec, 60°C 1min in 45 cycles.

Primers and probes were designed against p24 gag, and their sequences were as follows:

Forward: 5'-AAAAGAGACCATCAATGAGGAAGC-3'
Reverse: 5'-TGGTGCAATAGGCCCTGC-3'
Probe: 5'-FAM/CAGAATGGGATAGATTGCATCCAG-3'

Cell cycle analysis. At a given time point in culture, cell aliquots were labeled for 2 hours with BrdU provided in BD Pharmingen's BrdU Flow Kit. Following labeling, cells were permeabilized, stained, and fixed according to the manufacturer's instructions. Data were analyzed using a BD FACS Canto instrument with DiVa software 2.1 (Becton Dickinson).

Quantification of infectious units per million cells (IUPM). Mixed cultures of CFSE stained and unstained CD4 T cells (refer to Figure 4) were sorted at day 13 into CFSE-bright and CFSE-negative subsets. After washing, the sorted cells were resuspended in 5% RPMI media at 5×10^5 cells per mL and plated into duplicate wells of a 96-well flat-bottom plate coated with anti-CD3 and anti-CD28 antibodies, as described above (250uL of cell suspension/well). Infected cells were diluted into uninfected cells from the same donor that served as indicator cells to amplify growth of reactivated virus. Five-fold serial dilutions were made into 10^5 uninfected cells, in duplicate wells. Plates were sealed and incubated at 37°C in 5% CO₂ in air for 7 days. Unstimulated, cell controls were cultured following the same procedure using uncoated, tissue-culture treated plates. After 7 days, 100 uL of supernatant was

collected from each duplicate well and analyzed for soluble p24 by ELISA. Sample wells with p24 values $>50\text{pg/mL}$ were scored as positive. Infected cell frequencies were determined by the maximum likelihood method (70) and were expressed as infectious units per million CD4 cells.

Cell staining and flow cytometry analysis. The purity of CD4 lymphocyte preparations and the expression of CD25 and HLA-DR were monitored by direct staining flow cytometry, as described in Materials and Methods, Chapter II.

For cell proliferation assays, 10×10^6 cells were labeled with $10\mu\text{M}$ carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Carlsbad, CA) for 5 min at room temperature, quenched with 10 mLs of cold RPMI media with 10% human AB sera, placed on ice for 5 minutes, and then washed with 5 mLs warm RPMI media with 5% human AB sera. The samples were analyzed and sorted using a MoFlo cell sorter (DakoCytomation, Denmark) under biocontainment conditions. For intracellular p24 analysis, cells were washed in PBS, and fixed and permeabilized using Cytotfix/Cytoperm Buffer (BD Biosciences). After washing with Perm/Wash Buffer (BD Biosciences), cells were incubated for 30 min. with KC57-RD1 antibody (Beckman Coulter), specific for the p55, p24, and p17 antigens of HIV-1. Cells were washed again with Perm/Wash buffer and stored in 0.5% formaldehyde at 4°C until acquisition on a FACSCanto instrument (BD Biosciences). Data were analyzed with BD FACS DiVa software 2.1 (BectonDickinson).

CHAPTER IV

DETECTION OF HIV TRANSCRIPTIONAL ACTIVITY IN LATENTLY INFECTED, RESTING CD4 T CELLS

INTRODUCTION

Most studies aimed at understanding the mechanisms of how HIV-1 latency is maintained have utilized infected cell lines as models (42, 54). However, considering the profoundly quiescent state of resting memory CD4 T cells *in vivo*, continuously dividing cell lines may not provide an accurate representation of the latent reservoir *in vivo*. A few studies have examined highly purified populations of resting CD4 T cells taken from patients who had prolonged suppression of viral replication on HAART (32, 33). Despite the important observations gained from these studies with *ex vivo* samples, the extremely low frequency of latently infected cells obtained from the peripheral circulation (estimated to be 1 in 10^6 CD4 cells) could produce misleading results.

It was shown recently that resting CD4 T cells from patients on suppressive HAART produce abortive mRNA transcripts that lack a poly(A) tail (33). Although short transcripts dominated, processive polyadenylated HIV mRNAs were also present in both unspliced and multiply spliced forms. One caveat of this study was the extremely low frequency of infected CD4 T cells within the pool of total CD4 cells isolated from infected donors. Such sampling constraints may have skewed results toward very low levels of detected transcripts. Due to the low frequency of latently infected cells and the inability to differentiate between infected and uninfected subpopulations, this study also was unable to determine whether the processive, polyadenylated mRNAs were being translated into protein.

In contrast, the *in vitro* model developed by our research group addresses these problems by increasing the proportion of latently infected cells available for study. This chapter characterizes our cell model further, using biomolecular methods to examine the latently infected cells present at day 14 of culture. First we determined the metabolic state and the frequency of replication competent virus present in the latently infected CD4 cells. Second, we investigated whether the HIV provirus in a nonproductive, persistent infection is completely devoid of transcriptional activity (classical viral latency) or if low levels of gene transcription can be detected.

RESULTS

Detection of intracellular HIV Gag in resting infected CD4 cells after 14 days of culture.

In a series of early experiments, low levels of intracellular HIV Gag (p24/p55) were detected in the resting infected CD4 cell population at day 14 of culture. The subset of cells with Gag expression were found to be truly resting, however, and not transcriptionally active. An example of such experimental results is shown in Figure 4.1. Here, the vast majority of cells expressing Gag (7.5% of total culture) were in the G₀ phase of cell cycle (Fig. 4.1B; color coding within ICp24 cell subsets corresponds to cell DNA content, as depicted in 4.1A). In addition, the cells expressing p24 Gag did not contain the small fraction of transcriptionally active cells (0.5% BrdU+) found at day 14 (Fig. 4.1B). It is very important to note that these earlier experiments did not use addition of antiretroviral drugs to halt HIV replication and spread during the latter half of the culture period.

