ACTIVATED MACROPHAGES: IMPLICATIONS IN HIV-ASSOCIATED DISEASE PATHOGENESIS

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By

DEIRDRE ANNE KILLEBREW

Dissertation Committee:

Bruce Shiramizu, Chairperson

Martin Rayner

Alan Lau

Katlin Csiszar

Neil Frazer

ABSTRACT

Macrophages adapt their functional characteristics, as the needs of the local environment changes with compounding signals. Both monocytes and macrophages (M/MΦ) can be activated by an inflammatory insult, and have an impressive arsenal of over a hundred different secreted substances that regulate surrounding cells, or act as direct effectors. Cellular activation is a vital factor for HIV-1 infection, because CCR5 and CXCR4 expression make the conditions optimal for HIV-1 entry and replication.

HIV-1 integration occurs as a necessary step in the life cycle allowing for replication and transcription of the viral genome to take place. While HIV-1 is thought to integrate randomly, more recent studies have suggested that integration occasionally occurs in a less random fashion. HIV-1 infection and/or integration takes place in M/MΦ, which may be a common pathogenic link to HIV-1 associated diseases such as malignancies and neurological disorders.

HIV-1infected individuals are at risk for developing cancers. A common element among lymphomas is the inability to control a pathogen that acts as an ongoing immune activator. Thus, an increased risk of HIV-1 associated malignancies may result from from immunodeficiency, increased immune activation and possibly HIV-1 insertional mutagenesis. While there are data to show that non-random HIV-1 integration may occur, our goal was to identify preferential genomic sites where HIV-1 integration might be targeted leading to oncogenesis.

M/MΦ serve as HIV-1 reservoirs and may indirectly lead to HIV-associated dementia (HAD) via neurotoxic cytokine/chemokine production. It remains unknown if

peripheral M/MΦ are responsible for both circulating and cerebral spinal fluid (CSF) cytokines/chemokines. Our objective was to determine the relationship between cytokines in the periphery and the CNS among HAD patients. Different cytokines from plasma, CSF, and cultured peripheral M/MΦ supernatants were identified in subjects with HAD versus those with normal cognition, suggesting unique pathways leading to cytokine/chemokine release in the periphery versus the brain region. This may have implications in delineating a cause and effect in HAD pathogenesis. The focus of this dissertation is the role that activated macrophages play in HIV-1 pathogenesis.

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AIDS	Acquired Immunodeficiency Syndrome
BBB	Blood-Brain-Barier
C/C	Cytokine/Chemokine
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
EBV	Epstein-Barr Virus
ELISA	Enzyme-Linked Immunosorbant Assay
FIV	Feline Immunodeficiency Virus
KS	Kaposi's Sarcoma
KSHV	Kaposi's Sarcoma Herpesvirus
HAART	Highly Active Antiretroviral Therapy
HAD	HIV-1-Associated Dementia,
HAM	HIV-1-associated Malignancies
HHV8	Human Herpesvirus, type 8
HIV-1	Human Immunodeficiency Virus, type 1
H-NHL	HIV-1-associated Non-Hodgkin Lymphoma
HTLV-1	Human T-Lymphotrophic Virus, type 1
IPCR	Inverse-PCR
ITGβ4	beta (4) Integrin gene
LP-PCR	Linker-Primer PCR assay
MCP-1	Monocyte Chemoattractant Protein-1

M-CSF	Macrophage Colony Stimulating Factor
MDMs	Monocyte-Derived Macrophages
M/MΦ	Monocytes and Macrophages,
NC	Normal Cognition
NHL	Non-Hodgkin Lymphomas
PBMC	Peripheral Blood Mononuclear Cells
PCNSL	Primary Central Nervous System Lymphoma
PCR	Polymerase Chain Reaction
PEL	Primary Effusion Lymphoma
SINES	Short Interspersed Elements
SIV	Simian Immunodeficiency Virus
TLR1	Toll-like Receptor 1
VEC	Vascular Endothelial Cells

CHAPTER 1

INTRODUCTION

Macrophages are considered an extremely heterogenic group of cells, whose phenotype and capabilities depend on the anatomical site to which they migrate [1]. Development takes place in the bone marrow, where they progress: first as stem cells, then to committed stem cells, to monoblasts, to promonocytes, to bone marrow monocytes, and then eventually to peripheral blood monocytes. Blood monocytes circulate until directed into tissue, where specific tissue microenvironment signals "instruct" the monocyte to become a macrophage with specialized functions [1]. It is not fully understood how unactivated monocytes home-in on their residential tissues #1[2]. Tissue macrophages that are in a normal steady-state form are often referred to as "resident" macrophages [1]. Because different tissues support entirely different environments (pH, anaerobic, aerobic, sterile, etc.), it is not surprising that macrophages develop different phenotypes, however, there are some circulating monocytes that differentiate into multifunctional macrophages [1]. Thus, depending upon the microenvironment in which they reside, resident tissue macrophages will contribute different types of cytokines and cellular factors to an inflammatory innate immune response [2].

The anatomical site where monocyte or macrophage activation takes place is important in determining the functional role that the monocyte/macrophage portrays in the future. The tissue microenvironment significantly influences the development and function of the differentiated macrophage (Reviewed in[2]). Regardless of the state of

differentiation, both monocytes and macrophages can be activated by an inflammatory insult [2]. When macrophages become activated, they acquire increased functional abilities, including greater phagocytosis, upregulated cytokine and chemokine secretion, and higher MHC II expression [1]. Activation is also defined by a macrophage's decreased ability to proliferate, oxygen consumption, and release of proinflammatory mediators [1].

There are multiple pathways to macrophage activation, including the coined "classical" (IFN-g), "alternative" (IL-4 or IL-13), innate (Toll-receptor), and humoral (antibody and complement) pathways [3]. Macrophages are classically activated by IFN-g, through a cytokine cascade involving IL-12 and IL-18, produced by antigen-presenting cells [3]. An alternative and distinct activation pathway of macrophages exists through IL-4 and IL-13, produced generally in response to cellular and humoral, allergic and parasitic responses by TH2-type cells [3]. IL4 and IL-13 activate macrophages by increasing the expression of MHC class II and mannose receptors that encourage antigen presentation and endocytosis. IL-4 and IL-13 also upregulate the expression of selective cytokines, such as macrophage-derived chemokine (MDC) and thymus and activation regulated chemokine (TARC), that increase granuloma formation and the recruitment of more cells to the localized area [3]. IL4 and IL-13 increase monocyte responsiveness to IL-8 (64) [3]. IL-10 is the classic deactivator of macrophage cytokine production, especially TNF [3]. However, the pro- or anti-inflammatory actions of IL-10 are said to depend on the timing of the receptor-activating stimulus (127) [3]. The functional protein expression of macrophages changes over time. Both early expressed cytokines, up-regulated within the first 6 hours, and late cytokine expression, upregulated between 6 to

24 hours, following LPS stimulation, have been characterized in macrophages [4]. Macrophages adapt their functional characteristics, as the needs of the local environment changes with compounding signals.

The order in which a monocyte or macrophage is exposed to cytokines and other activating stimuli, makes a difference in which response will be given. D'Andrea et al exposed macrophages first to IL-4 then LPS, and found an upregulation of TNF-a and IL-12 [5]. However, when macrophages were exposed first to LPS and then IL-4, there was no enhanced inflammatory response [5]. Macrophages secreting proinflammatory cytokines will affect surrounding macrophages in a paracrine fashion, according to the type of cytokines/chemokine those macrophages were exposed to first.

Macrophages function during all stages of an immune threat. They have rapid phagocytic response tactics toward foreign invaders and metastasizing tumors [1]. This includes a low pH intracellular environment inside the phagolysosome that is toxic to most invaders. They also serve as one of the signals needed to activate T cells, by presenting processed antigen. Activated macrophages have an impressive arsenal of over a hundred different secreted substances that regulate surrounding cells or act as direct effectors [1].

Macrophages have the distinction of being a specific target for all lentiviruses, including HIV [6]. HIV's route of infection must include exhange of body fluid, such as through a mucosal surface, perinatally, or exposure to infected needles. Evolutionarily, it is logical that macrophages are an optimal target of HIV, because in mucosal transmission, cells of the macrophage lineage are usually the first to be infected [6]. HIV has been found in a very large array of cell types in the human body (ranging from a

variety of lymphocytes to tissue epithelial and cells of the mucosal lining), however the most consistently found infected cells are macrophage-lineage types and CD4+ T lymphocytes [6]. The primary receptor for most HIV isolates is CD4, and the secondary (entry) receptor, usually CCR5 (macrophage tropic) and CXCR4 (T cell tropic), determines the tropism of that particular HIV virion [6]. Cell tropism and efficiency of replication are determined by the variable region (V3) loop of the protein gp120 (reviewed in [6]). Therefore, point mutations in the V3 loop can vary cell tropism from one isolate to another, within a single host. The infection that takes place in CD4+ T cells and macrophages is very different; HIV infection in CD4+ T cells almost certainly leads to cell death, whereas infection in macrophages is not normally cytotoxic, and can continue for the length of the cell's life [6].

Cellular activation is a vital factor for HIV infection. Activated CD4+ T cells express both CCR5 and CXCR4, making conditions optimal for HIV entry and replication, likewise, there is very little replication in resting PBMC cultures [6]. Both resting and activated macrophages are efficiently infected with HIV, however, activation increases the amount of free nucleotides available for replication, and upregulates transcription activation factors, like NF-κB [6].

Retroviral integration of its DNA-copy into the host genome, is necessary for replication and expression of progeny virions [7]. Virions consist of two highly condensed single-stranded RNAs, packaged in a capsid and envelope with two replication enzymes, reverse transcriptase (RT) and integrase (IN)[8]. Immediately after infection, the genome is reverse transcribed into a double-stranded DNA copy. The product of reverse transcription is a blunt-ended, double-stranded, linear DNA. The left and right

ends of the viral DNA copy are segments of identical sequences, called long terminal repeats (LTRs), and named U3 and U5, respectively. At the very ends of both U3 and U5, there are small direct repeats that are polymerized to the host sequence upon integration. The viral copy DNA has its own short direct repeat terminal sequence, named R, located between the U3 and U5 LTR sequences on both ends. The dinucleotide sequence CA is a highly conserved characteristic of the inverted repeats; the "A" is universally joined to the host DNA [7-9]. In the HIV-1 LTR, the U3 region is 456 base pairs (bp) long, the R region is 97 bp, and the U5 is 82 bp in length[10]. The LTRs' functions seem multifaceted as important targets for cellular and viral protein regulation. Some characteristics of interest are: binding sites for cytokines and chemokines, sequences for a negative response element, a promoter, a repeat enhancer sequence, and the *tat1* response element, all localized to the U3 and R regions [10]. The first 15bp of the LTRs are called attachment (att) site sequences [9]. Studies on avian retroviruses, found that modifications to the att site less than 12bp from the end, especially to a "C" nucleotide 7bp in, have major effects on the promotion of full-site integration[11]. In HIV-1, bases 2-9 have been found to be critical for the IN protein's normal duties [12].

There are circularized proviral copies, with novel circle junction sequences created at the site of ligation, that do not get incorporated into the host genome[8]. However, studies show that the linear copy DNA undergoes integration. After viral DNA synthesis, sometimes while still in the cytoplasm, the 3' end undergoes a precise cleavage, where one or two nucleotides are removed from the 3'end, exposing the 3' OH group [9]. HIV only cleaves one nucleotide on each 3' end[9]. Recently, a study has shown that a DNA "flap," created during cDNA synthesis, mediates the active nuclear

import of HIV-1 DNA [13]. After the DNA copy is imported into the cell nucleus, the integration process proceeds. The recessed 3' ends of the viral DNA are then joined to the host DNA in a combined cleavage-ligation reaction[9, 14]. The IN protein mediates the 3'end processing and the strand transfer steps [14]. The 5' end joining process involves removing the overhanging 5' end viral nucleotides and ligating the resulting gaps [14]. Evidence indicates that IN directs 5' end joining as well. IN is able to reseal a nick in DNA, provided that there is at least one extra nucleotide at the 5' side of the nick [12, 15]. The integrated provirus becomes a permanent part of the cell's genome, and is transmitted vertically into every daughter cell [8].

Since the mid-1990's, highly active antiretroviral therapy (HAART) has been relatively successful in controlling the transmission and replication of HIV in patients fortunate enough to be receiving therapy. Characterization of HIV-1 infection includes immunodeficiency and overall immune activation. As patients live longer with HIV-1 infection as a result of effective HAART, an increase in HIV-1-associated diseases, such as malignancy and dementia, has been observed [16-18]. One common element among HIV-1-associated-malignancies and dementia, in this setting of immune dysfunction, is the inability to control a pathogen that acts as an ongoing immune activator.

In the following dissertation, I will describe in length, proposed mechanisms involving HIV-1 infection of monocytes/macrophages, the role of activated macrophages, and the relationship to cytokine/chemokine regulation. In this respect, the dissertation will focus on translating the concepts clinically for HIV-1 associated malignancies and HIV-1 associated dementia. Specifically, describing preferential HIV-1 integration in macrophages and HIV-associated malignancies, and how this relates to the pathogenesis

of HIV-associated non-Hodgkin lymphoma. I will also illustrate how the similar pathways can be related to HIV-associated dementia.

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CHAPTER 2

PREFERENTIAL HIV-1 INTEGRATION SITES IN MACROPHAGES AND HIV-ASSOCIATED MALIGNANCIES

D. A. Killebrew[1,2,3]✍, D. Troelstrup[1,2], and B. Shiramizu[1,2,4]

Retrovirology Research Laboratory[1], Hawaii AIDS Clinical Research Program[2]; Departments of Biomedical Sciences[3] and Pediatrics/Medicine[4], John A. Burns School of Medicine, University of Hawaii, Honolulu, HI

Address of all authors: 3675 Kilauea Ave., Young Bldg., 5th Floor, Honolulu, HI 96816; telephone: 808-737-2751; fax: 808-735-7047

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ABSTRACT

HIV-infected individuals are at risk for developing certain types of cancers. While there are data to show that non-random HIV integration may occur, our goal was to identify preferential genomic sites where HIV integration might be targeted leading to oncogenesis. Initially, a linker-primer PCR strategy was used to identify HIV-integration in isolated macrophages. Inverse-PCR was then used to analyze specimens from patients diagnosed with HIV-associated malignancies. From isolated macrophages, integration near a toll-like receptor on chromosome 4 was found. Necropsy tissues from 11 cases were analyzed with 1 tumor specimen found to have HIV integrated in chromosome 22q13.2 and within 300kb of HSCBCIP1 (CAP-binding protein complex interacting homologue). Tumor-specific primers were then used to screen uninvolved tissue from the same patient, which did not amplify the site-specific region. This report demonstrates that in both an *in vitro* system and human malignant tissue, specific viral integration can be identified.

INTRODUCTION

Since the beginning of the acquired immunodeficiency syndrome (AIDS) epidemic, the epidemiology of human immunodeficiency virus type 1 (HIV-1)-associated malignancies (HAM) has transiently changed as improvements in treatment have evolved [1-4]. While there has been a decrease in the incidence of HAM with the widespread use of antiretroviral (ARV) therapy in developed countries, the incidence is still relatively higher than in non-HIV-infected individuals[2, 3]. There is a spectrum of HAM that has been identified and therefore identifying a common etiology amongst the tumor types has been a challenge. It is well known that retroviral integration into the host genome is necessary for replication and expression of progeny virions [5]. While HIV-1 possesses this characteristic, lentiviral integration has primarily been random, and has not been considered to be a cause of human cancer.

Although many retroviridae are proven oncoviruses in animals, few are thought to cause cancer in humans. The common view of AIDS-associated malignancy is the result of an opportunistic proliferation due to an oncogenic stimulus and a depressed immune system. The human T-cell lymphotropic virus encodes a transactivating protein, tax 1, involved in upregulating the expression of growth factors, and has been linked to adult T-cell leukemia/lymphoma [6, 7]. After integration, the avian leucosis virus cis-activates the oncogene c-myc, and causes B-cell lymphoma in chickens [7, 8]. Murine leukemia virus also cis-activates c-myc, inducing transformation in mice. A direct causal role for a feline immunodeficiency virus (FIV) was suggested for a cat B-cell lymphoma [9]. A single FIV provirus integration site was found to occur the clonal B-cell population. The lentiviral integration was believed to occur prior to the B-cell transformation. Simian

immunodeficiency virus (SIV) infection creates a severe immunosuppression in macaques, much like that of HIV in humans [10]. In experimentally infected macaques, the SIV proviral sequence was detected in three separate tumors, one T-cell population and two oligoclonal. As part of an AIDS lymphogenesis mouse model experiment, SCID mice were injected with HIV-infected human body cavity based lymphoma macrophage cells [11]. Overall, 54.5% of the mice developed T-cell lymphomas in the spleen, liver, and bone marrow. Human macrophages were detected in the mice, up to six months post-infection. Unlike tumor transplantation experiments, these AIDS-related lymphomas arose through tumor induction. These studies are examples that lentiviral transformation is possible in animal cancers.

Experimental studies utilizing isolated HIV gene products have implied transforming activity of some viral products. Transgenic mice expressing HIV-1 *tat* gene products developed Kaposi's sarcoma (KS) skin lesions[12]; and were able to protect lymphoid, epithelial, and neuronal cells from apoptosis induced by serum starvation[13]. The HIV-1 tat protein is necessary for viral transcription, and is able to trans-activate cellular genes[13]. Extracellular HIV-1 tat has also been shown to stimulate human Kaposi's sarcoma cells. In a separate study, Mack, et al designed a construct linking the HIV 3'LTR to the genomic region directly upstream from the *c-fes/fps* transcription start site and demonstrated upregulation of the *c-fes* protein ten-fold above background, when expressed in a human cell line[14]. In subsequent experiments placing the LTR 2.6kb upstream of the *c-fes/fps* transcription start site; a three-fold upregulated expression was still detected, providing evidence for *c-fes* being cis-activated by the HIV-1 provirus. When the *tat* gene was added to the original construct, the upregulation was 87 times that

of background expression levels. These studies of upregulation by HIV gene products provide evidence for transformation capabilities.

Evidence is accumulating in support of HIV being a more direct effecter in oncogenesis Malignancy is a complicated process that usually involves an accumulation of abnormalities progressing to transformation and proliferation. The pathogenesis of Kaposi's sarcoma is controversial, with debates arising over the mode of transmission and the viral agent involved [15, 16]. Several herpes viruses have been implicated as a possible cause of KS, such as: Epstein-Barr virus, cytomegalovirus, and human herpes virus 8, also known as Kaposi's Sarcoma herpes virus[1, 17-19]. McGrath et al. reports finding HIV-1 integrated in macrophages surrounding an early KS lesion, but not in later, more developed lesions [20]. The integrated provirus and the expressed HIV-1 gene products may stimulate the surrounding macrophages to produce activating cytokines, and thus, lead to proliferation [20]. The concept of "sequential neoplasia"[20] may explain the absence of HIV-1 in B-cells and most HIV-associated tumors. HIV-integration in macrophages and T-cells could lead to a complicated interaction with B-cells via cell-cell signaling. The sequential model predicts that early forms of HIV-integrated, polyclonal macrophages, will initiate an environment, including cytokines and growth factors, that will aid in the transformation of the monoclonal B-cells. Any preexisting B-cell mutation or coinfection, would most likely further aid in the process of transformation. The sequential neoplastic model, helps to explain why an initial viral infection may lead to many different examples of AIDS-associated lymphoma.