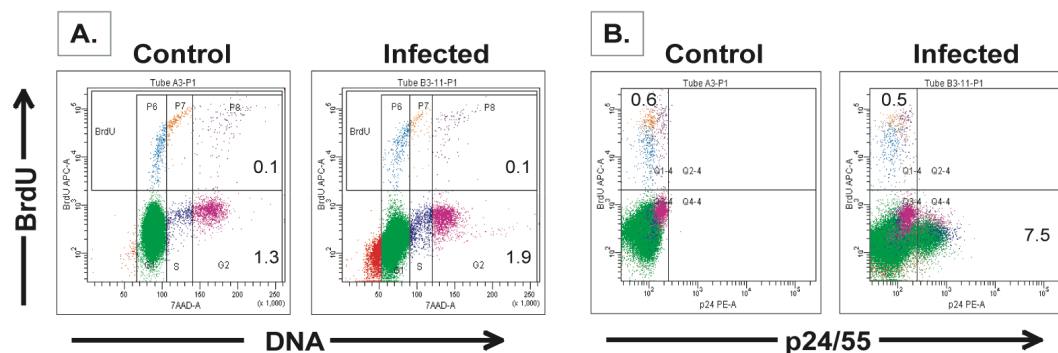


Figure 4.1: Detection of p24 Gag in CD4 cells at day 14 of culture. **A)** Infected and uninfected cells were labeled for BrdU incorporation overnight (BD kit). 7-AAD was then added to stain cellular DNA content, and analysis performed on a FACS Canto. **B)** Another cell aliquot was stained in parallel for intracellular HIV Gag (p24/p55).

Detection of HIV Gag in the infected quiescent cells could have resulted either from productive ongoing expression within cells, or from detection of excessive capsid protein (p24) in virions attached to the cell plasma membrane. To determine whether the detected Gag protein was due to virions bound to the surface of CD4 cells, an acid wash method (71) to remove bound virions from the cell membrane was performed prior to staining for p24 Gag expression. Infected cells treated with the acid wash procedure showed the same level of intracellular p24 expression as did infected cells that did not receive the wash (Figure 4.2, top panel). In addition, an aliquot of uninfected cells, exposed for 1 hour at 37°C to supernate taken from the peak of HIV infection, did not stain positive in the analysis for

intracellular p24 expression (Figure 4.2, bottom panel). These results demonstrated that HIV Gag detected in resting cells from day 14 of our infection model, represented true intracellular expression of Gag within cells.

Our experiments have not addressed whether the detected intracellular HIV Gag at day 14 of culture was the product of active ongoing translation or prior translation that was no longer active. To remove this variable from our cell model of viral latency and to circumvent continued rounds of HIV infection during the later phases of cell culture, subsequent work has utilized the addition of antiretroviral drugs (Nevirapine, anti-RT or Indinavir, anti-protease) to halt HIV replication and spread after day 7 of culture (Chapter III).

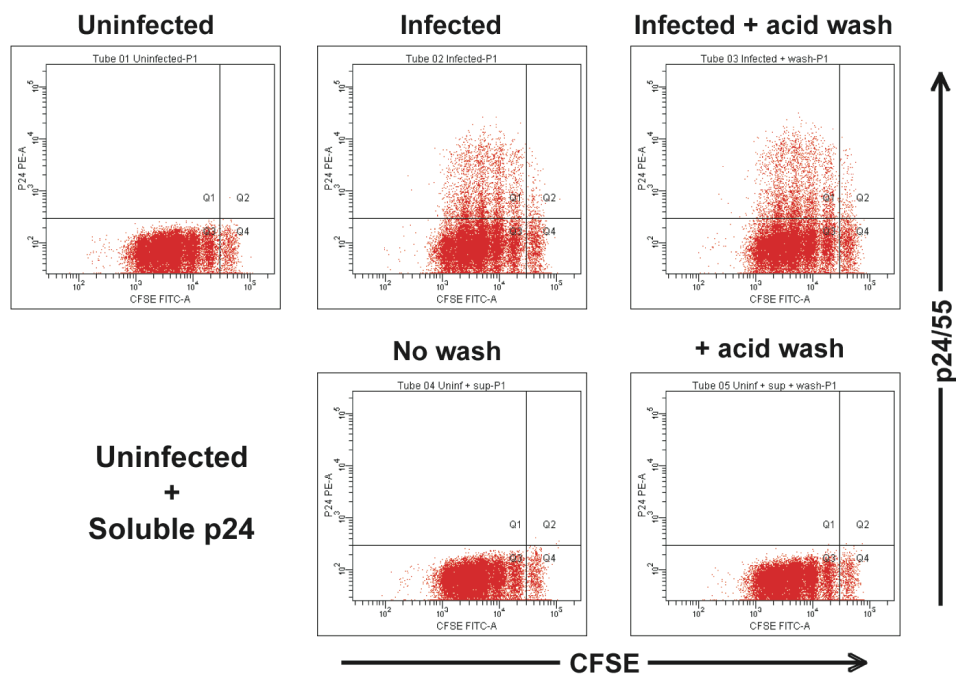


Figure 4.2. Detection of productive HIV infection using intracellular staining for p24/p55 Gag protein. **Top Row)** NL4-3-infected primary CD4 lymphocytes at day 7 after TCR stimulation. Cell divisions tracked with CFSE stain (X axis). ICp24-positive cells, with the presence of dim and bright subsets (Y axis). Some cells were treated by acid wash (pH 2) to strip any external virions from the cell surface (far right panel). **Bottom Row)** Uninfected CD4 cells (day 7) were exposed to soluble p24 collected from the parallel, infected culture (~150,000 pg in 1 ml) for 1 hr at 37° C. Half of the exposed cells were then treated by acid wash. Both cell aliquots were stained by the ICp24 method to determine whether cell membrane-bound virions could be detected. Levels of virion binding were confirmed by p24 ELISA on the total cell lysates.

A small fraction of latently infected CD4 cells in our model contains replication competent virus.

Other studies looking at persistent HIV infection *in vivo* have shown that the majority of integrated virus detected in CD4 T cells represents archived HIV DNA that is not replication competent (16). With this knowledge in mind, it became important for us to determine what fraction of HIV provirus found in CD4 cells from our latency model was replication competent.

To address this question, a second round of stimulation with immobilized anti-CD3/CD28 antibodies, in the presence or absence of antiretroviral drug to prevent new rounds of infection, was performed and cells were analyzed for intracellular Gag (ICp24) expression by flow cytometry. After 2 days of re-stimulation, a very small percentage of cells showed an increase in the expression of ICp24 by increasing the level of mean fluorescence intensity, MFI (Figure 4.3 top panel). The actual percentage of ICp24-positive cells did not appear to increase. Only the relative brightness of the stained ICp24 per cell increased, suggesting a substantial increase in the production of HIV Gag within the infected cells. Once again, it is important to note that in these experiments, antiretroviral drugs were not added to the initial acute infection culture to limit late infection events occurring after 7-10 days.

After 7 days of secondary cell stimulation and culture (Figure 4.3, bottom panel), the percent of cells with high levels of ICp24 expression (e.g. bright staining) increased slightly for the cell aliquot cultured in the absence of antiretroviral drug

(Stim). This was probably the consequence of spreading HIV replication, with new rounds of viral infection. In contrast, the aliquot of cells that was re-stimulated in the presence of antiretroviral drug (Stim + drug) showed a decrease in the percent and staining intensity of cells expressing ICp24. This was likely the result of cell death induced by the activation of productive HIV replication during cell re-stimulation

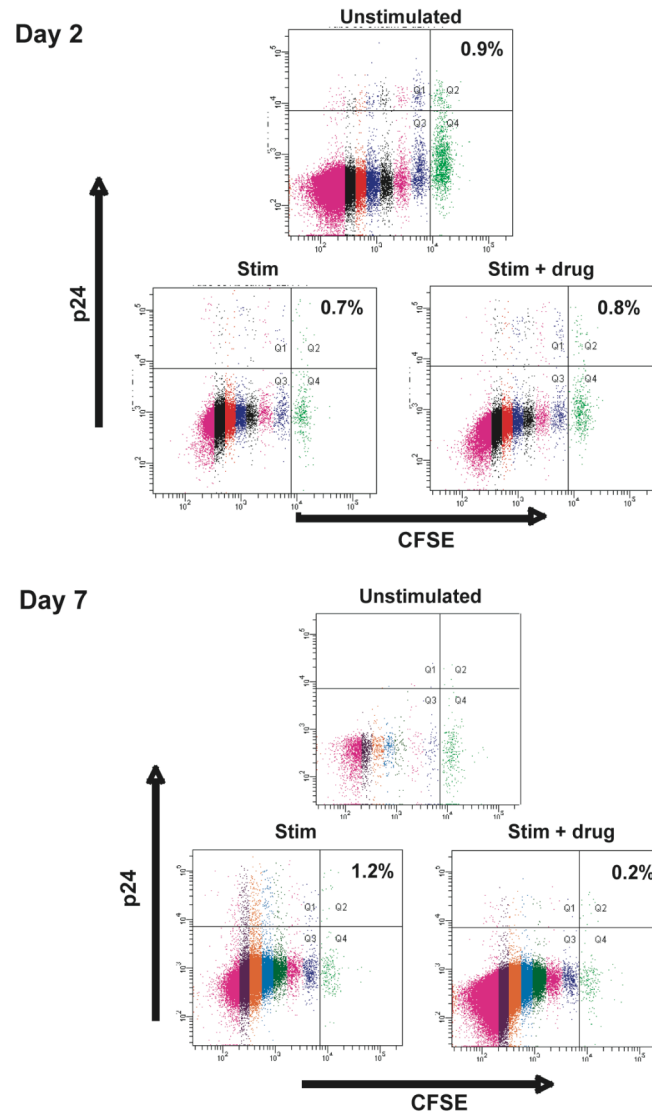


Figure 4.3: A small fraction of latently infected cells contain replication competent virus. Cells from day 14 of the *in vitro* model were stained for intracellular p24 (ICp24) expression following a second-round of stimulation with anti-CD3 and anti-CD28 antibodies and culture with or without the addition of antiretroviral drug. Cell aliquots from unstimulated and stimulated samples were analyzed by flow cytometry at two (top panel of 3 histograms) and seven (bottom panel of three histograms) days after re-stimulation.

Detection of unspliced and multiply spliced HIV transcripts in latently infected cells.

Persistent/latent HIV infection may involve the production of low levels of transcription, limited to early phase, multiply-spliced species of mRNA. In fact, reports from clinical studies have described the continued presence of multiply-spliced (MS) and unspliced (US) species of HIV RNA in peripheral blood CD4 T cells isolated from patients after long-term, therapy-induced suppression of viremia (76, 33).

To determine whether HIV provirus that is carried in quiescent, but persistently-infected, CD4 cells from our *in vitro* model is transcriptionally active, we analyzed the content of RNA extracted from infected CD4 cells throughout the 14 day culture period, with special emphasis on the end of culture when cells have returned to a resting state. In this series of experiments, antiretroviral drug (Indinavir) was added on day 7 of culture to prevent subsequent new rounds of infection. Interestingly, the relative levels of each HIV transcript species examined remained parallel throughout the culture period, with unspliced transcripts (gag) being the most abundant (Figure 4.4-A). At the end of culture on day 14, all the viral mRNA species were still detectable and the hierarchy of expression remained the same, with unspliced transcripts being the greatest. These results demonstrated that HIV latency, produced in our model system, is not devoid of viral gene transcription.