The significance of identifying integration near growth-regulating genes, and revealing how gene expression is influenced by the HIV LTR, lies in discovering

potential site-specific treatment strategies. The goal of the study was to identify preferential HIV integration sites using a previously described linker-primer PCR assay (LP-PCR) protocol which was specifically developed to detect and quantify integrated HIV DNA species[21]. Then an analysis of HIV-associated tumors was carried out to determine if preferential sites of integration could be found in clinical specimens.

MATERIALS AND METHODS

Identification of HIV Integration Using Isolated Macrophages

Monocyte/Macrophage Isolation and HIV-1 Infection.

Whole blood was obtained in potassium ethylenediaminetetraacetic acid tubes from HIV-seronegative volunteers. PBMCs were obtained using a standard Ficoll Hypaque separation protocol (Amersham Biosciences Corp., Piscataway, NJ, USA). Using a Monocyte Isolation Kit, monocytes were isolated by magnetic beads according to manufacturer's instructions (Miltenyi Biotec Inc, Auburn, CA, USA) and counted on a hemocytometer. Monocytes were then plated at a density of $4x10^5$ per cm^2 and cultured for 14 days to allow for differentiation into monocyte derived macrophages (MDMs). MDM media consisted of: a majority of Iscove's modified Dulvecco's medium (Irvine Scientific, Santa Ana, CA, USA), and 25% RPMI media (Sigma Inc., St. Louis, MO, USA), 4mM L-glutamine (Sigma Inc.), 100units/mL penicillin and 100μg/mL streptomycin (Sigma Inc.), 15ng/mL M-CSF (Sigma Inc.), and 10% Human Serum (Sigma Inc.). The cells were grown in a 37°C incubator, with 5% CO_2. On day 14, macrophages were infected with harvested HIV-1 viral supernatant (after .45μm filtration, Nalge Co., Rochester, NY, USA). The HIV-1 macrophage tropic isolate p89.6 (GenBank accession # U39362;NIH AIDS Research & Reference Reagent Program, Rockville, MD, USA) was previously batch grown and harvested in K562 cells (American Type Culture Collection, Manassas, VA, USA). Viral supernatant was pooled and frozen at –70°C until needed. A viral cocktail (1:1 viral supernatant and fresh media, plus 4μg of Polybrene per milliliter) was left on the macrophage monolayer for six hours. Cultures were washed three times with phosphate-buffered saline and then incubated with

MDM media. Samples, of target macrophages, were taken for two and a half days after infection and analyzed with a p24 assay (Beckman Coulter, Fullerton, CA, USA) and a quantitative lentiviral vector particle number real-time PCR assay [22, 23], to verify positive viral infection.

DNA Processing

Chromosomal DNA from primary cell culture macrophages was extracted72 hours after infection with HIV-1, using either phase-lock gel tubes (Eppendorf Inc., Hamburg, Germany) with phenol/chloroform/isoamyl alcohol (PCI; 25:24:1) or Gene-Clean "turbo" for genomic DNA (QBiogene, Carlsbad, CA, USA). The DNA was prepared following a modification of the LP-PCR protocol [21] (Figure 2.1). Briefly, 1μg of DNA was digested to completion using 20 units of *Bgl II* (New England Biolabs, Beverly, MA, USA), in 2XNEB Buffer #4, for 3 hours at 37°C, in a final volume of 20 μL. After primary digestion, the volume was increased to 1XNEB Buffer #4 and added: 20 mMTris-acetate, 0.1 mg of BSA, 1 mM dithiothreitol (DTT), and 10 units of *Nla III* (New England Biolabs); incubated at 37°C for 2 hours in a final volume of 40 μL. Two nucleotides (G and A) of the *Bgl II* overhang generated by digestion, were filled in with 5 U of Klenow (3'-5' exo-) (Stratagene Inc.,La Jolla, CA, USA). We modified buffer conditions to final concentrations of 7.5 mM DTT, 0.25 mM dGTP, and 0.25 mM dATP; incubated at 37°C for 30 min in a final volume of 50 μL.

We then re-extracted the digested chromosomal DNA. One microgram of digested DNA was ligated to 80 pmols of LU3 Linker with 1x ligation buffer (Promega Inc., Madison,WI, USA) and 80pmol of LU3 Linker by heating to 60°C for 10 minutes

and then snap-cooled. Following this, 9U of T4 DNA ligase (3U/μL) (Promega) were added and incubated overnight at 16°C. DNA was re-extracted in preparation for PCR.

PCR Amplification and Cloning

First round PCR was performed (Expand High Fidelity PCR System, Roche Inc., Indianapolis, IN, USA) with 50ng template DNA and primers: LU3LTF (5'-GCA TGA TCA ATG GGA CGA TCG CAT G) and U3210R (5'-TAG CAG GGT GTA GCA AGC TGT TGT CC). Nested PCR was performed on $1/50^{th}$ of the original PCR reaction with primers: LU3LTF and U3194R (5'-GCT GTT GTC CTC TCT GTT GTT CTC TCC TTC ATC). Cycling conditions were 1^{st} round: 94°C/5min; 25 cycles {94°C/30sec, 58°C/30sec, 72°C/1min}; and final cycle 72°C/10 min then held at 4°C. 2^{nd} round: 94°C/3min; 25 cycles {94°C/45sec, 58°C/30sec, 72°C/45sec}; and final 72°C/10min then held at 4°C. A region corresponding to the size greater than the positive control was cut out of the 2% TBE electrophoresis gel and purified (GeneClean). The PCR fragments were cloned into TOPO-TA cloning vector pCRII (Invitrogen Inc.,Carlsbad, CA, USA) and Top10 competent cells (Invitrogen Inc.). With HIV gene-specific primers to the 5' LTR, we ran positive selection colony PCRs to choose positive clones to be amplified with M13 primers and subsequently sequenced at the BioCore sequencing facility, University of Hawaii. Positive selection colony PCR primers were U3194R and U328F (5'-GAC AAG ATA TCC TTG ATC TGT GGG TCT ACC ACA CAC). Cycling conditions for positive selection colony PCR were: 94°C/3min; 30 cycles {94°C/1min, 58°C/45sec, 72°C/45sec}; and final cycle 72°C/10 min then held at 4°C. Cycling

conditions for M13 colony PCR were: 94°C/3min; 30 cycles {94°C/1min, 55°C/1min, 72°C/1min}; and final cycle 72°C/10 min then held at 4°C.

Sequence Analysis

Sequences were analyzed using *SeqWeb* software (Version 1.2, Accelrys, San Diego, CA) as well as NCBI's online analysis tools (www.ncbi.nlm.nih.gov, Bethesda, MD, USA). Sequences were analyzed using BLAST (NCBI website) for homology to HIV and Human sequences and referenced against the NCBI GenBank database. More precise analysis of HIV sequence data was done through the Los Alamos National Laboratory HIV databases site (hiv-web.lanl.gov/content/index, University of California for the US.Department of Energy, USA). To identify proximal host genes to the HIV-1 insertion site, we analyzed the genomic region 300 kb 5'upstream and 3'downstream, using the Golden Path Human Genome Draft BLAT function (www.genome.ucsc.edu) and referenced against the NCBI GenBank database [24].

Real-time PCR

When template was available, real-time PCR was performed to determine relative HIV copies per cell. Real-time reactions were performed on a iCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA), using iQ SYBR Green supermix (Bio-Rad). Real-time PCR amplifying a 245bp region of the human *c-Jun* gene (GenBank Accession number J04111), was performed on 17ng of the undigested, HIV-1 infected, MDM DNA with primers: hum5'jun (5'-CAT GGA GTC CCA GGA GCG GAT CA-3') and hum3'jun (5'-GCA ACT GCT GCG TTA GCA TGA GT- 3'). Cycling conditions were:

initial denaturing 94°C/15min; 40 cycles {94°C/30sec, 55°C/30sec, 72°C/30 sec}; and final cycle 72°C/3 min. Melt curve analysis included 90 steps of 0.5°C/10sec transition rate starting at 55°C, then final hold at 4°C. Real-time PCR, amplifying a 115bp region of the HIV-1 *gag* gene (GenBank Accession number K03455), was performed on 17ng of the undigested, HIV-1 infected, MDM DNA with primers: SK38 (5' -ATA ATC CAC CTA TCC CAG TAG GAG AAA T- 3') and SK39 (5' –TTT GGT CCT TGT CTT ATG TCC AGA ATG C- 3'). Cycling conditions were: initial denaturation 94°C/15min; 40 cycles {94°C/30sec, 60°C/30sec, 72°C/30 sec}; and final cycle 72°C/3 min, next a 90 step melt curve starting at 55°C with a 0.5°C/10sec transition rate, then final hold at 4°C. In order to determine the relative copies of HIV per cell, we divided the mean number of haploid copies of the HIV-1 *gag* gene, by the mean number of haploid copies of the human *c-Jun* gene. Final copy number was presented as HIV copies per diploid cell. There was not sufficient tumor DNA left over from the IPCR assay to perform real-time, copies per cell, analysis on patient samples.

Inverse Polymerase Chain Reaction (IPCR) of Tumor Specimens

Specimens

Twenty eight tumor specimens from HIV-infected patients were received from the NCI (National Cancer Institute) funded AIDS and Cancer Specimen Resource (ACSR) at the University of California at San Francisco (UCSF) as per guidelines established by the University of Hawaii Institutional Review Board. The samples consisted of the following tumor types: Ki-1 lymphoma, large cell lymphoma, Kaposi's sarcoma, high grade

lymphoma, and Burkitt's lymphoma. DNA was extracted from each of these samples using phase-lock gel tubes (Eppendorf Inc.) with PCI (25:24:1).

IPCR and Cloning

Following DNA extraction, the DNA quality was assessed by spectrophotometry and PCR using HLADQα house keeping gene [25]. Genomic DNA was digested with *PstI* (Promega Inc.) for six hours at 37°C. Following digestion, the restriction enzyme was heat inactivated at 65°C for 15 minutes. The DNA was diluted to 2 ng/mL and then circularly ligated with T4 ligase (Promega Inc.) for 18 hours at 15°C. Post-ligated DNA was purified using GeneClean III (Bio101, Carlsbad, CA).

Ligated DNA was amplified using two primer sets for inverse polymerase chain reaction (IPCR) and nested set polymerase chain reaction (PCR). A schematic of the IPCR strategy is shown in Figure 2.1. The outer primers: H (5'CCT TAA ATG CAT GGG TAA AAG TAG TRG), #115 (5'-TTG TTG GCT TCT TCT AAC TTC TCT GG); and the inner primers: J (5'-TGA TAC CCA TGT TTT CAG CAT TAT CAG), #116 (5'TGG TAC TAR CTT GWA GCA CCA TCC A)(R=A/G),(W=A/T). Cycling conditions were 1st round: 94°C/1min; 10 cycles {95°C/15sec, 62°C/30sec, 68°C/3min}; 35 cycles {94°C/15sec, 57°C/30sec, 68°C/3min}; and final cycle 68°C/8 min then held at 4°C. For the nested reaction, 1ul of the 1st round PCR product was used with the following cycling conditions: 94°C/1min; 35 cycles {94°C/15sec, 56°C/30sec, 68°C/3min}; and final 68°C/8min then held at 4°C. All PCR mixtures utilized the Expand High Fidelity PCR System (Roche Molecular Biochemicals). Positive and negative controls were used for each PCR run. Following nested set PCR, positive

samples were cloned into pCRII plasmid using the TOPO TA cloning kit with chemically competent TOP10 *E. coli* cells (Invitrogen Inc.). Clones were prescreened using nested set PCR primers (J and 116) prior to sequencing at the BioCore sequencing facility (University of Hawaii at Manoa).

Sequence Analysis

Sequences were analyzed using *SeqWeb* software (Accelrys, San Diego, CA) and NCBI's online analysis tools (www.ncbi.nlm.nih.gov). IPCR derived HIV insertion sites were evaluated using the Golden Path Human Genome Draft BLAT function (www.genome.ucsc.edu ; April freeze 2002) and referenced against the NCBI GenBank database [24].

Southern Blot Hybridization

Integrated sequence unique to the tumor tissue was confirmed using Southern Blot hybridization. Primers designed from the tumor tissue integrated DNA sequence were used to amplify DNA from both tumor (mediastinal fibrotic tissue) vs. non-tumor tissue (non-tumor pericardium tissue). For the PCR reaction, primers: sblotF (5'-TCA GGG AAG AAG CCT TGT GT-3') and sblotR (5'-TGC CAT TCT CCC TCA TTG AT) were used with 50ng of the specimen DNA, with the following cycling conditions: 94°C/1min; 35 cycles {94°C/15sec, 56°C/30sec, 68°C/3min}; and final 68°C/8min then held at 4°C. PCR products were loaded on a 2% agarose gel and blotted on to a positively charged membrane using downward capillary and alkaline transfer methods. The samples were probed with a DIG-labeled probe (DIG Oligonucleotide 3'-End Labeling Kit, Roche

Applied Science, Indianapolis, IN) derived from the tumor integration sequence containing 50% human and 50% HIV LTR regions: 5'-TTA GCC CTT CCA TAA CCT CAT ACT G.

RESULTS

HIV Integration in Isolated Macrophages

The isolation of macrophages and HIV infection were carried out in triplicate. A large number of colonies were generated following the infected macrophage DNA processing, Linker-Primer PCR and cloning. In order to improve the efficiency of identification of potential integration sites, colonies from each experiment run were screened for HIV. Internal HIV primers U3194R and U328F were used to screen the colonies: a total of 220 colonies from Linker-Primer PCR cloned inserts were screened from the three rounds of experiments. Out of the 220 colonies screened with positive selection PCR, a total of 23 matched the positive control for HIV 5' LTR. Of these 23 sequenced, only one contained HIV-1(GenBank U39362), our synthetic linker sequence, and human genomic DNA sequence. The other 22 sequences were all HIV-1 sequence, without any flanking human genomic DNA sequence.

The one successful linker-primer PCR sequence contained 25 base pairs of the linker-primer (LU3LT), as mapped out in Figure 2.2. The sequence also contained 60 base pairs of human genomic DNA sequence that has 100% homology to the coding region of Toll-like receptor 1, found on chromosome 4, band 4p14 within contig AC108044.5. This sequence also contained 24 base pairs of HIV sequence that has 100% homology to the 5' LTR (U3 region) and the HIV *nef* protein DNA coding region (Figure 2). The 24 base pairs of HIV sequence, aligned to position 129 – 152 within the 5' LTR (U3 region;GenBank U39362). The HIV portion of the PCR sequence ended prematurely 10 bases 5' to the primer sequence, U3194R, used to amplify the segment.

Characterized genes found within 100kb upstream or downstream of the integration site, on chromosome region 4p14 in the NCBI GenBank database, included Toll-like receptor 6 and 10. Sequences representing gene transcripts of hypothetical proteins, found within 300kb upstream or downstream of the integration site included: KLHL5 (Kelch-like 5), FLJ23235 (hypothetical protein FLJ23235), LOC92689 (hypothetical protein BC001096), KLF3 (Krupple-like factor 3), FLJ13197 (hypothetical protein FLJ13197) and LOC401126. The rationale for searching within the genomic regions 300 kb pairs upstream (5′) and downstream (3′) of the cloned HIV-1 insertion site was based on previous reports showing evidence of cellular transformation associated with retrovirus-mediated cis-activation of adjacent host cell genes within 90 to 270 kb of specific retroviral integration sites in animal models [26, 27].

An aliquot of the undigested DNA from the infected MDMs was analyzed by real-time PCR to determine relative HIV copies per cell. This sample of DNA template used for the linker-primer PCR was found to have an estimated 20 copies of HIV-1 per diploid cell. This method for determining copies of HIV per cell does not decipher between episomal and integrated forms.

IPCR of Tumor Specimens

Twenty-eight specimens from HIV-infected patients processed via the IPCR protocol were successfully analyzed with appropriate positive and negative controls. Successful amplification using IPCR and cloning was achieved in 1 of the 28 specimens. This particular case was a mediastinal Ki-1 lymphoma. This tumor specimen was found to have at least one integrated HIV copy (Figure 2.2). The integration was found in

chromosome 22 band 22q13.2 within contig NT 011521.1. Genes found within 300kb upstream or downstream of the integration site included: FLJ23588&HSCBCIP1(are aliases of the same gene; CAP-binding protein complex interacting protein 1), HMG17L1 (high-mobility group, non-histone chromosomal, protein 17-like 1), LOC129179, SULT4A1 (sulfotransferase family 4A, member 1), LOC150379 (GS2 like), LOC164691, C22orf20 (chromosome 22 open reading frame 20), CGI-51 (CGI-51 protein), LOC129178 (similar to bM150J22.1 – novel protein, ortholog of human C22orf1), LOC129177 (similar to cB13C9.1-adult brain protein 239), and LOC129176. The integrated sequence from the tumor specimen was unique to the malignant sample as evidenced by the failure to detect the same integration site in an uninvolved tissue taken from the same patient. Figure 2.3 demonstrates that the amplified band from the tumor specimen is positive, while the uninvolved tissue did not have an amplified band that hybridized to the tumor-specific probe. The product size indicated on the Southern blot is 192 base pairs.

DISCUSSION

The experiments successfully demonstrated that in both an *in vitro* system and human malignant tissue, specific viral integration can be identified. From the macrophage system, the sequencing information found the integration of HIV within a toll-like receptor on chromosome 4. Although the HIV portion of the PCR sequence was shorter than expected, it did lie 10 bases 5' to the primer (U3194R) which was used to amplify the segment. One possible reason we were unable to find our reverse primer, could be due to 3'-degradation of the PCR product prior to the cloning steps. This could explain why we were unable to find our nested primer within the sequence. The missing 129 basepairs of 5'end HIV sequence could possibly have occurred from a recombination event during integration of the HIV provirus, causing 5' proviral truncation within the human genome. Another explanation for the missing 129 base pairs of HIV sequence could be due to molecular manipulation. When analyzed with hairpin analysis software (http://biotools.idtdna.com/analyzer/oligocalc), the sequence presents multiple possibilities for hairpins (data not shown). In addition, deleted sequence may have been created from DNA looping and strand slippage during amplification.

Toll receptors are associated with the downstream signaling of cytokines through the initial cascade phosphorylation of serine kinases [28-32]. In general, Toll receptors use a pathway similar to interleukin-1 receptors [30]. Toll genes contain highly conserved structural motifs expressed in microbial pathogens[33]. Toll receptor 1 (TLR1) is highly expressed in leukocytes [34]. TLR1s are expressed as a transmembrane protein with a cytoplasmic tail containing a conserved region called the Toll/IL-1 receptor domain [31,

35]. Binding of the receptor will lead to a cytoplasmic signaling cascade in which the transcription factor NF-κB is activated to induce the production of pro-inflammatory cytokines[28, 29, 32]. NF-κB is a critical transactivator of HIV transcription, and also plays an important role in HIV replication in monocytes [7, 36, 37]. The presence of NF-κB binding sites in the U3 LTR, provides an evolutionary advantage of the integrated HIV provirus when the cell undergoes activation. [7]. More recent studies have shown that TLR stimulation by ligands can enhance HIV-1 replication under certain conditions [38, 39]. It is possible that the integration of HIV within the Toll 1 gene was a non-random event. Unfortunately, the LP-PCR protocol did not utilize clonal cells, so it was impossible to investigate the effects that this integration event had on expression of the Toll/IL-1 receptor, NF-κB, and HIV virus.