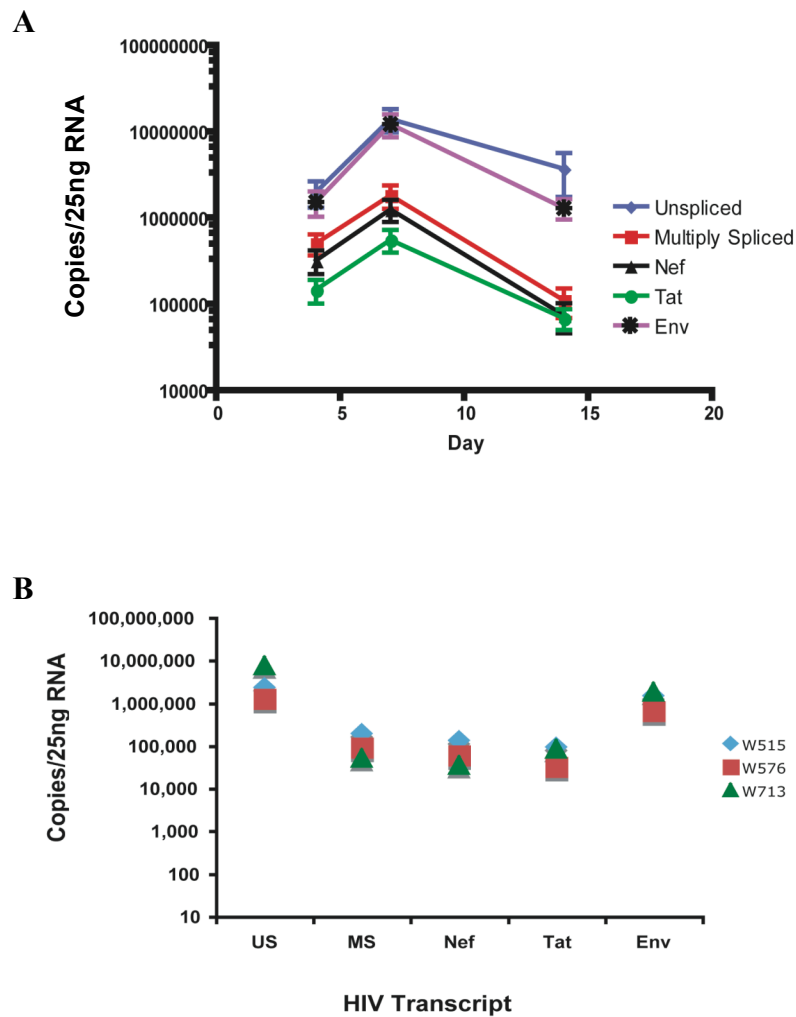


Figure 4.4: Unspliced and multiply-spliced HIV transcripts detected in CD4 cells from the *in vitro* model of latency. Total RNA was extracted from infected CD4 cell samples (days 4, 7, 14 of culture) and DNase treated. Real-time qRT-PCR analysis was performed using a multiplexed, standardized assay (see Materials and Methods). Data is expressed as copy number per 25 ng of input RNA. **A)** Time course of mRNA expression for each species of HIV transcript (color-coded legend). Data for three donors tested, error bars represent the standard error of the mean. **B)** Transcription levels of each viral mRNA species detected at day 14 of culture, for each of three donors tested.

DISCUSSION

The study of HIV latency in CD4 T cells requires a model with primary cells that adequately represent the latently infected cells found *in vivo*. The *in vitro* CD4 model developed in Dr. Spina's lab provides a powerful tool to study the mechanisms involved in both the establishment and the maintenance of latent HIV infection.

The detection of HIV Gag expression (ICp24) in latently infected, resting CD4 cells harvested from our long-term cultures was unexpected. Interestingly, the subset of cells expressing ICp24 at day 14 of culture (7.5% of the total population) was in the G₀ phase of the cell cycle and did not incorporate BrdU during overnight labeling. Detection of intracellular p24 expression in such cells raised the possibility that we were measuring excess virions, attached to the surface of the cells. Performance of acid washes to remove any bound virions confirmed that the detected ICp24 protein was indeed due to the presence of Gag protein within the cells. However, the possibility remains that this Gag protein was not newly synthesized, but rather maintained stably inside infected cells over hours to days. Further experiments are needed to address this question.

The detection of HIV Gag protein in latently infected, resting cells also indicated that HIV latent infection was most likely not devoid of viral transcriptional activity, as in "classical" proviral latency. Therefore, we applied a sensitive qRT-PCR assay for the detection of multiple different species of HIV mRNA to examine

the transcriptional activity of the HIV provirus in CD4 cells from our *in vitro* model. Both unspliced and multiply-spliced HIV RNA transcripts were found in latently infected cells. A relative hierarchy of expressed transcripts was seen, with unspliced *gag* transcripts being the most abundant followed by singly-spliced *env*, total multiply-spliced, *nef*, and *tat* species. These results were in agreement with findings from recent studies using T cells from infected patients (33). The stability of the HIV transcripts, detected in our cell model, was not investigated. Further work will be needed to determine whether all the detected HIV mRNA species are being continuously transcribed, even after infected cells return to a resting state. In addition, we do not know whether any of the multiply-spliced viral RNAs (e.g. *nef* and *tat*) may be translated into functional protein products.

The detection of viral mRNA transcripts in latently infected CD4 T cells suggests that HIV latency may not be maintained exclusively by a passive mechanism. Rather, HIV latent infection may be maintained actively through the interaction of viral factors, such as interfering RNA or regulatory proteins, with the transcriptional machinery of the T cell to prevent activation of the provirus into a productive replication cycle.

MATERIALS AND METHODS

Primary CD4 cell cultures. See Material and Methods Section, Chapter II.

Virus infection. See Materials and Methods Section, Chapter II.

Cell cycle analysis. Day 14 cells were labeled overnight (16 to 18 hours) with BrdU provided in BD Pharmingen's BrdU Flow Kit. After labeling, cell aliquots were permeabilized, stained, and fixed according to the manufacturer's instructions. Data were analyzed using a BD FACS Canto instrument with DiVa 2.1 software (Becton Dickinson).

Acid Wash. The method was based on a modification of a published procedure (71). Aliquots of approximately 10^6 cells were pelleted and resuspended in 0.5 mLs of RPMI media. Two mLs of cold acid wash (RPMI media with 2 M HCl, pH 2) were added and cells were placed in ice for 2 minutes. Cells were then washed once with RPMI media, and once with Dulbecco's PBS.

Re-stimulation of cultured CD4 cells. Following 14 days of culture, cells were harvested and washed with PBS, and viable cell counts were performed using Trypan blue dye exclusion. The recovered CD4 cells were resuspended in complete RPMI medium with 5% human AB serum (supplemented with 1mM L-glutamine, 50 U/mL

penicillin, 50ug/mL streptomycin), and distributed at a concentration of 10^5 cells/100uL/well in duplicate into 96-well flat-bottom plates that had been pre-coated with anti-CD3 plus anti-CD28 antibodies. The microcultures were incubated at 37 °C in 5% CO₂ in air for 2 to 7 days following stimulation. Cell samples were removed from culture, washed, and stained for intracellular p24 expression, as described in the Materials and Methods section of Chapter III.