The linker-primer integrated sequence was also found to be within 100kb of Toll-like receptor 6 and 10. The TLR 6 gene has a very similar ontology to the TLR 1 gene, in that both function through defense and immunity protein activity, interleukin-1 activating receptor activity, and activation of NF-κB, as well as many other inflammatory and immune-related functions. TLR 10 has similarly been found to function through interleukin-1 activating receptor activity, and inflammatory and immune processes.

Finding a HIV-1 provirus integrated upstream of genes HSCBCIP1 (cAMP-binding protein complex interacting protein 1) and HMG17L1 (high mobility group, nonhistone, 17-like1) is intriguing, which could lead to further studies on the influence of HIV *LTR* on gene expression. The HSCBCIP1gene is an alias of the protein DJBP, and involved in a transcription repression pathway that binds DNA through the recruitment of histone complexes [40]. HMG171 is a transcriptional activator that can bind to

chromatin and bend DNA, promoting the assembly of proteins on specific target DNA [41]. The function of HMG17L1 has been proposed by several studies and includes: a nuclear signal to determine whether cells undergo apoptosis or necrosis [41], extracellular signaling molecule targeting cytokine signaling targets [42], potent factor secreted by mononuclear phagocytes that causes inflammation and protease activity [43], and an activator of p53 sequence-specific DNA binding[44].

One approach taken in this study was to utilize the linker-primer PCR assay (LP-PCR) protocol, which was specifically developed to detect and quantify integrated HIV DNA species[21]. Chromosomal DNA preparations isolated from cells infected with HIV contain significant amounts of contaminating extrachromosomal HIV DNA. By using *Bgl II* restriction enzyme, the digestion selects against the amplification of the three main extrachromosomal HIV DNA forms. The digested extrachromosomal HIV DNA is thus prevented from religating by filling in two nucleotides (G and A) of the Bgl II recognition site. The chances of the Bgl II site occurring prior to an Nla III site in the cellular DNA sequence upstream of the 5'LTR is once every 16 integration events [21]. Thus, in theory 94% of all integrated forms of HIV should be able to be detected with this method. In our experience, the revised Vandegraaff technique appeared to be less than efficient, with only one linker-primer sequence out of 220 colonies containing the linker-primer, HIV sequence, and human genomic DNA sequence.

The original Vandegraaff protocol [21] utilized a clonal T-cell line. We believe, the use of primary macrophages, is the reason why the modified Vandegraaff protocol resulted in a much lower efficiency than reported in other studies using clonal cell lines [21, 45]. In our macrophage cells, each HIV-1 viral sequence and flanking genomic

sequence, represents a separate integration event; the cells were grown for 10 days – long enough to differentiate from monocytes, but not a sufficient time to multiply. Since PCR is an amplification process, it stands to reason that multiple copies would be easier to amplify than a variety of different sequences. Macrophages, in general require longer duration in culture for virus production to occur following infection [46]. This is contrary to most other hematopoietic cell types, and could be an interesting subject for future study. The low efficiency of the linker-primer assay, could be explained by limited integration events.

Identifying HIV-integration near growth-regulating genes would be important in understanding the pathogenesis of some types of HAM, but could also lead to discovering potential site-specific treatment strategies. One of the challenges in identifying site-specific integration is the low copy number of virus as well as the possible absence or presence of cells harboring integration, as suggested by the "sequential neoplasia" model [20]. Other investigators have also pursued similar studies with some success in identifying viral integration. In a study investigating HIV-1 proviral integration in peripheral blood lymphocytes, a pattern was found in repetitive Alu elements and an intron of the *BRCA1* gene[47]. This data supported previous experiments suggesting that HIV-1 integrates non-randomly into "Alu islands" [48]. Evidence for non-random HIV integration, possibly supports the idea that the integration process itself may have a function other than solely for replication.

In vivo experiments have shown that integration occurs preferentially in actively replicating cells [49]. Studies with a variety of retroviruses have shown that integration would take place within several hundred bp of a DNaseI-hypersensitive chromatin site

[50, 51]. 5'methylation of cytosine in runs of CpG, are associated with the 5' ends of housekeeping and tissue specific genes, and some 3' ends of tissue-specific genes [50]. In vitro data indicated that these methylated sites of active transcription are preferential targets of integration[50, 52]. Other data indicates that when preferred targets are tested as naked DNA, integration does result [49]. In general, in vitro studies support the idea that it is the local structure of the chromatin that determines the enhanced accessibility of a sequence for proviral insertion. Physical accessibility is indicated as a major component of preferential integration. One HIV-1 study found a "modest" preferential integration sequence, the sequence "CAATG", in both vivo and vitro analysis [53].

More recently, studies have shown that integration of HIV into transcriptional units is not random. One study, where integration was performed *in vitro* using a human cell-line (SupT1), integration sites of HIV-1 were found in clusters (hot spots) that were closer than randomly distributed [45]. In a recent study focusing on chronic HIV-1 infection of cultured human macrophages, a significant increase in expression of interferon-stimulated genes by microarray analysis was reported [54]. Overall, Meehan et al. found that 25% of the altered genes were involved in inflammatory responses, and 23% in transcriptional regulation [54]. Mack, et al. [24] reported an analysis of biopsy samples from HIV-1 infected patients, and found HIV integration events that were clonally expanded during HIV disease pathogenesis. HIV-1 was found to integrate non-randomly into transcriptionally active genes. In addition, proviruses were frequently found proximal to genes that encoded for cellular process proteins including: signal transduction networks, transcription, and translation. Evidence for non-random HIV integration into genes including important transcriptional control genes, confirms our

greater need to elucidate the means by which HIV is associated with malignancy. One mechanism by which animal retroviruses induce tumorgenesis is to integrate near cellular proto-oncogenes and promote overexpression [7]. Other animal retroviruses carry within their code transducing oncogenes; such as in the avian Fujinami sarcoma virus, the 16L avian virus, and the feline oncosarcoma virus, that carry the viral form of the oncogene fps (v-fps) [55, 56]. These viruses are known to cause myxosarcomas in chickens, and fibrosarcomas in chickens and cats [56].

Another oncogenic mechanism used by animal retroviruses, is to insertionally mutate in an essential coding region of an expressed gene, such as a receptor. Using inverse polymerase chain reaction (IPCR), a common integration site of HIV-1 was found upstream from the *c-fes/fps* oncogene, in six non-Hodgkin lymphomas (NHL) [14, 57]. The HIV-1 provirus was found integrated within the *fur* gene, upstream from the proto-oncogene *c-fes/fps* [57]. The unique cases described with viral integration upstream from the c-fes/fps oncogene are consistent with a theory that some cases of NHL may involve non-random HIV integration.

In conclusion, using two independent methods, our studies showed integration within an important immune functioning gene in the macrophage, as well as near a transcriptionally important gene in human tumor specimens. However rare, the ability to show integration events within tumor samples is of utmost importance. The position of the HIV LTR near, and thus most likely influencing, transcriptional control elements is of particular interest. Upon the development of a more efficient integration analysis protocol, we may be able to demonstrate more examples of directed integration of HIV.

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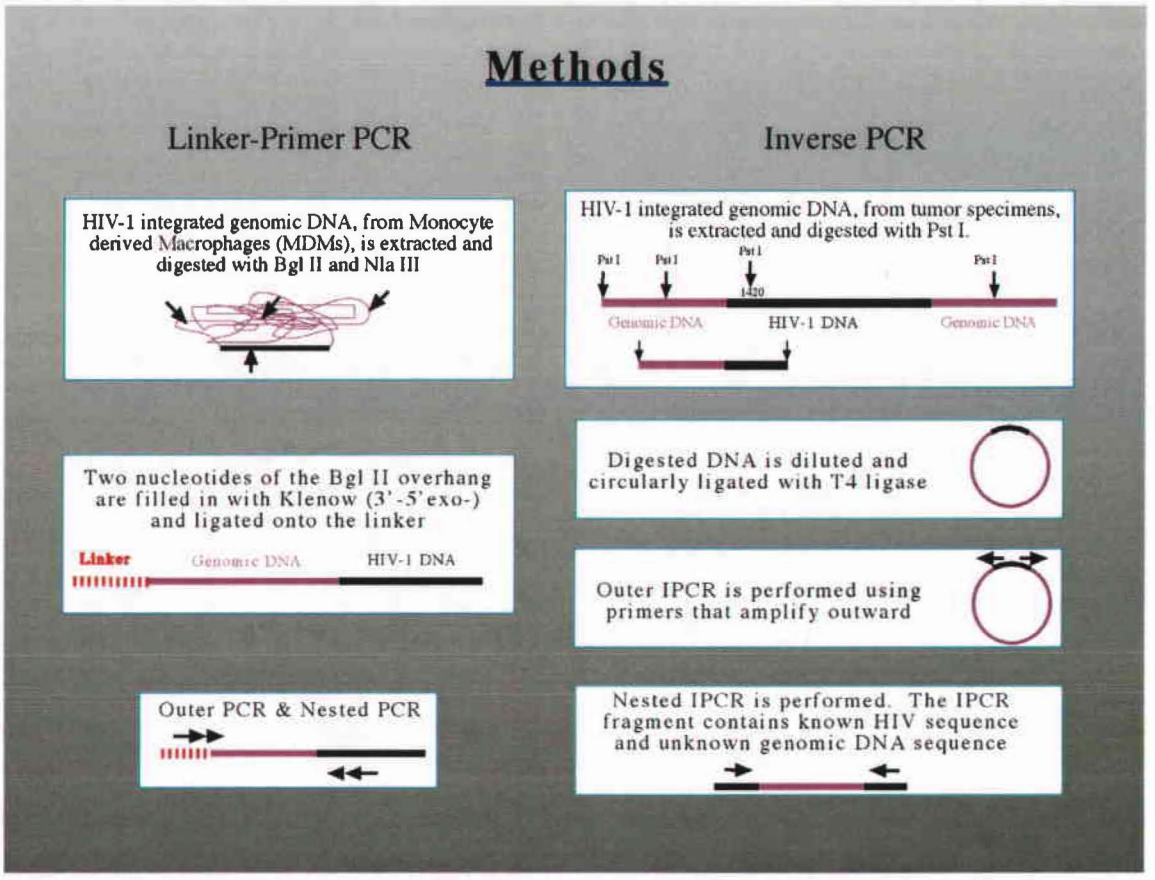

Figure 2.1. Illustration of the modified Vandegraff linker-primer PCR protocol and the Inverse PCR protocol. The linker-primer protocol involves: digestion of genomic DNA from Monocye derived Macrophages (MDMs), ligation of the linker onto the cut DNA, and outer and nested PCR. The Inverse PCR protocol involves: digesting genomic DNA, ligating the DNA into a circle, and performing outer and nested PCR.

HIV-1 Integrated Sequences

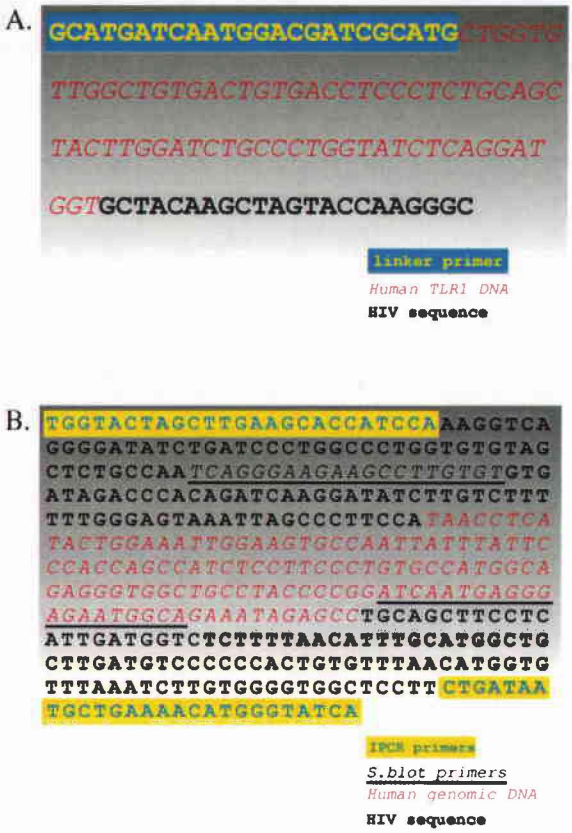

Figure 2.2. Map of the integrated HIV sequences. A. The linker-primer PCR sequence from macrophages, is shown with the linker-primer (in blue and yellow), Human Toll-like receptor 1 genomic DNA (in red), and integrated HIV-1 sequence (in black). B. The IPCR integrated HIV sequence from the tumor specimen is shown with the IPCR HIV primers (in yellow and blue), the Southern blot primers (underlined), HIV-1 integrated sequence (in black), and Human genomic DNA (in red).

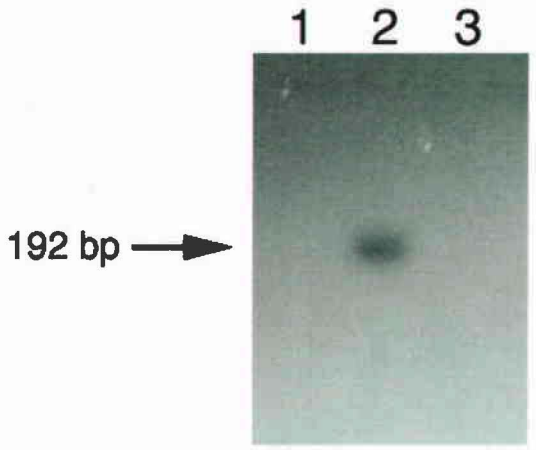

Figure 2.3. Southern blot hybridization of amplified products from malignant tissue (lane2) and uninvolved tissue (lane 3) from the same patient. Negative control (lane 1) was a PCR reaction set up without DNA.

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CHAPTER 3

PATHOGENESIS OF HIV-ASSOCIATED NON-HODGKIN LYMPHOMA

Deirdre Killebrew* and Bruce Shiramizu

University of Hawaii, Honolulu, HI

*Primary Author: Deirdre Killebrew, 3675 Kilauea Ave., Young Bldg, 5th Floor, Hawaii AIDS Clinical Research Program, Honolulu, HI 96816; 808-737-2751; Fax: 808-735-7047; email: dak@hawaii.edu

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ABSTRACT

In the current era of highly active antiretroviral therapy (HAART), the prevalence of HIV-associated non-Hodgkin lymphoma (H-NHL) is not as high as in the beginning of the acquired immunodeficiency syndrome (AIDS) epidemic, but still remains above that of non-HIV-infected individuals. Therefore, the epidemiology suggests that the pathogenesis of H-NHL may be multifactoral, involving the interaction of the immune system with HIV or other pathogens. Although HIV is a retrovirus, it is not characterized with the typical oncogenic potential associated with insertional mutagenesis. HIV integration occurs as a necessary step in the life cycle allowing for replication and transcription of the viral genome to take place. While HIV is thought to integrate randomly, more recent studies have suggested that integration occasionally occurs in a less random fashion. While the majority of H-NHL may be secondary to immune dysfunction, this non-random integration may be a factor leading to pathogenesis of a small subset of H-NHL since the pathways involved in malignancies usually require an accumulation of abnormalities leading to proliferation and transformation. A common element among lymphomas in this setting of immune dysfunction is the inability to control a pathogen that acts as an ongoing immune activator. Thus, an increased risk of H-NHL emerges from the combination of immunodeficiency, increased immune activation and possible HIV insertional mutagenesis. The focus of this review will be on the role viral pathogenesis plays in oncogenic transformation.

INTRODUCTION

In resource-rich countries, human immunodeficiency virus, type 1 (HIV-1) infection is relatively controlled with anti-retroviral therapy, thus these individuals are continuing to cope with a chronic disease. As patients live longer with HIV-1 infection, or are diagnosed with acquired immunodeficiency syndrome (AIDS), an increase in malignancies has been observed [1]. The epidemiology of HIV-1-associated malignancies (HAM), which includes Kaposi's sarcoma (KS) and HIV-1-associated non-Hodgkin lymphoma (H-NHL), has changed as improvements in antiretroviral therapies have developed [2-4]. While there has been a decrease in the incidence of HAM with the use of highly active antiretroviral therapy (HAART) in resource-rich countries, the incidence of some specific types of cancers is still relatively higher than in non-HIV-infected individuals [2, 3]. The risk of lymphoma in an HIV-infected individual is 100 times that of an uninfected person [5]. Lymphomas associated with HIV are usually aggressive, with the majority occurring in extralymphatic sites [6]. Although, the use of HAART is correlated with a significant decline in KS, the decrease in H-NHL cases is less dramatic [5].

An increased risk of H-NHL arises from the complex combination of immunosuppression and inflammatory activation. Other factors such as possible effects of HIV insertional mutagenesis are still unknown, but have been proposed. Identifying a common etiology, if there is one, among the AIDS-defining malignancies has been a challenge. One common element among lymphomas in this setting of immune dysfunction is the inability to control a pathogen that acts as an ongoing immune activator. Evidence is emerging that suggests that most of the malignancies that develop

during immunodeficiency are rare cancers associated with viral infection [7]. Retroviral integration into the host genome is necessary for replication and expression of progeny virions [8]. While HIV-1 could possibly cause insertional mutagenesis, historically, lentiviral integration has primarily been random, and has not been shown to be a direct cause of human malignancies. More recent studies have suggested that integration occasionally occurs in a less random fashion [9-12]. This non-random integration may be one of the factors leading to the pathogenesis of some subtypes of H-NHL since the pathways involved in malignancies usually require an accumulation of abnormalities leading to transformation and proliferation. The focus of this review will be on the role viral pathogenesis plays in oncogenic transformation.

HHV8/KSHV and Kaposi's Sarcoma

Before the HAART era, the most frequently diagnosed cancers in patients with AIDS were KS and non-Hodgkin B-cell lymphoma (NHL) [13, 14]. KS was, and still is, an extremely rare condition in an immunocompetent individual [15]. A patient's immune status directly affects the progression and clinical presentation of KS; i.e. the lower the CD4+ cell count, the faster the KS lesions grow and involve other organs [14, 15]. The histological appearance of KS has been described as lesions composed of endothelial cells and spindle cells mixed with mononuclear immune and red blood cells [15]. The human herpesvirus-8 (HHV8), also called Kaposi's sarcoma herpesvirus (KSHV) was found to be the direct epidemiologic agent of KS [16]. The inability of a patient with AIDS to control HHV8/KSHV and subsequently develop KS, indicates that this syndrome is an opportunistic malignancy with multiple etiologies [15]. HHV8/KSHV has

incorporated several eukaryotic cellular protein genes that could contribute to tumor formation, including: G-protein homologs, cyclin D, interleukin 6, bcl-2, latent nuclear antigen-1, viral cyclin, and interferon regulatory factor [3, 17]. The tumor microenvironment has been shown to be an essential aspect of KS progression [3]. The cross-talk of cytokines and chemokines between host cells and cancer cells can lead to further proliferation and survival of circulating malignant cells [18]. Both HIV-1 and HHV8/KSHV are sexually transmitted viruses, and both are the only known risk factor for developing KS [3].