Quantification of HIV transcripts by real-time qRT-PCR. CD4 cell samples were harvested from culture at sequential time points following infection and TCR stimulation. The HIV protease inhibitor, Indinavir, was added on day 5 of culture to prevent subsequent rounds of virus infection and spread. Total cellular RNA was extracted (Qiagen RNA Easy kit) from each sample and real-time qRT-PCR was performed using RNA standard controls to quantify the copy number of each of the targeted HIV mRNA species. Up to 1 µg RNA was isolated, reverse transcribed by random hexamer priming, and analyzed in TaqMan qPCR. Transcript copy numbers were normalized to 25 ng input RNA.

The real-time qRT-PCR method for quantification of NL4-3 viral transcripts was developed by Mary Lewinski, during a Ph.D. post-doctoral research project performed in Dr. Spina's lab. Specific TaqMan primer and probe sets targeting unspliced, multiply-spliced, Nef-encoding, Tat-encoding, and singly-spliced Env-encoding species of HIV RNA were designed. The amplification products (amplicons) spanned the splice donor (SD) – splice acceptor (SA) sites unique to the

RNA species of interest, with either the primers or probe annealing across the splice junction. Their sequences were as follows:

US RNA-gag-F: AAAAGAGACCATCAATGAGGAAGC
 US RNA-gag-R: TGGTGCAATAGGCCCTGC
 US RNA-gag-Probe: CAGAATGGGATAGATTGCATCCAGTGCA
 MS RNA-total-F: GCTCATCAGAACAGTCAGACTCATC
 MS RNA-total-R: TGTCGGGTCCCCTCGG
 MS RNA-total-Probe: CTTCTCTATCAAAGCAACCCACCTCCCAAT
 SS RNA-env-F: CGACTGGAAGAAGCGGAGA
 SS RNA-env-R: ATTACTATGGACCACACA ACTATTGC
 SS RNA-env-Probe: CGACGAAGAGCTCATCAGAACAGTCAGACTC
 RNA-nef-F: GGCGACTGGAAGAAGCGG
 RNA-nef-R: GGAGGTGGGTTGCTTTGATAGAG
 RNA-nef-Probe: AAGAGCTCATCAGAACAGTCAGACTCATCAAGCT
 RNA-tat-F: GGCGACTGAATTGGGTGTC
 RNA-tat-R: TCTACTGGCTCCATTTCTTGCT
 RNA-tat-Probe: TCCTCTGTCGAGTAACGCCTATTC

Using RNA derived from CEM T cells acutely infected with the NL4-3 HIV clone, cDNA amplicons for each of the designed splice variants were generated. The cDNA products were cloned into plasmids using a TOPO[®]-cloning vector system. Bacterial transformations were performed to screen clones for the inserted amplicons of interest, and maxi-preparations were made. Plasmids carrying the identified inserts were isolated, purified, and sequenced for specificity and proper orientation. Sense-oriented RNA standards were *in vitro*-transcribed, using the plasmid T7 promoter. The presence of a single band of the appropriate size was confirmed for each RNA amplicon standard, by denaturing agarose gel electrophoresis. The RNA standards were then DNase-treated, column purified, quantified by spectrophotometry, and diluted into a constant background of extracted RNA from uninfected CEM cells.

The designed primer and probe sets were tested and validated for their sensitivity and specificity of detection to each of the HIV target sequences.

CHAPTER V

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

CONCLUSIONS

The study of HIV-1 latency has been hindered severely by the lack of biologically relevant *in vitro* cell models that can mimic the state of viral latency found *in vivo*. In my thesis research project, I have used a novel primary T cell model of HIV latency to characterize both viral and cellular factors that are important for the establishment and maintenance of HIV latency in CD4 T cells.

From some of the earliest studies in this area, it was known that the activation state of T cells is intimately linked to the cell's susceptibility to HIV infection (7, 8, 25, 49). The first goal of my studies was to determine the stage of T cell activation in which cells were more likely to become infected and survive with an integrated provirus to latency. This data did not support a hypothesis put forward by others (77), that latently infected cells are mostly likely to develop from the infection of proliferating T cells during a narrow window of time as they revert back to a resting state. In our cell model, exposure to HIV followed closely by cell activation could have contributed to the development of latently infected cells in either of two ways: 1) fully-activated proliferating cells became productively infected sometime during the culture period, and then reverted back to a resting cell state; or 2) resting cells became infected while undergoing minimal activation which provided them a survival advantage. Further experiments, performed to address the question, demonstrated clearly that CD4 cells that do not divide, or divide only a few times,

following stimulation form the subpopulation of infected cells most likely to survive with latent infection.

The results from the first phase of my research project indicated that the vast majority of acutely infected, activated CD4 T cells are not able to survive multiple rounds of cell division in combination with the cytopathic effects of HIV to become a latently infected cell reservoir. Rather, the subset of CD4 T cells that exhibited minimal activation, in the presence of fully-activated and productively infected T cells, went on to survive with latent HIV infection. In the second phase of my research, I used a different experimental approach to examine whether the minimally activated CD4 cells in our model represented “bystander” cells during an immune T cell proliferative response. Other investigators had observed HIV infection of resting CD4 T cells *in vivo* (52) and hypothesized that cytokines present in the immune tissue milieu could provide a permissive environment for viral infection in resting T cells. To test this hypothesis, freshly isolated resting CD4 cells were labeled with the CFSE viable dye, cultured with unstained fully-activated, HIV-infected CD4 cells from the same donor, and then tracked over time for levels of cell activation and viral infection. The results showed that the majority of latently infected CD4 cells, found at the end of culture, originated from the direct infection of non-proliferating “bystander” T cells. This data suggest that direct infection of resting (non-proliferating) CD4 memory T cells may be the major contributor to development of the latently infected T cell reservoir *in vivo*.

In the final section of my thesis project, I examined whether HIV latency that developed in our CD4 cell model represented a classical mode of viral latency, where no viral products are produced, or whether some level of viral transcription takes place. Applying a qRT-PCR method specially adapted for our culture system, experiments detected the presence of multiple different species of HIV RNA in the latently infected CD4 cells at the end of culture. These results agreed with findings from *in vivo* studies that detected HIV RNA transcription in resting cells from the lymphoid tissue of infected patients (52). The detection of viral RNA transcription in latently infected cells raises the possibility that HIV latency may be maintained by an active mechanism, in which viral products interact with cell processes to influence the states of viral latency and reactivation.