HIV-associated Non-Hodgkin Lymphoma

H-NHL is characterized as a high-grade malignancy of B-cell origin, with a poor prognosis, and having extensive extranodal involvement [14]. There are three types of H-NHL: systemic (nodal and extranodal), primary central nervous system (PCNSL), and body cavity-based, or also known as, primary effusion lymphoma (PEL). Approximately 80% of all H-NHLs are considered to be systemic, defined as not being limited to the CNS (PCNSL) or other anatomic regions (PEL) [19]. A majority of systemic H-NHL cases occur outside the lymph nodes (extranodal). Close to 20% of H-NHL cases are PCNSL [19]. PEL is the rarest H-NHL type, approximating 3% of all cases [19].

The introduction of HAART has lowered the incidence of systemic lymphomas. However, the reduction has not been as successful as noted with the incidence of KS. H-NHL is the second most common neoplasm worldwide, but in regions where HAART is available, NHL remains one of the most common HIV-associated malignancies, and the most lethal lymphoma when considering the frequency of HIV-associated lymphomas in

developed countries [13, 15, 20, 21]. With HAART, the most notable decline in incidence of NHL in HIV-positive individuals, has been in the PCNSL [13, 21-23].

Duration of HIV-1 infection and the level of immunosuppression since seroconversion, as measured by nadir CD4 cell counts, are both predictors of NHL development [14, 17]. One study showed that as CD4 cell count fell below 350 cells/μL, the development of lymphoma became more probable [17]. Extreme immunosuppression (CD4 cell count as low as 30 cells/μL) has a higher association with subsets of H-NHL, such as primary central nervous system lymphomas (PCNSL), and PEL [15]. In general, any type of immunosuppression, acquired or otherwise, is associated with an increased risk of NHL [13].

Viral association with H-NHL

The association of HHV8/KSHV with lymphoma was described for PEL, a rare type of NHL, and considered unique due to its uncommon clinical and molecular features [13, 24] (see Table 3.1). When tested for the presence of HHV8/KSHV, PEL are found to be 100% positive, suggesting a contributory role of HHV8/KSHV in PEL [3, 17]. PEL are found in a variety of body cavities, such as the pleural cavity, with no involvement of lymph nodes or spleen [25]. PEL cells are of B-cell genotype, but usually lack typical B-cell lineage antigens, CD19 and/or CD20. Some of these malignant B-cells display CD38 only [24]. They also frequently express CD45 and other activation associated cell markers [20]. PEL tumors can be monoclonal or polyclonal, and roughly 70% are co-infected with Epstein-Bar virus (EBV), although nearly all lack *c-myc* translocations [3, 20, 24]. Growths of these tumors in vitro are mediated by IL-6 and VEGF [26, 27].

MIP-1 may also play a role in PEL pathogenesis by attracting other cytokine and chemokine producing cells into the lymphoma microenvironment [28].

Half of the AIDS-related systemic lymphomas are not associated with oncogenic viruses, and are negative for EBV and HHV8 [3, 17]. In these lymphomas, it is proposed that chronic antigenic stimulation by HIV infection, leads to oligoclonal B-cell expansion [29-32]. Due to T-cell depletion, the absence of T-cell control over B-cell proliferation, could lead to a population of B-cells that are at increased risk of random somatic mutations that develop into transformation and outgrowth of B-cell clones [29-32]. A few studies have shown the presence of B-cell polyclonal lymphomas in HIV-1 infected patients [33-36]. Although B-cell polyclonal lymphomas have been found in iatrogenic and EBV mediated malignancies, polyclonal malignancies represent a new type of HIV-associated lymphomas [34]. McGrath et al. deemed the polyclonal B-cell lymphoma "a new class of HIV-associated disease" because the tissue samples were free of EBV and *c-myc* proto-oncogene rearrangement [34]. It was also reported, that there is a high correlation between the most aggressive lymphoproliferative samples with the highest HIV-1 serum levels [34]; indicating a possible activating role for HIV-1 in the lymphoma process. HIV could conceivably contribute to neoplastic development through impairment of cell-mediated immunity and dysregulation of cytokine loops [13].

Herndier et al. (1992), found the existence of an aggressive monoclonal T-cell lymphoma in a patient infected with HIV-1 [35]. Although T-cell NHL is occasionally found, the presence of HIV p24 antigen expression and evidence of HIV-1 integration had not previously been reported. Viral integration in malignant cells is more characteristic of the human T-lymphotropic virus type I (HTLV-1)-associated leukemias.

However, in this particular case, Southern blot analysis only found HIV-1 integration, not HTLV-1[35]. There was also absence of EBV and human herpes virus-6 (HHV-6), ruling out their possible role in this particular malignancy. The unusual finding of HIV integration in tumor tissue suggests the possibility of cellular oncogene stimulation or insertional mutagenesis.

Histological markers in H-NHL

B-cells are vulnerable to transformation at several stages during the differentiation process [3]. Markers or identifiers of B-cell stimulation in the form of high serum globulin and HIV p24 antigens help to identify HIV-infected people that are at high risk for developing NHL [30]. Also, genetic markers may identify additional groups of people at high risk for developing H-NHL. Caucasian and African American ethnic groups have a higher proportion of the cytokine stromal cell derived factor 3'A variant [37]. An individual carrying the 3'A variant has a predisposition towards H-NHL that is double in risk compared to heterozygotes and quadruple the risk in homozygotes, respectively. People who encode the chemokine receptor deletion (CCR5Δ32), do not express CCR5, and are resistant to development of NHL and HIV infection [37]. Roughly 40% of AIDS-associated NHL have been found to possess mutations in the tumor suppressor gene p53, a protein that regulates cell replication through its role in cell cycle control [17]. BCL6 is a histological marker for both AIDS-Burkitt's lymphoma (30% EBV positive) and AIDS-diffuse-large-cell lymphoma (50 –70% EBV positive) [3, 23]. The B-cell differentiation in these systemic NHL lymphomas is arrested in the germinal-center centroblasts and the early centrocyte stages. Other AIDS-associated

cancers characterized as post-germinal-center B-cell lymphomas include: Hodgkin's disease, Castleman's Disease, PEL, and multiple myeloma. These lymphomas express MUM1 and syndecan-1, but not BCL6 [3, 23]. EBV-associated B-cell lymphomas are caused by both mutations and translocations of genes at various stages of differentiation, and associated with expression of EBV latent genes [38]. EBV latency type 3 antigens, such as the transforming latent membrane protein-1, and 2, and EBNA-2, have been shown to possess transforming ability on cultured cells [17]. The oncogenic potential of HHV8 infection in B-cells is less understood [3].

Molecular studies have shown that systemic H-NHL are monoclonal and polyclonal; or oligoclonal if a predominant rearrangement in the immunoglobulin J_H gene is absent [24]. In contrast, the majority of PCNSL are large cell, monoclonal B-cell lymphomas that are clonally infected with EBV, although typically lacking the c-myc translocation [24].

Transformation Potential of Retroviruses

Historically lentiviruses have not been thought of as human cancer-causing pathogens. Although many retroviruses are proven oncoviruses in animals, few are thought to cause cancer in humans. The pathogenesis of AIDS-associated malignancies is generally thought to be the result of an opportunistic proliferation due to an oncogenic stimulus and a depressed immune system. Feline immunodeficiency virus (FIV) has been suggested to be the causative agent for one type of cat B-cell lymphoma [39]. A single FIV provirus integration site was found in the clonal B-cell population. The lentiviral integration was believed to occur prior to the B-cell transformation. Simian

immunodeficiency virus (SIV) infection creates a severe immunosuppression in macaques, much like that of HIV in humans [40]. In experimentally infected macaques, the SIV proviral sequence was detected in three separate tumors, one T-cell population and two oligoclonal. As part of an AIDS lymphomagenesis mouse model experiment, SCID mice were injected with HIV-infected human body cavity based lymphoma macrophage cells [41]. Overall, 54.5% of the mice developed T-cell lymphomas in the spleen, liver, and bone marrow. Human macrophages were detected in the mice, up to six months post-infection. Unlike tumor transplantation experiments, these AIDS-related lymphomas arose through tumor induction. These studies are examples that lentiviral transformation is possible in animal cancers.

Experimental animal studies utilizing isolated HIV gene products have implied transforming activity. Mice that transgenically expressed the isolated HIV-1 *tat* gene product, developed histologically identical Kaposi's sarcoma (KS) skin lesions[42]; and were able to protect lymphoid, epithelial, and neuronal cells from apoptosis induced by serum starvation [43]. The HIV-1 tat protein is necessary for viral transcription, and is able to trans-activate cellular genes [43]. Extracellular HIV-1 *tat* has also been shown to stimulate human Kaposi's sarcoma cells.

Evidence is accumulating suggesting that HIV may have a more direct effecter role in some types of human oncogenesis. McGrath et al. reports finding HIV-1 integrated in macrophages surrounding an early KS lesion, but not in later, more developed lesions. The integrated provirus and the expressed HIV-1 gene products may stimulate the surrounding macrophages to produce activating cytokines, and thus, lead to proliferation [44]. Using inverse polymerase chain reaction, a common integration site of

HIV-1 was found upstream from the *c-fes/fps* oncogene, in six separate non B-cell lymphomas [12, 45]. The lymphoma histologies consisted of: one T-cell, three mixed lymphocyte and macrophage populations, one angioimmunoblastic lymphadenopathy, and one large cell lymphoma ascites tumor population. The HIV-1 provirus was found integrated within the *fur* gene sequence, upstream from the proto-oncogene *c-fes/fps* [12]. *Fur*'s translational gene product, furin, was found to contain a cysteine-rich region that had significant homology to the human insulin receptor and the human epidermal growth factor [46]. *Fur* and *fes/fps* are reported to be transcribed in the same direction, and when compared to another species (feline) the distance between the two genes are roughly the same, ~1.1kb [47]. The close proximity of the two transcription units, conserved in evolution in human and cat, may have functional implications. Alternatively, the furin amino acid sequence has also been reported to have high homology to the yeast KEX2 endoprotease [48]. The common integration site of HIV-1 found upstream from the *c-fes/fps* oncogene is especially relevant for tumorgenesis considerations because the *c-fes/fps* oncogene encodes a protein-tyrosine kinase that has been implicated in controlling transformation of hematopoietic cells [12]. The 92kD *c-fes/fps* protein signals through macrophage activating cytokines (IL-3, GM-CSF, and M-CSF). Integrated macrophages could lead to a complicated interaction with B-cells and T-cells via cell-cell signaling. Several animal retroviruses have acquired the *c-fes/fps* oncogene sequence [49]. Up-regulation of the *c-fes/fps* protein has been shown in cells with the integrated HIV-1 provirus [1]. Western blot analysis determined the *c-fes* protein was in a phosphorylated,

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activated form in the clonal macrophages associated with the analyzed tumors. Evidence of six separate cases of HIV-1 integration upstream from the *c-fes/fps* oncogene is indicative that the above-mentioned cases of non-B-cell lymphomas developed non-randomly.

The LTRs' functions seem multifaceted as important targets for cellular and viral protein regulation. Some characteristics of interest are: binding sites for cytokines and chemokines, sequences for a negative response element, a promoter, a repeat enhancer sequence, and the *tat1* response element, all localized to the U3 and R regions[50]. HIV proviral LTRs contain recognition motifs for the NF-κB families of transcriptional transactivators [51]. This is potential evidence that HIV has evolved mechanisms to initiate cellular activation pathways to amplify expression of its own genome. HIV entry into the cell induces macrophage colony-stimulating factor (M-CSF) upregulation [52]. In turn, M-CSF enhances the susceptibility of macrophages to HIV-1 infection, as well as facilitating the cells own replication. NF-κB and M-CSF function as HIV replication enhancers, in conjunction with their lymphoproliferative properties. An experimental construct linking the HIV 3'LTR to the genomic region directly upstream from the *c-fes/fps* transcription start site, demonstrated ten-times background upregulation of the *c-fes* protein, when expressed in a human cell line[1]. Experiments moving the LTR 2.6kb upstream of the *c-fes/fps* transcription start site still found a three-fold upregulated expression; giving evidence to the idea of *c-fes* being cis-activated by the HIV-1 provirus. When the *tat* gene was added to the original construct, the upregulation was 87 times that of background expression levels. These studies of upregulation from HIV LTR constructs provide evidence for transformation ability.

Preferential HIV Integration

The integrated provirus becomes a permanent part of the cell's genome, and is transmitted vertically into every daughter cell [53]. One way animal retroviruses induce tumorgenesis is to integrate near cellular proto-oncogenes and promote overexpression [54]. Another oncogenic mechanism used by animal retroviruses, is to insertionally mutate an essential coding region of an expressed gene, such as a receptor. *In vivo* experiments have shown that integration of retroviral DNA occurs preferentially in actively replicating cells [55]. Studies with a variety of retroviruses have shown that integration takes place within several hundred base pairs of a DNaseI-hypersensitive chromatin site [56, 57]. 5'methylation of cytosine in runs of CpG, are associated with the 5' ends of housekeeping and tissue specific genes, and some 3' ends of tissue-specific genes [56]. *In vitro* data indicated that these methylated sites of active transcription were preferential targets of integration [56, 58]. Other data indicate that when preferred targets are tested as naked DNA, integration does occur [55]. In general, *in vitro* studies support the idea that it is the local structure of the chromatin that determines the enhanced accessibility of a sequence for proviral insertion. Physical accessibility is indicated as a major component of preferential integration. However, an avian retrovirus has been shown to preferentially integrate into very specific target sequences [59]. One HIV-1 study found a "modest" preferential integration sequence, the sequence "CAATG", in both *in vivo* and *in vitro* analysis [60]. HIV-1 had previously been found to preferentially integrate near L1Hs repetitive elements [61], and also more recently near different Alu

elements [9]. L1Hs and Alu sequences are two of the most common repeated elements in the human genome [61].

It is generally thought that HIV-1 integrates into the human genome randomly. However, in a study investigating the flanking regions around the HIV-1 provirus in peripheral blood lymphocytes, a pattern of integration was found in repetitive Alu elements and an intron of the *BRCA1* gene [9]. This study supports previous data suggesting HIV-1 integrates non-randomly into "Alu islands" [62]. Alu sequences are a type of short interspersed element (SINES) that usually have some homology to the restriction enzyme recognition site "AluI;" they are usually found in noncoding regions, and are believed to be retrotransposed throughout most mammalian genomes [63]. Evidence for non-random HIV integration, supports the idea that the integration process itself may have a function other than solely for replication.

Identifying HIV-integration near growth-regulating genes may elucidate the pathogenesis of some types of H-NHL, regardless of any association with other oncogenic viruses. Studies have shown that integration of HIV into transcriptional units is not random. In one study, where integration was performed *in vitro* using a human cell-line (SupT1), hot spots (clusters) of integration sites of HIV-1 were found that were closer than randomly distributed [11]. Sequence analysis of 524 sites of HIV integration, found 68% of the integration events occurred within genes. Human endogenous retroviruses (HERVs) were negatively correlated with HIV integration, given that only 8.3% were found outside of genes. In a recent study focusing on chronic HIV-1 infection of cultured human macrophages, a significant increase in expression of interferon-

stimulated genes by microarray analysis was reported[2]. Overall, Meehan et al. found that 25% of the altered genes were involved in inflammatory responses, and 23% in transcriptional regulation [2]. Mack, et al. reported an analysis of biopsy samples from HIV-1 infected patients, and found HIV integration events that were clonally expanded during HIV disease pathogenesis [10]. HIV-1 was found to integrate non-randomly within transcriptionally active genes. In addition, proviruses were frequently found proximal to genes that encoded for cellular process proteins including: signal transduction networks, transcription, and translation. Evidence for non-random HIV integration into genes including important transcriptional control genes, confirms our greater need to understand the means by which HIV is associated with malignancy. The "sequential neoplasia model" is a theory that helps to explain how HIV-1 infected macrophages may stimulate a microenvironment into being favorable for oncogenesis [12, 44, 45] (see Figure 3.1). The integrated provirus and the expressed HIV-1 gene products may stimulate the surrounding macrophages to produce activating cytokines, and thus, lead to proliferation [44]. Macrophages are among the first cell types infected with HIV and tend to act as long-term reservoirs that are difficult to eradicate [52]. Through cytokines, chemokines, and growth factors, they function as regulatory cells that control the pace and intensity of disease progression [52]. The idea of "sequential neoplasia" may explain the absence of HIV-1 in B-cells and in a small subset of HIV-associated tumors [44]. Integrated macrophages and T-cells could lead to a complicated interaction with B-cells via cell-cell signaling. The depletion of T-cells could create an

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absence of control over B-cell proliferation, leading to a population of B-cells that are at increased risk of random somatic mutations that develop into transformation and outgrowth of B-cell clones. The sequential model predicts that early forms of integrated, polyclonal macrophages, will initiate an environment, producing cytokines and growth factors, which will aid in the transformation of the ultimately monoclonal B-cells. In time, polyclonal B-cell lymphomas may develop dominant clones that eventually evolve into monoclonal B-cell cancers [45]. Any preexistent B-cell mutation or coinfection, would most likely further aid in the process of transformation. The sequential neoplastic model helps to explain why an initial viral infection may lead to many different examples of AIDS-associated lymphoma.

CONCLUSIONS

An increased risk of H-NHL arises from the complicated interconnection of immunosuppression and inflammatory activation. The idea that HIV itself plays a role in the development of malignancy should not be ignored. Evidence for non-random HIV integration, supports the idea that the integration process itself may have a function other than solely for replication. Macrophages are important reservoirs of HIV, and are essential to an immune system's integrity. The tumor microenvironment is a delicate balance of cell signals and regulation. HIV could conceivably contribute to neoplastic development though impairment of cell-mediated immunity and dysregulation of cytokine loops. The constant state of activation demonstrated by an immune system infected with HIV is an example of the positive feedback loop predicted by neoplastic models and other hypotheses of the direct effects of HIV in malignancy. A better understanding of how HIV-1 fits into the spectrum of tumor viruses needs to be defined.

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Table 3.1: Features of HIV-associated Lymphoma

H-NHL	Cells	Clonality	Characteristics	Co-infection	Location
Systemic	B-cells	Mono & polyclonal	Lack genetic mutations associated with most malignancies (p53, Ras, RB1); Burkitt's and diffuse large-cell-lymphoma: Express BCL6, and c-myc translocations	Typically not associated with oncoviruses	80% Extranodal Involvement
PCNSL	B-cells	Mostly monoclonal	Majority large cell lymphomas lacking *c-myc* translocations; low CD4 cell counts	Most EBV-associated	CNS
PEL	B-cells	Mono & polyclonal	Lack B-cell antigens (CD19, and/or CD20) or *c-myc* translocations; CD45, MUM1, CD38, & Syndecan-1; low CD4 cell counts	HHV8 100% EBV 70%	Body cavities (Pleural, Peritoneal, Pericardial, etc.)

H-NHL = HIV-associated Non-Hodgkin's Lymphoma; Systemic=H-NHL not localized to PCNSL or body cavity (PEL); PCNSL= Primary Central Nervous System Lymphoma; PEL = Primary Effusion Lymphoma

Table 3.1: A quick reference guide to some of the features of the HIV-associated lymphomas discussed in this review. All information given on the cell types, clonality, detailed characteristics, co-infection likelihood, and location in the body are referenced in the body text of this review.

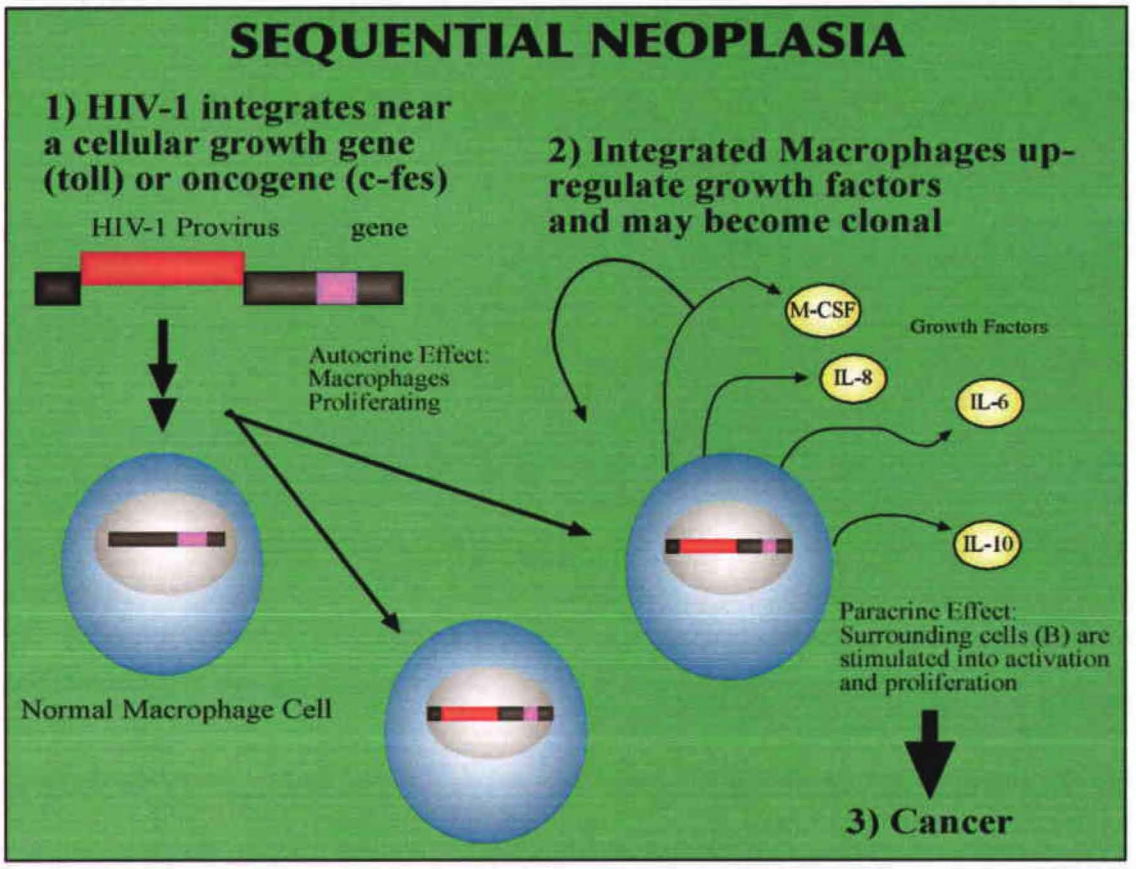

Figure 3.1: The Sequential Neoplasia Model. This model helps to explain how HIV-1 infected macrophages may stimulate a microenvironment into being favorable for oncogenesis. The integrated provirus and the expressed HIV-1 gene products may stimulate the surrounding macrophages to produce activating cytokines, and thus, lead to proliferation of B-cells and most HIV-associated tumors.

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CHAPTER 4

DISCORDANT PLASMA AND CEREBRAL SPINAL FLUID CYTOKINES/CHEMOKINES IN RELATION TO HIV-ASSOCIATED DEMENTIA

[1,2]Deirdre Killebrew, [1]David Troelstrup, [1,3]Victor Valcour, [1]Andrew Williams, [1]Joleen Aguon, [1]Dahlia Sapalo, [1,4]Cecilia Shikuma, [1]Silvia Ratto-Kim, [1,2,4,5]Bruce Shiramizu

[1]Hawaii AIDS Clinical Research Program; [2]Retrovirology Research Laboratory; [3]Dept. of Geriatrics; [4]Dept. of Internal Medicine; [5]Dept. of Pediatrics; University of Hawaii John A. Burns School of Medicine, 3675 Kilauea Ave., Young Bldg., 5th Floor, Honolulu, HI 96816

Correspondence: Deirdre Killebrew, 3675 Kilauea Ave., Young Bldg., 5th Floor, Honolulu, HI 96816; Tel: 808-737-2751; Fax: 808-735-7047; email dak@hawaii.edu

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ABSTRACT

Monocytes and macrophages serve as HIV-1 reservoirs and may indirectly lead to HIV-associated dementia via neurotoxic cytokine/chemokine production. It remains unknown if peripheral monocytes and macrophages are responsible for the presence of circulating and cerebral spinal fluid cytokine/chemokine. The purpose of this evaluation was to determine the relationship between inflammatory and chemoattractant cytokine/chemokine in the periphery and the CNS among individuals with HIV-associated dementia and normal cognition. To accomplish this, we utilized specimens from the *Hawaii Aging with HIV Cohort* to assay plasma, cerebral spinal fluid, and cultured peripheral monocyte and macrophage supernatant cytokine/chemokines from individuals with HIV-associated dementia and normal cognition by ELISA, relative real-time PCR, and protein macroarrays. To further characterize the activated cells that may be responsible for HIV-associated dementia, inverse-PCR was used to identify sites of viral integration. Different mediators of inflammation, and chemoattraction from monocyte and macrophage supernatants, plasma, and cerebral spinal fluid were identified in HIV-associated dementia versus normal cognition. This data suggests unique pathways leading to cytokine/chemokine release in the periphery versus the brain region. This may have implications in delineating a cause and effect in HIV-associated dementia pathogenesis.

INTRODUCTION

HIV-associated dementia (HAD) continues to cause morbidity in the highly active antiretroviral (HAART) era [1-4]. However, the etiology and pathogenesis of HAD remains unclear. The immune system and HAART are generally able to control the underlying retroviral infection in plasma among patients who are compliant to the complicated regimens. However, there exist viral reservoirs and mechanisms whereby HIV-1 persists in cellular compartments, which may ultimately play a role in the pathogenesis of HAD. Through continued macrophage activation, microglia and CNS perivascular cells have the potential to produce inflammatory, and chemoattractant-related chemokines/cytokines (C/C), which may be important to causing HAD [5]. Under normal conditions, C/C from activated cells can be beneficial to neurons [6], however in a different environmental milieu, these secretory products could be directly or indirectly neurotoxic through related neuropathogenic pathways leading to HAD [6-9].

Presence of macrophage activation, macrophage secretory products, and abundance of macrophages appear to corellate better with HAD than HIV-1 RNA levels (Reviewed in[10]). HAD is most likely the result of viral toxins, and activated monocytes and macrophages (M/MΦ) and astrocyte secretory products, inflicting damage upon neurons.

Monocytes/macrophages expressing the activated markers, $CD16^{+}$and $CD69^{+}$, appear to be increased in subjects with HAD compared to individuals without HAD and HIV-1 seronegative subjects [11]. In animal models, acute SIV infection is associated with increased circulating $CD16^{+}$ monocytes and $CD16^{+}$ perivascular macrophages in the brain [12, 13]. Monocytes/macrophages, including those with $CD14^{+}/CD16^{+}$ phenotype,

originate in the bone marrow and upon activation display greater phagocytocis, upregulated cytokine and chemokine secretion, and higher MHC II expression [14]. Monocytes/macrophages function as immunoregulatory cells by releasing C/C, in response to HIV or HIV products, including mediators of inflammation and chemoattraction [2, 15-19]. These peripheral mediators may cross into the CNS and influence the central environment, although, it is unclear whether the levels detected in the cerebral spinal fluid (CSF) reflect circulating levels [20, 21]. Thus, the importance of any differences in the C/C levels in the periphery and CSF is not known, but may reflect a complex interaction of M/MΦ mediators involved in the neuropathogenesis of dementia.

Macrophages and resident microglia play a role in HIV-1 persistence [22-24]. However, given that the overall number of M/MΦ in the CNS is relatively low before AIDS and HAD develop, how exactly the M/MΦ HIV-1 infectivity rate relates to HAD is unclear [15]. In general, the CNS region is a sequestered compartment within the brain with a specific population of cells. However, blood monocytes have been found to traffic into the brain under homeostatic conditions (Reviewed in[15]). As a correllate of HAD development, an increase in activated M/MΦ trafficing into the CNS coincides with the detection of abundant pro-inflammatory, chemotactic, and chemoattractant cytokines and chemokines (Reviewed in[10]). These C/C, through a positive-feedback loop, recruit additional M/MΦ and release potentially neurotoxic substances in the CNS.

To understand the link between HAD and C/C secreted by M/MΦ, the current study was designed to examine C/C levels in plasma, CSF, and supernatants of isolated $CD14^+/CD16^+$ M/MΦ from patients with HAD and normal cognition (NC). The

objective was to explore levels of inflammatory or chemoattractant mediators from activated macrophages and their potential relationship to HAD. The information would be helpful in identifying relationships of the C/C, and thus eventually help discover potential treatment or preventative approaches in targeting cytokine-regulated pathways leading to HAD.

METHODS

Specimens and Patient Population

Specimens were obtained following informed consent from participants in the *Hawaii Aging with HIV Cohort*, a longitudinal prospective study designed to investigate the neuroepidemiology of HIV in older individuals [25, 26]. Since October 2001, older (50+ years old) and younger (20-40 years old) HIV-1-seropositive participants in Hawaii have been recruited using broad community-based methods to maximize representation of the HIV-1-seropositive population. Exclusion criteria include major neurological or psychiatric illness, learning disability, major head injury, brain opportunistic infection, and primary language other than English. Neurological and neuropsychological tests were performed on each participant. Samples were taken an average of 2.5 months after cognitive assessment. Lumbar puncture was performed on all participants willing to undergo the procedure at entry and without contra-indication. Diagnoses of HAD and NC were determined by consensus conference of neurologists and neuropsychologists using the American Academy of Neurology 1991 criteria [27]. Participants were invited to come in for a blood draw based on convenience of local, and their category of either normal cognition or demented cognition.

Specimen Processing and Isolation of Activated Monocytes/Macrophages (M/MΦ)

Peripheral blood mononuclear cells (PBMC), plasma, and cerebral spinal fluid were obtained at study entry visit. Enrichment for monocytes was accomplished by density gradient centrifugation with Percoll (Amersham Biosciences, Piscataway, NJ). Cells were counted on a hemacytometer, and labeled with $CD16^+$ magnetic beads

(Miltenyi Biotec Inc., Auburn, CA). Labeled cells were then passed through the AutoMacs (Miltenyi Biotec Inc.) cell separation machine, and both $CD16^+$ and $CD16^-$ cell populations were harvested then plated separately at a density of $3x10^6$ per cm^2 and grown in a 37°C incubator, with 5% CO_2. Cells were washed 24 hours after plating to purify for adherent monocytes. Monocytes were cultured for 10 days to allow for differentiation into monocyte-derived macrophages (MDMs) in a media consisting of: 25% RPMI media (Sigma Inc., St. Louis, MO), Iscove's modified Dulvecco's medium (Irvine Scientific, Santa Ana, CA), 4mM L-glutamine (Sigma Inc.), 100units/mL penicillin and 100μg/mL streptomycin (Sigma Inc.), 15ng/mL MCSF (Sigma Inc.), and 1ng/mL IL3, and 10% Normal Human Serum (Sigma Inc.). The media also included MCSF and IL3 for the first 7 days then , on day 7 after plating, the media components were changed to exclude MCSF and IL3; the MDMs were grown for 3 more days without cytokines before sampling began, and without cytokines for the rest of the time the cells were in culture. On day 10 after plating, supernatant samples were taken from all cultured $CD16^+$ and $CD16^-$ MDMs, and frozen until ready for assaying. Samples were taken at the same time each day for 5 consecutive days. Digital photographs were taken of each patient's cultured sample on day 10 after plating.

Enzyme-Linked Immunosorbant Assay (ELISA)

Human Quantikine ELISA Kits (R&D Systems, Inc., Minneapolis, MN) were used according to manufacturer's instructions to assay for interleukin-6 (IL-6), IL-8, IL-10, TNFα, soluble FAS (sFAS), soluble tumor necrosis factor receptor I (sTNFRI), sTNFRII, and monocyte chemoattractant protein-1 (MCP-1). Frozen plasma and

supernatants were prepared for ELISA. There was less supernatant available for ELISA assays than plasma, and therefore less supernatant available to repeat samples that produced inconsistent results. Thus, we show a higher number of data points for plasma ELISA assays than supernatant assays.

RNA Isolation and Relative Real-Time PCR

After 15 days in culture, total RNA and DNA were extracted from MDMs following the Qiagen RNA "Micro" Extraction Kit, and the Qiagen DNA "Micro" Extraction Kit protocols (Qiagen Inc., Valencia, CA). RNA samples were amplified following the Roche microarray target amplification kit protocol (Roche Diagnostics Co., Indianapolis, IN); a protocol that results in amplified reverse-transcribed (RT) cDNA. IL-8 and MCP-1 primers and hybridization probes were designed by the Roche LightCycler Probe Design software, assuring that either one of the primers or the internal probes spanned the exon junction, to avoid amplification of genomic sequences. Both primers and probes were manufactured by Roche. The standard curves for both target genes were generated using increasing concentrations of lymphoblast cell line K562 (Roche) RT-cDNA. The G6PDH reference gene detection mix and standard curve template was supplied in the LightCycler G6PDH Housekeeping Gene Set (Roche). Real-time PCR was performed on the LightCycler (Roche) in glass capillary tubes, with a total volume of 20μl. Fast Start DNA Master Hybridization Probes Reaction Mix (Roche) (2μl/reaction) served as the source of buffer, Taq polymerase, dNTPs, and $MgCl_2$. Additional $MgCl_2$was added for a final concentration of 5 mM for all reactions. A final primer concentration of 0.5μM was used for each primer in all reactions (IL8F:

ATGACTTCCAAGCTGGC, IL8R: AGGGTTGTGGAGAAGT; MCP1F: GCTGTGATCTTCAAGACCATTGTG, MCP1R: GAACACTCACTCCACAACC). The final hybridization probe concentration of 0.3μM was used for each probe in all reactions (IL8-Red640: ATTTATCAAAGAACTGAGAGTGATTGAGAGT, IL8-Fluorescein: ACCACACTGCGCCAAC; MCP1-Red640: GCAGAAGTGGGTTCAGGATTCC, MCP1-Fluorescein: GGACCACCTGGACAAGCA). Unknown cDNAs (50ng) were assayed in duplicate with both target genes (IL8 and MCP1) and the reference gene (G6PDH) with the following cycling conditions: initial denaturation and Taq activation of 95° 10 minutes; 45 cycles of 95° 10 seconds, 56° 15 seconds, 72° 15 seconds; with a final cool down cycle of 40° for 30 seconds. An automated photometric detector in combination with the Roche LightCycler 4.0 software, monitors the fluorescent dye emissions, created when the 640 dyed and Fluorescein dyed probes hybridize to the target sequence. By determining where the unknown sample crosses the amplification threshold point on the standard curve, the initial relative concentration of target gene is extrapolated. The final normalized ratio is calculated by the program comparing each target to reference genes ratio to the normalizing calibrator ratio of 1; the calibrator control reaction corrects for variation between reactions.

Protein Macroarray

Using the Human Cytokine Antibody Array III (Ray Biotech, Norcross, GA) as per manufacturer's guidelines, a membrane protein array format is used to detect multiple chemokines/cytokines of interest. While cytokines are traditionally detected by ELISA,

the array format has the advantage of detecting many chemokines/cytokines simultaneously with increased sensitivity. As little as 4 pg/ml of MCP-1 can be detected using the protein array format compared to 40 pg/ml of MCP-1, which is generally the lower limit of detection with ELISA. Overall, the detection range for the multiple chemokines/cytokines is at least 100-fold greater compared with ELISA. The variability within the same membrane of duplicates for each chemokine/cytokine ranges from 0 to 10% in duplicated experiments in contrast to ELISA with variations on the order of 20%. The protein array membrane utilizes a biotinylated antibody with a labeled-streptavidin. Stored paired plasma and CSF were retrieved for analysis. Briefly, the protein membrane array was conditioned with the blocking buffer and incubated at room temperature followed by exposure to the specimen. Membranes were then washed with the washing buffer and prepared for detection. The primary biotin-conjugated antibody was then added followed by washing. Then diluted HRP-conjugated streptavidin was added to the membrane, incubated, and washed. The detection reaction was set up immediately following the antibody exposure. Detection buffers were added and then prepared for exposure using a Chemilumiimager (Roche, Indianapolis, IN). The relative intensities of the chemokine/cytokine are normalized using the internal controls provided on the same membranes. Both positive and negative controls are included on every membrane, which are also used to orient the array for analysis.

Flow Cytometry for Activated Monocyte Subsets

PBMCs isolated as described above were stained to demonstrate the phenotypic cell surface markers with the following monoclonal antibodies (BD Biosciences, Palo

Alto, CA): Isotype controls (anti-mouse IgG1/IgG2a/IgG2a), CD14PE, and CD14/CD16/anti-HLA-DR using a 3-color acquisition panel in a FACS Caliber flow cytometer (BD Biosciences). Approximately 75,000 total events were acquired and analyzed for each sample. Monocyte population was gated on a forward scatter vs. side scatter dot plot (FSC vs. SSC) with the gated population confirmed to be >95% $CD14^{+}$.

Inverse Polymerase Chain Reaction (IPCR) to Identify HIV Site-Specific Integration

Specimens screened for relative high HIV DNA copy underwent analysis for viral integration using Inverse-PCR (IPCR), as previously described [28]. Briefly, DNA (1 microgram) was digested with *Pst I* (Promega, Madison, WI) and religated with T4 DNA ligase (Promega). Genomic/HIV hybrid sequences were amplified using nested PCR with Expand High Fidelity Taq Polymerase system (Roche Biomedical) using primers: outer primers, LTR 1-115 5'-TTG TTG GCT TCT TCT AAC TTC TCT GG-3' and *gag* 1-H 5'-CTT TAA ATG CAT GGG TAA AAG TAG TRG-3'(R=A/G); inner primers, LTR 2:-116 5'-TGG TAC TAR CTT GWA GCA CCA TCC A-3' (W=A/T) and *gag* 2- J 5'-TGA TAC CCA TGT TTT CAG CAT TAT CAG-3'; and cycling conditions: 94° 1 minute, 10 cycles of 94° 15 seconds, 62° 30 seconds, 68° 3 minutes, 35 cycles of 94° 15 seconds, 57° 30 seconds, 68° 3 minutes with a final elongation of 68° 8 minutes for the outer primers; and 94° 1 minutes, 35 cycles of 94° 15 seconds, 56° 30 seconds, 68° 3 minutes with a final elongation of 68° 8 minutes for the inner primers. Amplification was performed using GeneAmp System 9700 thermalcycler (Perkin Elmer, Emeryville,

CA). PCR products were cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA), screened with inner IPCR primers 116/J, and sequenced.