These results from my research project have several implications for the development of novel treatment strategies targeting the elimination of the latently infected T cell reservoir. A few studies have tested the idea of non-specifically activating resting T cells in the hope of purging latently infected cells in the presence of suppressive HAART (55). However, non-specific T cell activation can have several detrimental effects, such as increased rates of apoptosis and long-lasting CD4 T cell depletion (56, 57). More selective treatment approaches, such as targeting the memory T cell subset (58) could be more promising, but will still lead to the indiscriminate killing of uninfected memory T cells. Therefore, this research area must address the need for identification of potential biological markers that are

expressed exclusively in HIV-infected non-dividing, minimally activated CD4 cells to provide more stringent targets for therapy development.

FUTURE DIRECTIONS

One of the most important findings from my thesis research has been the identification and characterization of the subset of memory CD4 T cells in which latent HIV-1 infection is most likely to become established and maintained. The identification of specific biologic markers that are associated with infection of this subpopulation of non-dividing, minimally activated T cells is a next logical step in the process of being able to selectively target latent infection. It has been shown previously that different subsets of CD4 T cells possess different susceptibilities to HIV infection (59, 60). Recently, a secreted cellular factor with pleiotropic functions in innate immunity, ps20, has been identified as a signature protein that renders certain subsets of memory CD4 T cells permissive to HIV-1 infection (61). The use of our *in vitro* cell model to identify factors similar to ps20 in non-dividing CD4 cells that are prone to latent HIV infection could lead to the development of antiviral or cytopathic reagents to preferentially target latently infected T cells.

The detection of HIV RNA transcripts present in latently infected CD4 cells from our infection model also warrants further investigation into the mechanisms by which HIV-1 latency is maintained. Several cellular and viral factors may be

involved in the maintenance of the integrated provirus in a non-productive state and its host cell in a resting non-activated state. The next step in this research area would be the examination of latently infected CD4 T cells for the presence of viral proteins translated from the corresponding mRNA transcripts. Even if protein products are not detected, viral RNA itself may interact and interfere with cellular processes, as is the case in other viral infections (62, 63).

The *in vitro* primary T cell model of HIV-1 infection that is described and applied in the research studies presented here provides an invaluable tool for the study of HIV latency. This cell model has been optimized to yield sufficient numbers of latently infected T cells that can be utilized in further work to characterize the molecular mechanisms critical to the establishment and maintenance of HIV latency. In addition, this cell model can be used to test the efficacy of novel treatment strategies for the purging and elimination of the latently infected T cell reservoir, providing a practical yet crucial contribution to therapy design if the goal of HIV eradication in infected patients is to be achieved.

REFERENCES

1. Gayle H. An overview of the global HIV/AIDS epidemic, with a focus on the United States. *AIDS*. 2000 Sep;14 Suppl 2:S8-17.
2. Dybul M, Fauci AS, Bartlett JG, Kaplan JE, Pau AK, Panel on Clinical Practices for Treatment of HIV. Guidelines for using antiretroviral agents among HIV-infected adults and adolescents. *Ann Intern Med*. 2002 Sep 3;137(5 Pt 2):381-433.
3. Sierra S, Kupfer B, Kaiser R. Basics of the virology of HIV-1 and its replication. *J Clin Virol*. 2005 Dec;34(4):233-44.
4. Frankel AD, Young JA. HIV-1: Fifteen proteins and an RNA. *Annu Rev Biochem*. 1998;67:1-25.
5. Chan DC, Kim PS. HIV entry and its inhibition. *Cell*. 1998 May 29;93(5):681-4.
6. Stevenson M. HIV-1 pathogenesis. *Nat Med*. 2003 Jul;9(7):853-60.
7. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature*. 1995 Jan 12;373(6510):123-6.
8. Zack JA, Arrigo SJ, Weitsman SR, Go AS, Haislip A, Chen IS. HIV-1 entry into quiescent primary lymphocytes: Molecular analysis reveals a labile, latent viral structure. *Cell*. 1990 Apr 20;61(2):213-22.
9. Perelson AS. Modeling viral and immune system dynamics. *Nat Rev Immunol*. 2002 Jan;2(1):28-36.
10. LaBonte JA, Patel T, Hofmann W, Sodroski J. Importance of membrane fusion mediated by human immunodeficiency virus envelope glycoproteins for lysis of primary CD4-positive T cells. *J Virol*. 2000 Nov;74(22):10690-8.
11. Stewart SA, Poon B, Jowett JB, Chen IS. Human immunodeficiency virus type 1 vpr induces apoptosis following cell cycle arrest. *J Virol*. 1997 Jul;71(7):5579-92.
12. Meyaard L, Otto SA, Jonker RR, Mijster MJ, Keet RP, Miedema F. Programmed death of T cells in HIV-1 infection. *Science*. 1992 Jul 10;257(5067):217-9.

13. Geleziunas R, Xu W, Takeda K, Ichijo H, Greene WC. HIV-1 nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. *Nature*. 2001 Apr 12;410(6830):834-8.
14. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature*. 1998 Dec 17;396(6712):690-5.
15. Silvestri G, Sodora DL, Koup RA, Paiardini M, O'Neil SP, McClure HM, et al. Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. *Immunity*. 2003 Mar;18(3):441-52.
16. Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, et al. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature*. 1997 May 8;387(6629):183-8.
17. Chun TW, Finzi D, Margolick J, Chadwick K, Schwartz D, Siliciano RF. *In vivo* fate of HIV-1-infected T cells: Quantitative analysis of the transition to stable latency. *Nat Med*. 1995 Dec;1(12):1284-90.
18. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: Function, generation, and maintenance. *Annu Rev Immunol*. 2004;22:745-63.
19. Marrack P, Mitchell T, Bender J, Hildeman D, Kedl R, Teague K, et al. T-cell survival. *Immunol Rev*. 1998 Oct;165:279-85.
20. Mackay CR. Migration pathways and immunologic memory among T lymphocytes. *Semin Immunol*. 1992 Feb;4(1):51-8.
21. Akbar AN, Terry L, Timms A, Beverley PC, Janossy G. Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. *J Immunol*. 1988 Apr 1;140(7):2171-8.
22. Birkeland ML, Johnson P, Trowbridge IS, Pure E. Changes in CD45 isoform expression accompany antigen-induced murine T-cell activation. *Proc Natl Acad Sci U S A*. 1989 Sep;86(17):6734-8.
23. Kawashima H. Roles of sulfated glycans in lymphocyte homing. *Biol Pharm Bull*. 2006 Dec;29(12):2343-9.