Statistical Analysis

Statistical analysis was performed using Student t-test, and Mann-Whitney Rank Sum Test to calculate the p-values. In addition to looking at each individual C/C, the analysis of the macroarray data was expanded to include the best 2 and 3 variable models to determine best predictor of HAD. All C/C were ranked according to R^2 variance levels, however, only those with p-values of 0.05 or less, are presented, and were not adjusted for multiple comparisons.

RESULTS

Plasma and PBMCs for ELISAs were obtained from 14 individuals with HAD and 15 individuals with normal cognition. Supernatants were obtained from the $CD14^+/CD16^+$ cells in culture from specimens in which an adequate number of cells was obtained (approximately 13% of the specimens). There were 23 matched CSF and plasma specimens used in the protein array (macroarray) assay. Participants were primarily Caucassian males with an averge of 14.1 years of education (Table 4.1).

Plasma and supernatant MCP-1 and IL-8 levels were elevated in individuals with HAD compared to individuals with normal cognition (NC) ($p<0.05$) (Figures 4.1 and 4.2){MCP-1 supernatant n= 4 NC, 6 HAD}{IL8 supernatant and plasma n=10 NC, 11 HAD}. From a subset of individuals within the cohort, selected at random, activated macrophages were assessed by $CD14^+/CD16^+$ markers. HIV-seronegative individuals and non-demented HIV-1-infected individuals typically have <11% $CD14^+/C16^+$ in their circulation, therefore we analyzed the data using two groups [11]. One group consisted of those with <11% $CD14^+CD16^+$ {MCP-1 n=4; IL-8 n=7} and the other group were those with ≥11% $CD14^+/CD16^+$ {MCP-1 n=6; IL-8 n=12}. Supernatant MCP1 levels were elevated in individuals with HAD versus NC ($p<0.05$), as well as in the group with higher activated cells ($p=0.026$) (Figures 2 and 3). IL-8 levels were also elevated in individuals who had >11% $CD14^+/CD16^+$ markers ($p=0.025$) (Figure 4.3). No other correlations between cytokine/chemokine levels and HAD in the plasma and/or supernatants (IL-6, TNFα, sFAS, sTNFRI, sTNFRII) were identified using Student t-test or Mann-Whitney Rank Sum Test. All ELISA protein levels were also analyzed by logistic regression as single variables, as shown in Table 4.2. Cytokines were ranked

according to variance value (R^2) and presented in Table 4.2 if p-values were 0.05 or less. The C/C predictive values with respect to HAD are ranked respectively: $CD16^+$ supernatant IL-8, $CD16^+$ supernatant MCP-1, $CD16^-$ supernatant MCP-1, plasma IL-8, plasma FAS, plasma MCP-1, plasma TNFRII.

To further investigate C/C expression in relation to HAD, relative real-time PCR analysis was performed for the target genes IL-8 and MCP-1. Normalized ratios are a measure of the relative target gene (IL-8 and MCP-1) expression compared to the reference gene (G6PDH) expression for each participant sample. Some samples did not have a detectable amplification threshold for the target genes, even though the reference gene was detectable at normal levels. Because of the limited sample amount, we could only repeat the assay on these specimens using a similar concentration of starting template. Therefore, for the analysis, if the repeated samples were still not detectable by the target gene, but the housekeeping gene was in acceptable parameters, we considered these samples extremely low in target expression. The majority of the participant samples demonstrated lower IL-8 expression compared to the housekeeping, G6PDH, gene. NC samples (n=2/3) expressed IL-8 at a lower level than G6PDH. HAD samples (n=8) expressed IL-8 at a lower level than G6PDH in 6/8 samples. MCP1 was also lower in NC samples (n=2/3) than G6PDH. However, a majority (6/8) of the HAD samples (n=8) expressed higher MCP-1 levels. Because of the limited sample size (n=11), statistical analysis was not useful in identifying trends for future studies.

Expanding the analysis using the macroarray increased the number of C/C to test the plasma and CSF. The analysis of the 42 cytokines/chemokines (Table 4.3) detected by macroarray was performed individually for plasma and CSF specimens using single,

two, and three variables to identify the best combinations for predicting HAD compared to NC. The initial analysis using a single variable for plasma and CSF identified IL-4 and IL-10, respectively predicted HAD, Table 4.4. The best two and three-variable models predicting HAD from plasma and CSF can be viewed in Table 4.4.

Identification of HIV-1 integration sites was performed on 4 specimens with relatively high HIV-1 DNA copy per cell by IPCR [28]. From 2 of the 4 specimens, HIV-1 integration was mapped within the beta (4) integrin gene. These particular specimens were also found to have high IL8 levels, high MCP1 levels, and were associated with high $CD14^{+}/CD16^{+}$ activated M/MΦ.

DISCUSSION

Chemokines/cytokines (C/C) associated with HAD are secreted by M/MΦ and microglia in the CNS, and are sites of productive HIV-1 infection in the brain compartment [29-32]. The "Trojan Horse" hypothesis theorizes HIV-1 enters the CNS via M/MΦ crossing the blood-brain-barier (BBB) [18, 33]. Thus, M/MΦ likely contribute directly and indirectly to HAD by introducing virus to the CNS and through production of inflammatory cytokines.

HIV-1 cleaved envelope glycoprotein products, gp120 and gp41, have neurotoxic effects in the CNS. While Gp41 is directly toxic to neurons [34], gp120 indirectly results in damage through macrophages and microglia and the production of inflammatory cytokines and arachidonic acid [2]. It has been proposed that activated macrophages and microglia, regardless of HIV-1 infection, are the source of multiple infammatory C/C, and that these products lead to neuronal damage [9, 16, 18, 31, 35, 36]. Activated M/MΦ in the CNS can potentially act in a positive feedback loop, where they produce C/C, serving as messengers between other cells to further produce more C/C that are toxic to neurons [18]. The level of C/C production in the CNS, has been correlated to the severity of neurological symptoms [8].

Pro- and anti-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-10, and soluble TNF receptor II (sTNFR) are elevated in inflammatory conditions [37, 38]. Other factors such as FAS, FAS-Ligand, and MCP1, have been associated with CNS pathology [37, 39, 40]. Expression of various proinflammatory mediators in the CSF and plasma has been studied in relation to HIV-1 and HAD [21, 41-48]. In the current study, increased MCP-1 and IL-8 levels were

associated with HAD. Furthermore, MCP-1 levels in the $CD14^+/CD16^+$ supernatants and mRNA from patients with HAD were increased, as were plasma IL-8, IL-8 in $CD14^+/CD16^+$ supernatants, and plasma FAS, and plasma TNFRII.

MCP-1 and IL-8 are cellular adhesion products and chemoattractant proteins and may be particularly important to trafficking to the CNS. MCP-1 is a beta-chemokine, known for its chemoattractant ability and the ability to activate monocytes [49]. MCP-1 is upregulated in astrocyte and microglia cultures when exposed to HIV-1 tat protein [49, 50]. In a recent genetic study of HAD, polymorphisms in MCP-1 protein produced a five-fold increase in the risk of developing HIV-associated cognitive dysfunction [51]. As a paracrine signaling cytokine, MCP-1 may act peripherally as well as centrally. In a macaque model, the effect of SIV proteins increasing MCP-1 was enhanced by IL-4 and dependent on the tropic-strain of the virus, i.e. macrophage-tropic [52]. In these macaques, IL-4 increased replication efficiency in X4 SIV virus and not in R5 SIV virus. The enhanced X4 rplication in macrophages, in turn, upregulated secretion of MCP-1 [52].

We hypothesize that MCP-1 and IL-8 can contribute to HAD through recruitment of more infected and/or activated M/MΦ, which is enhanced by IL-4. In turn, the activated M/MΦ act as viral shuttles and produce more neuronal toxins [18]. While IL-8 is generally a proinflammatory cytokine produced in response to inflammation, it is also known to trigger monocytes to adhere to vascular endothelium [53, 54]. IL-8 is produced by MDMs, microglia, and astrocytes in the brain and M/MΦ are known to upregulate IL-8 production in the presence of HIV tat protein [55] . IL-8 output can potentially to lead to a positive feedback loop, because IL-8 stimulates HIV-1 replication [55]. In rats,

increased levels of IL-8 inhibits long-term potentiation in the hippocampus, where most learning and memory take place [56].

It is possible that a cascade of events takes place leading to an accumulation of perivascular activated M/MΦ, which has been described in relation to HAD [57, 58]. One of the pathways leading to accumulation of perivascular macrophages is dependent on abnormal circulating monocyte subsets [5, 11, 59]. In a model we propose from our findings, circulating activated macrophages are affected by IL-8 and MCP-1 in a feedback loop, which further stimulates CNS vascular endothelial cells (VEC) to produce more IL-8 and MCP-1 (Figure 4.4). The VEC MCP-1 binds to circulating M/MΦ CCR2 receptor, allowing for migratory influx of M/MΦ into the CNS. This recruitment of more M/MΦ to the perivascular region may be a source of activated macrophages and/or virus into the CNS, contributing further to the pathogenesis of HAD [57, 60].

Overall, the best predictors of HAD, as depicted in the protein array results, have pro-inflammatory activity. Although IL-4 has historically been considered anti-inflammatory, alternative activation of macrophages has been established with IL-4/IL-13 signaling pathways, independent of the "classical", IFN-γ, macrophage activation cascade [61-63]. Alternate activation cascades are still being elucidated. However, the local presence of cytokines, that range in presumed function, such as IL-10, TGFβ and M-CSF, have been reported as part of the environment that alternate activation takes place [62]. One of the more notable pro-inflammatory cytokines identified in the array, IFN-γ, is known as a powerful activator of macrophages, and ultimately empowers cytotoxicity [63]. Finding pro-inflammatory activators and chemoattractants as best predictors of

HAD in both the plasma and CSF lends credence to the theory that the pathogenesis of HAD is fueled by the neurotoxic environment, rather than the viral assault itself.

To further characterize the activated cells that may be responsible for HAD, IPCR was used to identify sites of viral integration. Others have recently reported success in identifying site-specific HIV-1 integration near or within transcriptional units, including HAD cases [64-68]. Similar findings demonstrating integration into transcriptional units have also been found [65, 66, 68]. HIV-1-integration has been found within genes that encode for factors associated with signal transduction, apoptosis, and transcription regulation, as well as proximal to genes that encode for receptor-associated, signal transduction-associated, transcription-associated, and translation-associated proteins. From the two specimens in which HIV-1 integration was mapped within the beta (4) integrin gene (ITGβ4), we also demonstrated that the other parameters from the same specimens consistently demonstrated high IL-8 levels, high MCP-1 levels, and were associated with high $CD14^+/CD16^+$ activated M/MΦ. While not previously described in HAD, ITGβ4 belongs to the laminin family of receptor proteins involved in adhesion and motility of cells [69, 70]. The significance of viral integration within ITGβ4 remains unclear but if increased expression of the protein is found in relation to viral integration, then this may be a significant finding in understanding migration patterns of activated M/MΦ.

We present here a study exploring C/C from blood plasma, cultured cell supernatants and CSF in relation to HAD. The limitations of this study include small sample size, and incomplete data on individual HAART regimens. Using this exploratory

experimental design, the protein array, relative real-time PCR, and integration analyses provide encouraging results that can be utilized in the future.

The pathogenensis of HAD is not completely clear, but the proposed pathways involve: monocytes trafficking into the perivascular region, secretion of neurotoxic substances, and immune activation, may all be possible key events [5, 19, 31, 71]. Our findings support the theory that C/C, such as MCP-1, IL-4, and IL-8, possibly acting synergistically with activated M/MΦ, may be important in the pathogenesis of HAD. While it remains to be proven that the activated peripheral monocytes are precursors of CNS perivascular macrophages, selective expansion of cells with these markers have been noted in the perivascular space of SIV-infected macaques and humans [57, 72]. Additional studies show activated cells bearing $CD14^{+}/CD16^{+}$ markers infected with HIV-1 in brain specimens from patients with HIV-1 encephalopathy [57]. The number of activated M/MΦ found in the CNS are a better predictor of HAD than viral load [23]. While the factors that drive M/MΦ to the perivascular region are unclear, we hypothesize that MCP-1 and IL-8, act together with IL-4 as chemoattractant proteins released by the activated cells, trafficking additional M/MΦ to the CNS. The different mediators of inflammation, and chemoattraction from M/MΦ supernatants, plasma, and CSF identified in this study, all indicate the formation of a closed feedback loop, where the signals for inflammation seem to be amplified above the suppressors.

By focusing on activated macrophages, in a variety of experiments, we explored the relationship of the source of many of the proinflammatory C/C to HAD. We present multiple methods used to explore the activated nature of M/MΦ, including a suprising HIV-1 integration pattern. Many of our findings can be confirmed by current literature,

however, the role of the peripheral macrophage in HAD is still unclear, and therefore, any corraborating evidence is useful in elucidation.

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Table 4.1. Demographics of Participants

N		Normal Cognition	HAD
		Mean (SD)	
41	**Age (years)**	45.2 (+/-12.01)	51.5 (+/- 7.84)
	Years of Education	13.7 (+/- 2.36)	14.4 (+/- 1.90)
	CD4 Nadir	325.2 (+/- 246.45)	206.2 (+/- 160.70)
	Gender		
	Female	18.7%	4.3%
	Male	81.3%	95.7%
	Ethnicity		
	Caucasian	56.3%	60.9%
	Asian/Pacific Islander	18.7%	21.7%
	Other	25.0%	17.4%
	Viral Prevalence		
	Undetectable	56.3%	39.1%
	Patient Treatment		
	HAART	81.3%	82.6%

Table 4.2. Ranking of ELISA cytokines by predictive capacity with respect to HAD

Variable	N (NC/HAD)	X^2	p	R^2
CD16+ supernatant IL8[A]	19 (10/9)	6.252	0.01	0.75
CD16+ supernatant MCP1[A]	8 (3/5)	7.751	0.01	0.73
CD16- Supernatant MCP1	9 (4/5)	5.891	0.02	0.65
Plasma IL8	20 (10/10)	6.615	0.01	0.65
Plasma FAS	14 (6/8)	6.000	0.01	0.47
Plasma MCP1	15 (6/9)	4.083	0.04	0.28
Plasma TNFRII	15 (6/9)	3.753	0.05	0.24

[A]Due to the small number of samples, the distribution of data points may or may not be skewed; therefore, it is unclear as to if the large R^2 value is a true reflection of the data.

Table 4.3. Cytokines & Chemokines Assayed by Protein Array

1. Angiogenin	15. IL4	29. MDC (monocyte-derived chemokine
2. EGF	16. IL5	30. MIG (monokine induced by IFNγ)
3. ENA-78 (CXCL5)	17. IL6	31. MIP1_ (macrophage inflammatory protein)
4. GCSF	18. IL7	32. Oncostatin M
5. GMCSF	19. IL8	33. PDGFB
6. GRO (growth-regulated	20. IL10	34. RANTES
7. GRO	21. IL12	35. SCF (stem cell factor)
8. I309 (CCR-8R ligand)	22. IL13	36. SDF1 (stromal-derived factor)
9. IFNγ	23. IL15	37. TARC (thymus/activation-regulated
10. IGFI	24. Leptin	38. TGFβ1
11. IL1b	25. MCP1	39. Thrombopoietin
12. IL1α	26. MCP2	40. TNFα
13. IL2	27. MCP3	41. TNFβ
14. IL3	28. MCSF	42. VEGF

Table 4.4. Best Predictors of HAD vs. Normal Cognition Comparing Plasma and CSF Mediators Using Protein MacroArray

Predictor Models for Plasma

Single Variable	ChiSq	*p*	R^2
IL4	3.87	0.05	0.24
Two Variables	**ChiSq**	***p***	**R^2**
IL4 IFNγ	6.27	0.04	0.36
IL-α IL4	6.26	0.04	0.36
ENA78 **IL4**	**6.12**	**0.05**	**0.35**
Three Variables	**ChiSq**	***p***	**R^2**
ENA78 **IL1α** IL4	**8.88**	**0.03**	**0.48**
ENA78 **IL4** IFNγ	**8.86**	**0.03**	**0.48**
ENA78 **IL1β** **IL4**	8.29	0.04	0.45
I309 **IL1α** IL4	7.71	0.05	0.51
IL1α IL4 **MDC**	7.62	0.05	0.45

Predictor Models for CSF

Single Variable	ChiSq	*p*	R^2
IL10	5.23	0.02	0.20
MCP1[A]	**3.10**	**0.05**	**0.72**
Two Variables	**ChiSq**	***p***	**R^2**
IL10 TARC	7.99	0.02	0.29
IL1α **TARC**	**6.66**	**0.04**	**0.26**
GRO IL10	6.60	0.04	0.24
IL10 **MCP2**	**6.31**	**0.04**	**0.23**
Three Variables	**ChiSq**	***p***	**R^2**
GRO **IL10** **TARC**	**9.88**	**0.02**	**0.35**
IL10 **MCP2** **TARC**	**9.62**	**0.02**	**0.34**
IL10 **MCSF** **TARC**	**9.15**	**0.03**	**0.34**
IL10 **RANTES** **TARC**	**8.72**	**0.03**	**0.31**
IL8 **IL10** **MCP2**	8.60	0.04	0.32
IL10 **MCP2** **MIP1γ**	8.58	0.04	0.31
IL10 **TARC** **TNFα**	**8.49**	**0.04**	**0.31**
IL10 **MIP1γ** **RANTES**	**8.43**	**0.04**	**0.31**
IL10 **MCP2** Ang	**8.33**	**0.04**	**0.32**
IL1β **IL10** **TARC**	**8.30**	**0.04**	**0.31**

[A]Due to the small number of samples, the distribution of data points may or may not be skewed; therefore, it is unclear as to if the values are a true reflection of the data.

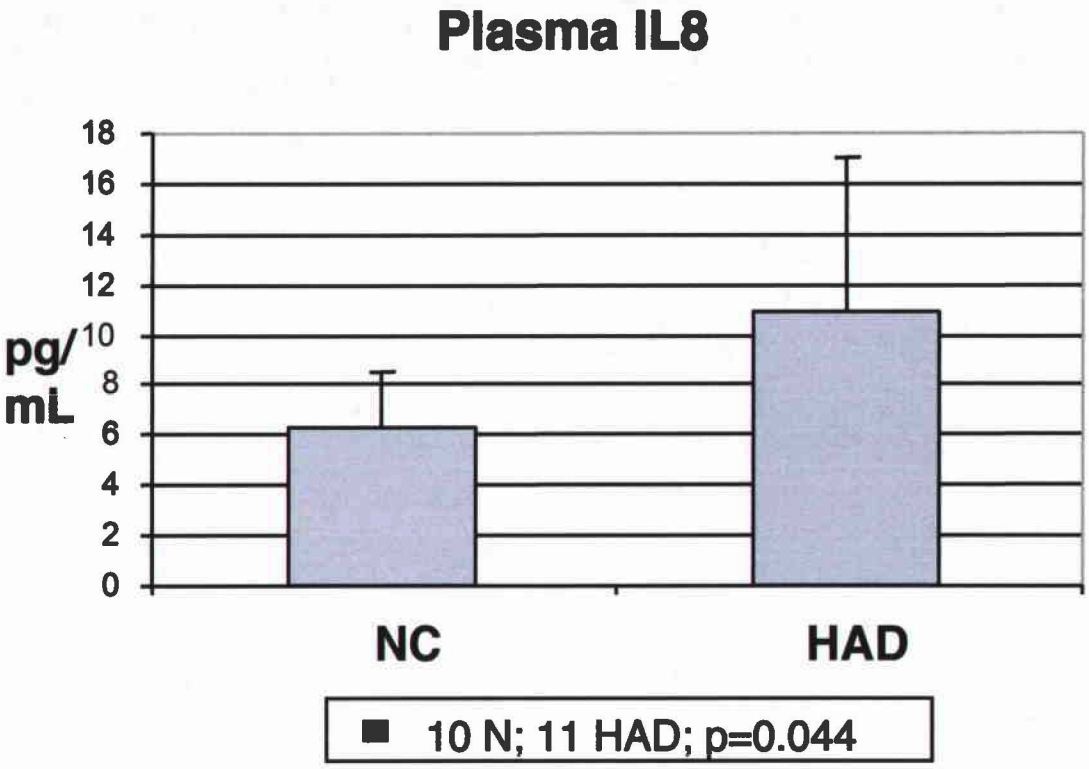

Figure 4.1. Plasma IL8 Levels by ELISA. Significant plasma IL8 levels from individuals with normal cognition (N) compared to HIV-associated dementia (HAD), p=0.044.

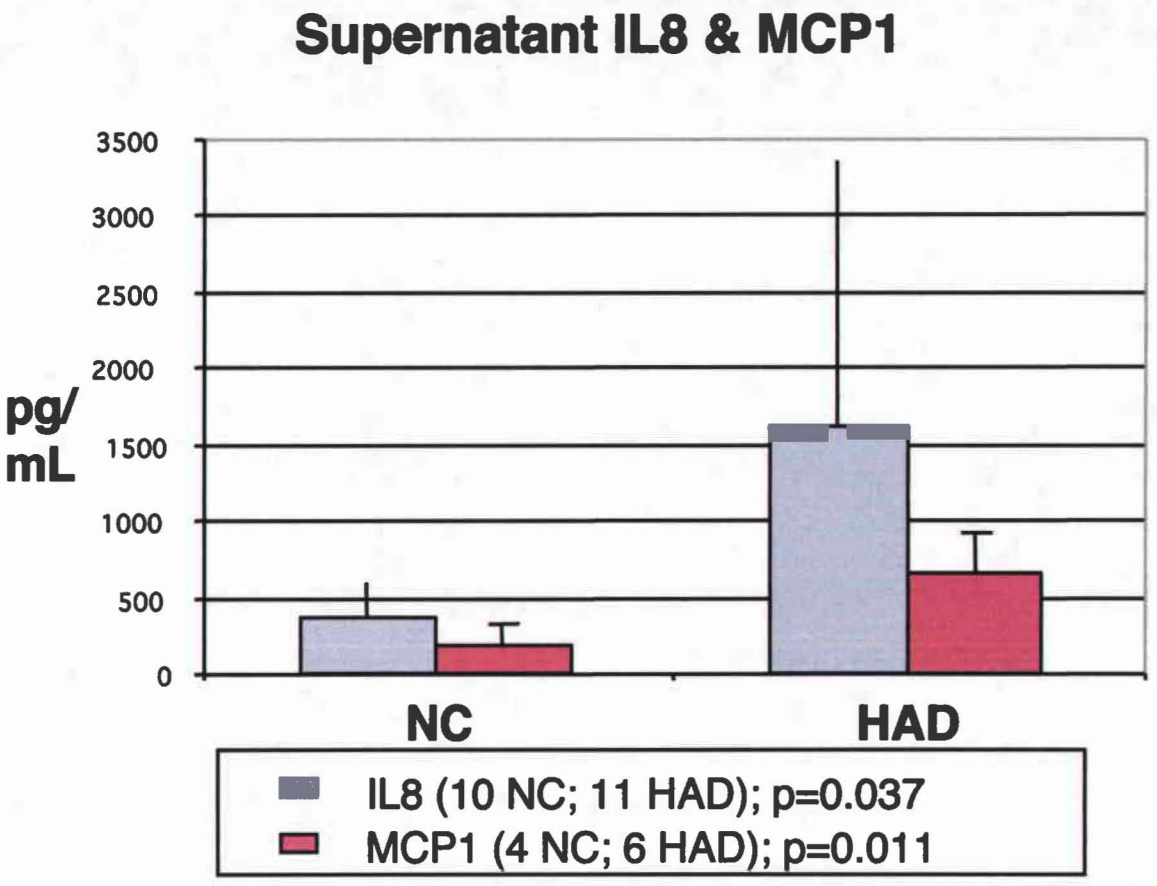

Figure 4.2. Supernatant IL8 and MCP1 Levels by ELISA. Supernatants isolated from isolated cultured $CD14^+/CD16^+$ cells (activated M/MΦ) from individuals with normal cognition (N) compared to HIV-associated dementia (HAD) showing significant differences with IL8 (p=0.037) and MCP1 (p=0.011)

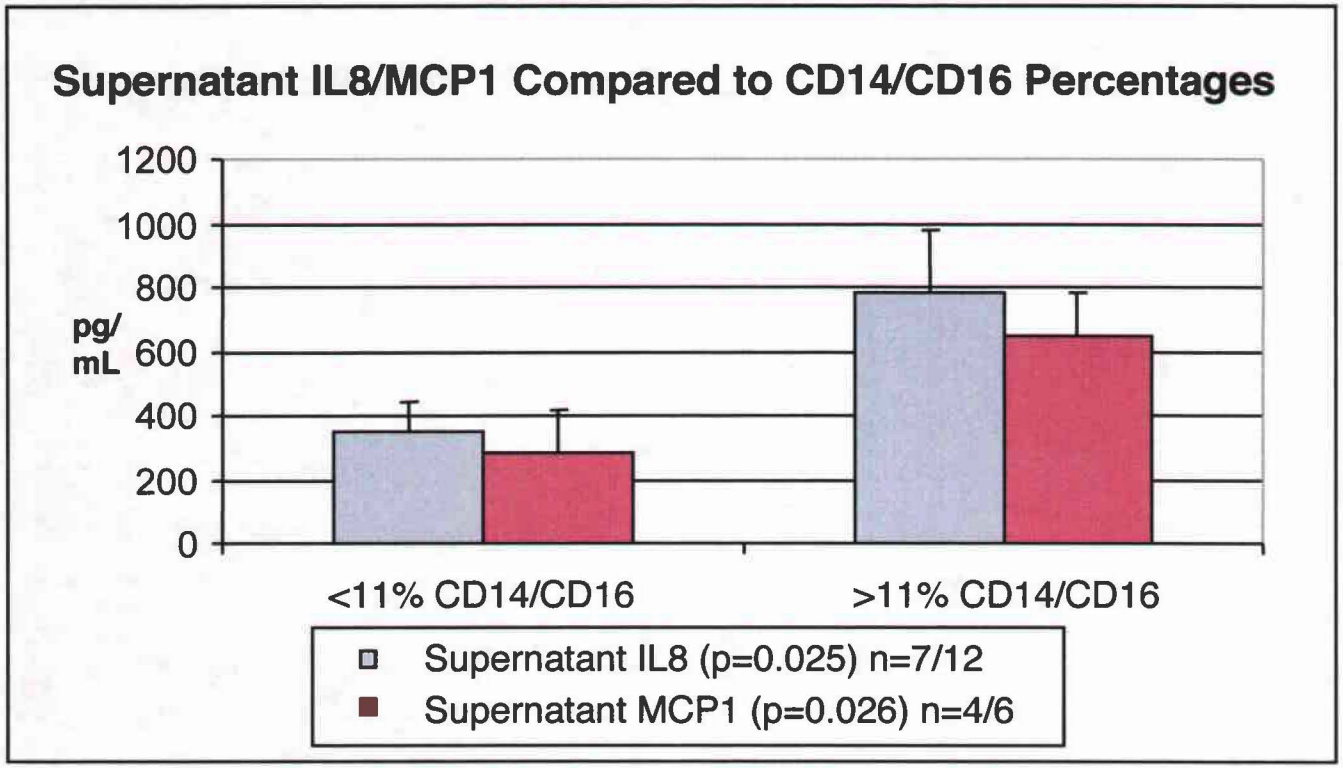

Figure 4.3. Comparison of IL8 and MCP1, sorted by activation level of M/MΦ. Dividing individuals with $CD14^+/CD16^+$ less than (<) or greater/equal to (≥) 11%, supernatant levels of IL8 and MCP1 are increased in individuals with higher percentage of $CD14^+/CD16^+$ cells (p=0.025 and 0.026, respectively).

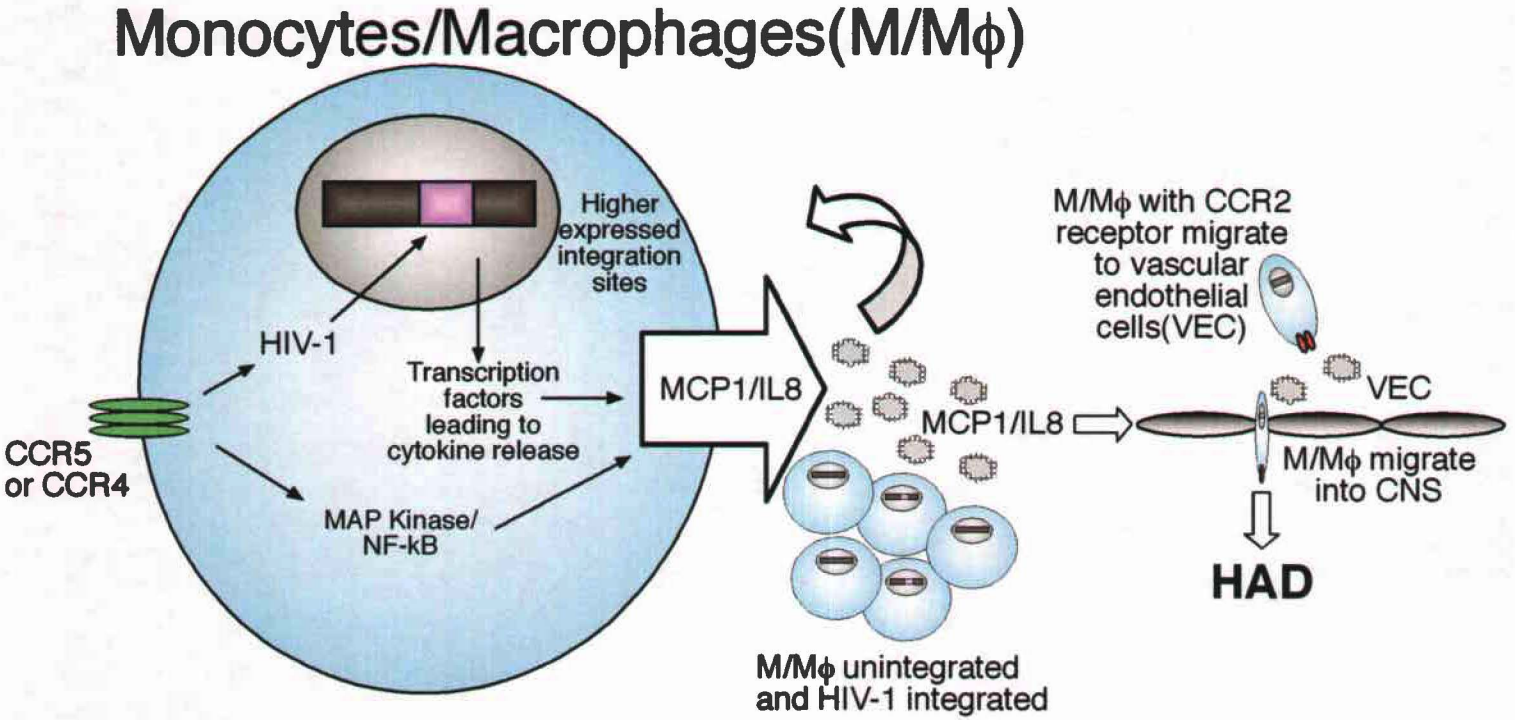

Figure 4.4. Proposed HAD Model Involving Cellular Adhesion Products and Activated M/MΦ. Circulating activated macrophages are affected by IL8 and MCP1, which are produced by M/MΦ and can also be affected through a feedback loop. Other sources of IL8 and MCP1 by CNS vascular endothelial cells further recruit M/MΦ to CNS perivascular regions setting up pathways leading to HAD.

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CHAPTER 5

UNPUBLISHED EXPERIMENTS

MACROPHAGES, CYTOKINES, AND INTEGRATION

Relevant Background

An increased risk of H-NHL arises from the complicated interconnection of immunosuppression and inflammatory activation. The idea that HIV-1 itself plays a role in the development of malignancy should not be ignored. Evidence for non-random HIV-1 integration, supports the idea that the integration process itself may have a function other than solely for replication [1-6]. Macrophages are reservoirs of HIV-1, and are essential to an immune system's integrity. The tumor microenvironment is a delicate balance of cell signaling and regulation. HIV-1 could conceivably contribute to neoplastic development though impairment of cell-mediated immunity and dysregulation of cytokine loops. The constant state of activation demonstrated by an immune system infected with HIV-1 is an example of the positive feedback loop predicted by neoplastic models and other hypotheses of the direct effects of HIV in malignancy [1]. The unique cases described with viral integration upstream from the *c-fes/fps* oncogene are consistent with a theory that some cases of NHL may involve non-random HIV-1 integration [2-4, 6]. Using two independent methods, our studies showed HIV-1 integration within an important immune functioning gene in the macrophage genome, as well as near a transcriptionally important gene in human tumor specimens. However rare, the ability to show integration events within tumor samples is of utmost importance.

Macrophages, Cytokines, and Integration Results

Our linker-primer PCR protocol, resulted in the discovery of an integrated HIV-1 sequence, within the coding region of Toll-like receptor 1 (figure 5.1), after residue 612 of the amino acid sequence (figure 5.2). This region is believed to be within a cytoplasmic domain [7], 12 residues upstream of the TIR-domain encoding the MyD88 binding region [8-10]. Found within 300kb, up or downstream of the integrated HIV-1 sequence on chromosome 4, are the known genes: Toll-like receptor 6, Toll-like receptor 10, Kruple-like factor 3, and Kelch-like 5.

In our IPCR tumor samples, the HIV-1 provirus was found integrated within an intron on chromosome 22, 200kb upstream of genes HSCBCIP1 and HMG17L1. Within 300kb up or downstream of the IPCR HIV-1 integration, the following known genes are encoded: 239AB, a functional sulfotransferase enzyme gene, and adiponutrin.

From two HIV-1-associated dementia patient specimens, with high levels of $CD14^{+}/CD16^{+}$ activated monocytes and macrophages, HIV-1 integration was mapped within introns 4 and 26, of the beta (4) integrin gene (ITGb4). Found within 300kb, up or downstream of the integrated HIV-1 sequence on chromosome 17, are the known genes: RECQ5, SEN54L, galactokinase, H3F3B, and CDK3.

Macrophages, Cytokines, and Integration Discussion

After taking a closer look at the HIV-1 integration within the Toll-like receptor 1 gene, there are several more points of interest worth discussion. In general, Toll receptors are vital to innate and adaptive immunity, and use a pathway similar to

interleukin-1 receptors [8, 10-12]. Toll receptors are associated with the downstream signaling of proinflammatory cytokines through the initial phosphorylation cascade of serine kinases [10, 12-15]. The intracellular TIR-domain binds to the adaptor molecule, MyD88, which ultimately leads to the nuclear localtization of NF-kB and thus the activation of multiple cytokines , including IL-1, IL-8 and B7.1 [8, 10, 11]. Toll genes contain highly conserved structural motifs expressed in microbial pathogens [16]. Toll receptor 1 (TLR1) is highly expressed in leukocytes [17]. The Toll-like family of receptors are considered to be one of the immune system's main anti-microbial defense tools, because of the broad spectrum of pathogen-associated molecular markers the TLRs recognize [8, 18, 19].

Toll receptors are type I transmembrane proteins, with an extra cellular leucine-rich repeats domain (LRR), and a cytoplasmic domain homologous to the cytoplasmic domain of the human interleukin IL-1 receptor [9]. Mutation experiments have proven that the TIR domain is vital for cytoplasmic signaling, and point mutations can affect the binding of the adaptor protein MyD88 [10, 20]. Our finding of the HIV-1 integration 12 amino acids N-terminus of the start of the TIR domain poses an interesting question: Would the transcription of the Toll gene be completely negated by this viral integration, or could the HIV-1 transcription sites continue and possibly enhance transcription? The issue is unclear in the literature. Different length transcripts resulting from splice variants or multiple transcription sites have been documented for TLR1 (www.ncbi.nlm.nih.gov/entrez/query). A chimaeric mouse CD4 extracellular domain and human TLR1 transmembrane and cytoplasmic domains, produced an active receptor able to cause the activation of NF-kB. This experiment also demonstrated that the

CD4/human TLR1 chimaeric construct, was able to induce the expression of IL-1, IL-8 and B7.1 in the presence of IFN-gamma [9]. However, more recent studies have found extracellular and cytoplasmic domains, other than TIR, to be just as essential for NF-kB downstream signaling [20, 21]. In TLR3, the cytoplasmic "linker" region, not homologous, but similar to our TLR1 HIV-1 integration region, was found to be vital for localization and downstream signaling [21]. Without knockout expression analysis of TLR1, it is hard to say, whether an HIV-1 integration would stop transcription, or possibly enhance it. However, the Vaccinia pox virus has been found to antagonize TLR1, and other Toll and IL-1 receptor, TIR domains [22]. It is conceivable that preventing expression of a fundamental innate and adaptive immune gene, such as TLR1, would be to the evolutionary advantage of HIV-1 viral propagation.

The linker-primer integrated sequence was also found to be within 100kb of Toll-like receptor 6 and 10. The TLR 6 gene has a very similar ontology to the TLR 1 gene, in that both function through defense and immunity protein activity, interleukin-1 activating receptor activity, and activation of NF-κB, as well as many other inflammatory and immune-related functions [8, 23-25]. TLR 10 has similarly been found to function through interleukin-1 activating receptor activity, and inflammatory and immune processes [18].

Within 300kb of the linker-primer integrated sequence: Kruple-like factor 3 lies upstream of the Toll genes, and Kelch-like 5 lies downstream of the Toll genes. Kruple and Kelch were originally found to be developmental body plan genes in *D. melanogaster*. In humans, Kruple is believed to be a negative transcription regulator of the Polymerase II gene [26]. The expressed protein of the Kelch gene, in humans, is

believed to bind actin, and have a role in actin cytoskeleton organization and biogenesis [27].

In our IPCR tumor samples, the HIV-1 provirus was found integrated within an intron, 200kb upstream of genes HSCBCIP1 (cAMP-binding protein complex interacting protein 1) and HMG17L1 (high mobility group, nonhistone, 17-like1), on chromosome 22. The HSCBCIP1gene is an alias of the protein DJBP, and involved in a transcription repression pathway that binds DNA through the recruitment of histone complexes [28]. HMG171 is a transcriptional activator that can bind to chromatin and bend DNA, promoting the assembly of proteins on specific target DNA [29]. The function of HMG17L1 has been proposed by several studies and includes: a nuclear signal to determine whether cells undergo apoptosis or necrosis [29], an extracellular signaling molecule targeting cytokine signaling targets [30], a potent factor secreted by mononuclear phagocytes that causes inflammation and protease activity [31], and an activator of p53 sequence-specific DNA binding [32].