24. Bukrinsky MI, Sharova N, Dempsey MP, Stanwick TL, Bukrinskaya AG, Haggerty S, et al. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc Natl Acad Sci U S A*. 1992 Jul 15;89(14):6580-4.
25. Spina CA, Guatelli JC, Richman DD. Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes *in vitro*. *J Virol*. 1995 May;69(5):2977-88.
26. Bukrinsky MI, Stanwick TL, Dempsey MP, Stevenson M. Quiescent T lymphocytes as an inducible virus reservoir in HIV-1 infection. *Science*. 1991 Oct 18;254(5030):423-7.
27. Shen A, Siliciano JD, Pierson TC, Buck CB, Siliciano RF. Establishment of latent HIV-1 infection of resting CD4(+) T lymphocytes does not require inactivation of vpr. *Virology*. 2000 Dec 5;278(1):227-33.
28. Unutmaz D, KewalRamani VN, Marmon S, Littman DR. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. *J Exp Med*. 1999 Jun 7;189(11):1735-46.
29. Ho DD, Moudgil T, Alam M. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. *N Engl J Med*. 1989 Dec 14;321(24):1621-5.
30. Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Natl Acad Sci U S A*. 1998 Jul 21;95(15):8869-73.
31. Furtado MR, Callaway DS, Phair JP, Kunstman KJ, Stanton JL, Macken CA, et al. Persistence of HIV-1 transcription in peripheral-blood mononuclear cells in patients receiving potent antiretroviral therapy. *N Engl J Med*. 1999 May 27;340(21):1614-22.
32. Hermankova M, Siliciano JD, Zhou Y, Monie D, Chadwick K, Margolick JB, et al. Analysis of human immunodeficiency virus type 1 gene expression in latently infected resting CD4+ T lymphocytes *in vivo*. *J Virol*. 2003 Jul;77(13):7383-92.
33. Lassen KG, Bailey JR, Siliciano RF. Analysis of human immunodeficiency virus type 1 transcriptional elongation in resting CD4+ T cells *in vivo*. *J Virol*. 2004 Sep;78(17):9105-14.
34. Lassen KG, Ramyar KX, Bailey JR, Zhou Y, Siliciano RF. Nuclear retention of multiply spliced HIV-1 RNA in resting CD4+ T cells. *PLoS Pathog*. 2006 Jul;2(7):e68.

35. Han Y, Lassen K, Monie D, Sedaghat AR, Shimoji S, Liu X, et al. Resting CD4+ T cells from human immunodeficiency virus type 1 (HIV-1)-infected individuals carry integrated HIV-1 genomes within actively transcribed host genes. *J Virol.* 2004 Jun;78(12):6122-33.
36. Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, et al. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science.* 1997 Nov 14;278(5341):1295-300.
37. Wong JK, Hezareh M, Gunthard HF, Havlir DV, Ignacio CC, Spina CA, et al. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science.* 1997 Nov 14;278(5341):1291-5.
38. Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, et al. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med.* 1999 May;5(5):512-7.
39. Strain MC, Gunthard HF, Havlir DV, Ignacio CC, Smith DM, Leigh-Brown AJ, et al. Heterogeneous clearance rates of long-lived lymphocytes infected with HIV: Intrinsic stability predicts lifelong persistence. *Proc Natl Acad Sci U S A.* 2003 Apr 15;100(8):4819-24.
40. Pomerantz RJ, Trono D, Feinberg MB, Baltimore D. Cells nonproductively infected with HIV-1 exhibit an aberrant pattern of viral RNA expression: A molecular model for latency. *Cell.* 1990 Jun 29;61(7):1271-6.
41. Fischer M, Joos B, Wong JK, Ott P, Opravil M, Hirschel B, et al. Attenuated and nonproductive viral transcription in the lymphatic tissue of HIV-1-infected patients receiving potent antiretroviral therapy. *J Infect Dis.* 2004 Jan 15;189(2):273-85.
42. Jordan A, Bisgrove D, Verdin E. HIV reproducibly establishes a latent infection after acute infection of T cells *in vitro*. *EMBO J.* 2003 Apr 15;22(8):1868-77.
43. Sahu GK, Lee K, Ji J, Braciale V, Baron S, Cloyd MW. A novel *in vitro* system to generate and study latently HIV-infected long-lived normal CD4+ T-lymphocytes. *Virology.* 2006 Nov 25;355(2):127-37.
44. Burke B, Brown HJ, Marsden MD, Bristol G, Vatakis DN, Zack JA. Primary cell model for activation-inducible human immunodeficiency virus. *J Virol.* 2007 Jul;81(14):7424-34.
45. Shen A, Zink MC, Mankowski JL, Chadwick K, Margolick JB, Carruth LM, et al. Resting CD4+ T lymphocytes but not thymocytes provide a latent viral reservoir in a simian immunodeficiency virus-macaca nemestrina model of human

immunodeficiency virus type 1-infected patients on highly active antiretroviral therapy. *J Virol*. 2003 Apr;77(8):4938-49.

46. Saleh S, Solomon A, Wightman F, Xhilaga M, Cameron PU, Lewin SR. CCR7 ligands CCL19 and CCL21 increase permissiveness of resting memory CD4⁺ T-cells to HIV-1 infection: A novel model of HIV-1 latency. *Blood*. 2007 Sep 19.

47. Han Y, Wind-Rotolo M, Yang HC, Siliciano JD, Siliciano RF. Experimental approaches to the study of HIV-1 latency. *Nat Rev Microbiol*. 2007 Feb;5(2):95-106.

48. Vatakis DN, Bristol G, Wilkinson TA, Chow SA, Zack JA. Immediate activation fails to rescue efficient human immunodeficiency virus replication in quiescent CD4⁺ T cells. *J Virol*. 2007 Apr;81(7):3574-82.

49. Stevenson M, Stanwick TL, Dempsey MP, Lamonica CA. HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO J*. 1990 May;9(5):1551-60.

50. Swiggard WJ, Baytop C, Yu JJ, Dai J, Li C, Schretzenmair R, et al. Human immunodeficiency virus type 1 can establish latent infection in resting CD4⁺ T cells in the absence of activating stimuli. *J Virol*. 2005 Nov;79(22):14179-88.

51. Choi J, Walker J, Talbert-Slagle K, Wright P, Pober JS, Alexander L. Endothelial cells promote human immunodeficiency virus replication in nondividing memory T cells via nef-, vpr-, and T-cell receptor-dependent activation of NFAT. *J Virol*. 2005 Sep;79(17):11194-204.

52. Zhang Z, Schuler T, Zupancic M, Wietgreffe S, Staskus KA, Reimann KA, et al. Sexual transmission and propagation of SIV and HIV in resting and activated CD4⁺ T cells. *Science*. 1999 Nov 12;286(5443):1353-7.

53. Wu Y, Marsh JW. Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. *Science*. 2001 Aug 24;293(5534):1503-6.

54. Stanley SK, Folks TM, Fauci AS. Induction of expression of human immunodeficiency virus in a chronically infected promonocytic cell line by ultraviolet irradiation. *AIDS Res Hum Retroviruses*. 1989 Aug;5(4):375-84.

55. Chun TW, Engel D, Mizell SB, Hallahan CW, Fischette M, Park S, et al. Effect of interleukin-2 on the pool of latently infected, resting CD4⁺ T cells in HIV-1-infected patients receiving highly active anti-retroviral therapy. *Nat Med*. 1999 Jun;5(6):651-5.

56. van Praag RM, Prins JM, Roos MT, Schellekens PT, Ten Berge IJ, Yong SL, et al. OKT3 and IL-2 treatment for purging of the latent HIV-1 reservoir *in vivo* results

in selective long-lasting CD4⁺ T cell depletion. *J Clin Immunol*. 2001 May;21(3):218-26.

57. Sereti I, Herpin B, Metcalf JA, Stevens R, Baseler MW, Hallahan CW, et al. CD4 T cell expansions are associated with increased apoptosis rates of T lymphocytes during IL-2 cycles in HIV infected patients. *AIDS*. 2001 Sep 28;15(14):1765-75.

58. Saavedra-Lozano J, Cao Y, Callison J, Sarode R, Sodora D, Edgar J, et al. An anti-CD45RO immunotoxin kills HIV-latently infected cells from individuals on HAART with little effect on CD8 memory. *Proc Natl Acad Sci U S A*. 2004 Feb 24;101(8):2494-9.

59. Ciuffi A, Bleiber G, Munoz M, Martinez R, Loeuillet C, Rehr M, et al. Entry and transcription as key determinants of differences in CD4 T-cell permissiveness to human immunodeficiency virus type 1 infection. *J Virol*. 2004 Oct;78(19):10747-54.

60. Vyakarnam A, Matear PM, Martin SJ, Wagstaff M. Th1 cells specific for HIV-1 gag p24 are less efficient than Th0 cells in supporting HIV replication, and inhibit virus replication in Th0 cells. *Immunology*. 1995 Sep;86(1):85-96.

61. Alvarez R, Reading J, King DF, Hayes M, Easterbrook P, Farzaneh F, et al. WFDC1/ps20 is a novel innate immunomodulatory signature protein of human immunodeficiency virus (HIV)-permissive CD4⁺ CD45RO⁺ memory T cells that promotes infection by upregulating CD54 integrin expression and is elevated in HIV type 1 infection. *J Virol*. 2008 Jan;82(1):471-86.

62. Goodrum F, Reeves M, Sinclair J, High K, Shenk T. Human cytomegalovirus sequences expressed in latently infected individuals promote a latent infection *in vitro*. *Blood*. 2007 Aug 1;110(3):937-45.

63. Samols MA, Skalsky RL, Maldonado AM, Riva A, Lopez MC, Baker HV, et al. Identification of cellular genes targeted by KSHV-encoded microRNAs. *PLoS Pathog*. 2007 May 11;3(5):e65.

64. Spina CA, Kwoh TJ, Chowders MY, Guatelli JC, Richman DD. The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. *J Exp Med*. 1994 Jan 1;179(1):115-23.

65. Koelsch KK, Liu L, Haubrich R, May S, Havlir D, Gunthard HF, et al. Dynamics of total, linear nonintegrated, and integrated HIV-1 DNA *in vivo* and *in vitro*. *J Infect Dis*. 2008 Feb 1;197(3):411-9.

70. Myers LE, McQuay LJ, Hollinger FB. Dilution assay statistics. *J Clin Microbiol.* 1994 Mar;32(3):732-9.
71. Chowers MY, Pandori MW, Spina CA, Richman DD, Guatelli JC. The growth advantage conferred by HIV-1 nef is determined at the level of viral DNA formation and is independent of CD4 downregulation. *Virology.* 1995 Oct 1;212(2):451-7.
72. Briggs JA, Simon MN, Gross I, Krausslich HG, Fuller SD, Vogt VM, et al. The stoichiometry of gag protein in HIV-1. *Nat Struct Mol Biol.* 2004 Jul;11(7):672-5.
73. Calvelli T, Denny TN, Paxton H, Gelman R, Kagan J. Guideline for flow cytometric immunophenotyping: A report from the national institute of allergy and infectious diseases, division of AIDS. *Cytometry.* 1993 Oct;14(7):702-15.
74. Nicholson J, Kidd P, Mandy F, Livnat D, Kagan J. Three-color supplement to the NIAID DAIDS guideline for flow cytometric immunophenotyping. *Cytometry.* 1996 Sep 15;26(3):227-30.
75. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, et al. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol.* 1986 Aug;59(2):284-91.
76. Fischer M, Wong JK, Russenberger D, Joos B, Opravil M, Hirschel B, et al. Residual cell-associated unspliced HIV-1 RNA in peripheral blood of patients on potent antiretroviral therapy represents intracellular transcripts. *Antivir Ther.* 2002 Jun;7(2):91-103.
77. Stebbing J, Gazzard B, Douek DC. Where does HIV live? *N Engl J Med.* 2004 Apr 29;350(18):1872-80.
78. Spina CA, Prince HE, Richman DD. Preferential replication of HIV-1 in the CD45RO memory cell subset of primary CD4 lymphocytes in vitro. *J Clin Invest.* 1997 Apr 1;99(7):1774-85.