Within 300kb upstream of the IPCR HIV-1 integration, the gene 239AB is encoded. 239AB is believed to play a role in central nervous system development and function [33]. Further downstream from the HSCBCIP1 and HMG171 genes, lies a functional sulfotransferase enzyme gene, believed to catalyze the sulfate conjugation of many hormones, and neurotransmitters specific to the brain region [34]. Even further downstream lies adiponutrin, encoding a protein for a triacylglycerol lipase that mediates triacylglycerol hydrolysis in adipocytes, and is most likely involved in the balance of energy usage and storage in adipocytes [35].

To further characterize the activated cells that may be responsible for HAD, IPCR was used to identify sites of viral integration. On chromosome 17, HIV-1 integration was mapped within introns 4 and 26, of the beta (4) integrin gene (ITGb4), from two patient specimens. We also demonstrated that the other parameters from the same patient specimens consistently demonstrated high IL-8 levels, high MCP-1 levels, and were associated with high $CD14^{+}/CD16^{+}$ activated monocytes and macrophages (M/MΦ). While not previously described in HIV-1-associated dementia (HAD), ITGb4 belongs to the laminin family of receptor proteins involved in adhesion and motility of cells [36, 37]. This gene encodes the integrin beta 4 subunit, which tends to associate with the alpha 6 subunit, and is believed to play a role in invasive carcinoma [38-40].

Heading upstream from ITGb4, the gene RECQ5 encodes for an ATP binding and DNA repair and helicase activity protein [41]. Also found within 300kb upstream of ITGb4, is the SEN54L gene, a subunit of the tRNA splicing endonuclease, which catalyzes the splicing and removal of introns, a vital process required for cell growth and division [42]. Downstream from ITGb4 lies the gene for galactokinase, an important enzyme for the metabolism of galactose. Gene mutations of galactokinase are associated with cataract formation [43]. Further downstream, the gene H3F3B encodes a nuclear protein, one of a pair of four core histones (H2A, H2B, H3, and H4) that form an octamer, around which roughly 146 bp of DNA is wrapped in repeating units, called nucleosomes [44]. Within 300kb of the ITGb4 integration site lies the gene CDK3, suggested to be a protein that can phosphorylate histone H1 and interacts with a type of cyclin; and therefore, may be involved in cell cycle control [45].

The HIV-1 provirus contains: trans-activators (such as *tat*), LTR binding sites for

cellular transcriptional control elements (such as NF-kB and Sp1), LTR enhancer sequences, and LTR regulatory regions that can have either a positive or negative regulatory effect on up or downstream genes [46]. Therefore, at this point we can only surmise, that the integration of HIV-1 could have a positive or negative regulatory effect on the genes within the range of influence. Many of the genes mentioned are immune or cell cycle functioning genes. In the activated environment of lymphoma and HIV-associated dementia (HAD), any influence over these genes would be significant. HIV integration, by definition, is a mutagen. Macrophages in an activated environment would still be a heterogenous group, however, their similarity lies in the fact that their activated functional response, would necessitate a pattern of activated expression, regardless of the route. A differential pattern of expression, would most likely equal different integration sites. Thus, a pattern of integration can still be forged, by taking a closer look at expression.

Others have recently reported success in identifying site-specific HIV-1 integration near or within transcriptional units, including HAD cases [5, 47-50]. Similar findings demonstrating integration into transcriptional units have also been found [5, 48, 50]. HIV-1-integration has been found within genes that encode for factors associated with signal transduction, apoptosis, and transcription regulation, as well as proximal to genes that encode for receptor-associated, signal transduction-associated, transcription-associated, and translation-associated proteins. As documented here, the sphere of influence that HIV-1 may have, includes an extremely large spectrum of genes. As HIV-1 is a known activator of macrophages, it is reasonable to conclude that integration could

have a severe impact on the type, and variety of functional responses given, in an environment where immune activation goes unregulated.

The pathogenensis of HAD is not completely clear, but the proposed pathways involve: monocytes trafficking into the perivascular region, secretion of neurotoxic substances, and immune activation, of which all are likely key events [51-54]. Our findings support the theory that chemokines and cytokines, such as MCP-1, IL-4, and IL-8, possibly acting synergistically with activated M/MΦ, may be important in the development of HAD.

A population of tissue macrophages will display a functional phenotypic pattern, however, each individual macrophage will display a slightly unique pattern depending on a variety of factors, including activation progression [55]. In the literature, there is a small amount of information suggesting the idea that once the stimulating source is removed, monocyte/macrophages will revert to their original non-activated form (reviewed in [55]). With our clinical samples, we first separated out the activated monocytes, and then grew them in culture for 7 – 10 days for differentiation into macrophages. Have these differentiated macrophages taken on an activated phenotype in response to their activated precursors, or have they developed into steady-state macrophages? Current staining experiments in our laboratory are working to answer this. However, if the activated phenotype is downregulated, when studying activated macrophages, how to get around the time needed for differentiation is a conundrum.

While it remains to be proven that the activated peripheral monocytes are precursors of CNS perivascular macrophages, selective expansion of cells with activation markers have been noted in the perivascular space of SIV-infected macaques and humans

[56, 57]. Additional studies show activated cells bearing $CD14^{+}/CD16^{+}$ markers infected with HIV-1 in brain specimens from patients with HIV-1 encephalopathy [56]. A subset of CD14+CD16+ monocytes were found to have higher proliferation levels in patients with HIV-1 infection, HAD, and sepsis when proceeded by production of activating cytokines, such as TNFa, M-CSF, or IL-6 [58]. Opposite to our results, Weber et al. (2000) showed that CD14+CD16+ monocytes revealed little CCR2 expression and therefore strongly impaired MCP-1 induced transendothelial chemotaxis [58]. The authors suggested that the lower MCP-1 production may have been a result of preactivation or reduced mRNA production [58]. Our methods were similar to theirs, except RNA extraction was with Rnazol, and ours was with a Qiagen kit. Since the Qiagen kit involves lysis directly on the plate, it is easier to believe more RNAs would be extracted with the Qiagen method. Although Weber et al. (2002) found decreased expression of CCR2 in CD14+CD16+ monocytes, increased expression of CCR5 MIP-1a/RANTES was shown, indicating an alternate chemotaxis pathway [58]. Weber et al. (2000) CD14+CD16+ monocytes, were also found to have elevated pro-inflammatory TNFa levels, but not anti-inflammatory IL-10 [58]. Our CSF protein array results indicated elevated levels of TNFa and IL-10 levels. Although, elevated TNFa levels could create an environment where activated monocytes either upregulate MCP-1 or MIP-1a, drawing more lymphocytes to the area, IL-10 represents a late member of the feedback loop, initiated to keep recruitment under control.

In our protein array experiments, the single best predictor of HAD in plasma was IL-4. This is an exciting finding, and can be thought about from several different angles. IL-4 and IL-10 have historically been grouped together as TH2-type responses (allergic,

cellular and humoral responses to extracellular pathogens), generally thought of as deactivating [59]. As mentioned before with IL-10, deactivating signals would not be contrary to our theories, considering late response, negative feedback is expected. However, if both IL-4 and IL-10 are acting in this manner, then it is curious that the different body compartments (periphery versus brain regions), would have different elevated sources for a similar response. However, if we consider IL-4 to be acting as an "alternative activator", the picture of the HAD predictor models from plasma, becomes very different [59]. It would then be recognized that, from plasma (representing the periphery), all the cytokines at high enough expression levels to predict HAD are pro-inflammatory/activating. This picture would lead credence to the idea that the struggle of pro- versus anti-inflammatory (neuro-degenerative versus neuro-protective) signals within the brain compartment in HAD, is at the least, aggravated by similar issues taking place in the periphery.

By focusing on activated macrophages, in a variety of experiments, we explored the relationship of the source of many of the proinflammatory cytokines and chemokines to HAD. The number of activated M/MΦ found in the CNS are a better predictor of HAD than viral load [60]. While the factors that drive M/MΦ to the perivascular region are unclear, we hypothesize that MCP-1 and IL-8, act together with IL-4 as chemoattractant proteins released by the activated cells, trafficking additional M/MΦ to the CNS. The different mediators of inflammation, and chemoattraction from M/MΦ supernatants, plasma, and CSF identified in this study, all indicate the formation of a closed feedback loop, where the signals for inflammation seem to be amplified above the suppressors.

Macrophages, Cytokines, and Integration Conclusions

In conclusion, using two independent methods, our studies found integration sites within an important immune functioning gene in the macrophage genome, near a transcriptionally necessary gene in a human tumor specimen, and within introns of a laminin family receptor protein, involved in adhesion and motility of cells. The position of the HIV LTR near, and thus most likely influencing the function of these genes is of particular interest.

An increased risk of H-NHL arises from the complicated interconnection of immunosuppression and inflammatory activation. Evidence for non-random HIV integration, supports the idea that the integration process itself may have a function other than solely for replication. Macrophages are important reservoirs of HIV, and are essential to an immune system's integrity. The tumor microenvironment is a delicate balance of cell signals and regulation. HIV could conceivably contribute to neoplastic development though impairment of cell-mediated immunity and dysregulation of cytokine loops.

Using multiple methods, and a variety of experiments, we explored the activated nature of M/MΦ. By focusing on activated macrophages, we have come closer to establishing the source of many of the proinflammatory cytokines and chemokines contributing to HAD. This dissertation has focused on translating the clinical concepts for HIV-1 associated malignancies and HIV-1 associated dementia, in respect to activated macrophages.

NEGATIVE DATA: SEQUENCE ANALYSIS OF SITES OF INTEGRATION (SASI)

Relevant Background

A retrovirus is dependent upon integration of a DNA copy of the RNA genome into host cellular DNA as a means to replicate its own genome [61]. While the human immunodeficiency virus type 1 (HIV-1) possesses this characteristic, in general, the integration takes place randomly. In some cases, there is evidence for non-random integration of the virus and this has been described in a cohort of HIV-associated malignancies suggesting a role for HIV-1 integration in types of HIV-associated malignancies [2, 6]. Since the beginning of the acquired immunodeficiency sydrome (AIDS) epidemic, a high incidence of lymphoma has been described [62]. In some HIV-associated tumors, HIV p24 expression has been associated with a common HIV integration site upstream from the *c-fes* oncogene [6]. The evidence of HIV integration within tumor cells and upstream from an oncogene, provides evidence for HIV as a tumorgenic agent. The possible mechanisms of HIV transformation range from promotor activity to insertional mutagenesis. Our goal was to identify possible preferential genomic sites where HIV integration is targeted.

SASI Hypothesis

1) The occurrence of HIV-associated cancers, is related in part to the integration site of HIV.
2) By creating an in vitro model, we will identify preferential genomic sites, possibly genes, where HIV integrates.

3) Identified preferential sites of integration can identify potentially expressed genes, which can be used as targets for characterizing tumor specimens.

SASI Specific Aims

1) Using the technique, sequence analysis of sites of integration (SASI), we will analyze HIV-1 integration sites to identify a preferential region for integration

SASI Research Design Rationale

Our long range goal was to identify possible preferential genomic sites where HIV integration is targeted. A pattern of preferential integration of HIV-1 would suggest that the act of integration provides both a means for replication, and possibly a step towards immortalization of some HIV-1 clones.

Although previous studies have demonstrated a common site of HIV-1 integration [6, 63], to date only a small number of samples have been analyzed. These studies utilized IPCR, and in the process revealed some disadvantages of the technique [64]. IPCR has been known to amplify circular HIV clones, and cause confusing results in the data. Also, analyzing a large library of HIV proviral clones through IPCR is too time consuming. The proposed procedure was a modification of the serial analysis of gene expression (SAGE) technique [65]. The revised method is called: sequence analysis of sites of integration (SASI). One of the major modifications to the original technique was to site-mutate the HIV-1 LTR to include a type IIs restriction enzyme site, *BpmI*. Type II restriction sites involve enzymes that cut 14 and 16 nucleotides downstream of the recognition sequence. By inserting the *BpmI* site into the U5 LTR of HIV-1, we

anticipate being able to isolate short segments of cellular DNA. Upon digestion with *BpmI*, the digested fragments should contain viral DNA and a small segment of cellular sequence at the site of integration. If successful, SASI would have allowed us to analyze hundreds to thousands of integration sites.

SASI Research Design Methods

1. Creation of New *BpmI* Site in LTR

Several site directed mutagenesis protocols were attempted in order to mutate the HIV-1 U5 LTR, these included: Transformer Site-Directed Mutagenesis Kit (Clontech Laboratories, Palo Alto, CA), Segmental PCR Mutagenesis, Single-PCR Mutagenesis, and the Quick Change XL Site Directed Mutagenesis Kit (Stratagene Inc.). Ultimately, we created a new *BpmI* Site in the HIV LTR using the Quick Change XL Site Directed Mutagenesis Kit (Stratagene Inc.) on the HIV-1 macrophage tropic isolate p89.6 (GenBank accession # U39362; NIH AIDS Research & Reference Reagent Program, Rockville, MD, USA).

Table 5.1: Transformer Site-Directed Mutagenesis Kit Protocol

	[66]	[67]	1X(ul)		Cycles	Temp C°	Time
template	.068ug/ul	10ng	.15ul		1	95	1min
Buffer	10X		5				
PrimerF	20uM	~400pm	1			95	50s
PrimerR	20uM	~400pm	1		18	60	50s
DNTP			1			68	24m30s
Quick sol			3				
Pfu Poly			1		1	68	7min
H2O			37.85				

After the PCR, the samples were incubated at 37°C for 1 hour, and then 4ul of sample was used to transform into XL Gold cells. Colonies were picked and plasmid preps were done with Qiagen (Valencia, CA) plasmid prep kits.

2. Transfection and Cell Propagation

We used a $CaCl_2$ method to transfect the mutated p89.6 HIV plasmid (called m89.6) into two human myelogenous leukemia cell lines, K-562 and 28SC (American Type Culture Collection, Manassas,VA), and primary PBMCs . We originally tried transfecting with electroporation (2 KV), but cell viability was poor, therefore, ultimately we consistently used the $CaCl_2$ method of transfection. The $CaCl_2$ protocol was modified from Current Protocols. ~8ug of plasmid DNA was used (no carrier DNA), to transfect into ~$8X10^5$ total cells per 60mm plate. According to protocol, media was changed about 2 – 4 hours before transfection.

Table 5.2: $CaCl_2$protocol

Tube	1A(*m89.6)	1B(*p89.6 = control)	
H2O	835ul	840	*depends on concentration of
plasmid DNA	40 μL (equal to 8μg)	35	-adjust H2O accordingly
2M CaCl = total 1000ul	125	125	

Tube	2A	2B
2xHepes	1000	1000

With an aerator, bubbles were created in tubes (2) with HEPES, followed by the addition of water and DNA to the HEPES tubes. 1mL of this transfection buffer was immediately added to each of the 4 plates of cells. Cells were incubated at 37°C over night, and media changed after 24 hours. We collected samples for HIV-1 p24 antigen assays (Beckman

Coulter, Fullerton, CA, USA) daily. High levels of virus production was usually detected on day 3, however, sometimes a 2nd transfection was necessary.

Viral supernatants were harvested for approximately 8 days, after viral production verification. We used the recommended growth media for the cells: Iscove's modified Dulbecco's medium with 4mM L-glutamine, 1.5g/L sodium bicarbonate, and 10% heat inactivated FBS bovine serum (Sigma Inc., St. Louis, MO). The cells were grown in a 37°C incubator, with 5% CO_2.

3. Infection

We tried to infect fresh K562 and 28SC cells, both human myeloid leukemia cell lines, but ultimately needed to use monocyte-derived-macrophages (MDMs). Whole blood was obtained in potassium ethylenediaminetetraacetic acid tubes from HIV-1 seronegative volunteers, as per guidelines established by the University of Hawaii Committee on Human Studies (Institutional Review Board). PBMCs were obtained using a standard Ficoll Hypaque separation protocol (Amersham Biosciences Corp., Piscataway, NJ, USA). Monocytes were isolated by CD14 magnetic beads according to manufacturer's instructions (Miltenyi Biotec Inc, Auburn, CA, USA) and counted on a hemocytometer. Monocytes were then plated at a density of $4x10^5$ per cm^2, and then further purified by adherent plate method, by washing with PBS 24 hours after plating. Monocytes were cultured for 14 days to allow for differentiation into macrophages. MDM media consisted of: a majority of Iscove's modified Dulbecco's medium (Irvine Scientific, Santa Ana, CA, USA), and 25% RPMI media (Sigma Inc., St. Louis, MO, USA), 4mM L-glutamine (Sigma Inc.), 100units/mL penicillin and 100µg/mL streptomycin (Sigma Inc.), 15ng/mL M-CSF (Sigma Inc.), and 10% Human Serum

(Sigma Inc.). The cells were grown in a 37°C incubator, with 5% CO_2. On day 14, macrophages were infected with harvested HIV-1 viral supernatant (after .45μm filtration, Nalge Co., Rochester, NY, USA). The mutated HIV-1 macrophage tropic isolate, m89.6, was batch grown and harvested in K562 cells. Viral supernatant was pooled and frozen at –70°C until needed. A viral cocktail (1:1 viral supernatant and fresh media, plus 4μg of Polybrene per milliliter) was left on the macrophage monolayer for six hours. Cultures were washed three times with phosphate-buffered saline and then incubated with MDM media. Supernatants from the macrophages, were taken for two and a half days after infection and analyzed with a p24 assay (Beckman Coulter, Fullerton, CA, USA) and a quantitative lentiviral vector particle number real-time PCR assay [68, 69], to verify positive viral infection.

4. Cellular DNA Isolation

Genomic DNA from the HIV-1 infected cells, was isolated using GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, Piscataway, NJ), on Day 3 after infection. Preparations were performed according to the manufacturer's optimized protocol for cell culture DNA extractions. Copies of HIV per cell were computed using Real-Time PCR of extracted DNA with comparison to two standards, OM.1 (one copy of HIV per cell) and cJun (one allele per chromosome)[70].

5. Restriction Digest

Digestions were carried out in separate aliquots, with EcoRI and PstI (New England Biolabs, Beverly, MA) to cut the DNA into workable pieces (Figure 5.3A). Protocol includes: 6ug of DNA, 10U EcoRI OR PstI, 1X Buffer, and H2O up to 50ul, with incubation at 37°C overnight. EcoRI was inactivated at 65°C for 20 minutes, or PstI

at 80°C for 20 minutes. After incubation, an aliquot was saved for gel visualization, the rest of the digested DNA was purified with GeneClean (Bio101, Vista, CA).

6. LTR isolation

The DNA pieces were denatured, annealed, and extended with an incorporated KpnI site biotinylated primer (Table 5.3 in Appendix). Theoretically, the primer should have bound to a sequence within the HIV-1 LTR.

Table 5.4: Protocol for extension reactions

	[stock]	[final]	1X(ul)		Cycles	Temp °C	Time
template		420ng	X		1	95	4min
Buffer	10X	1X	5				
PrimerF	20uM	1uM	2.5			95	45s
DNTP	4000uM	200uM	2.5		25	54	45s
Quick sol			3			72	1min
Pfu Poly	2.5U/ul		1				
H2O			X		1	72	10min

Prime-a-Gene Labeling System (Promega Co., Madison,WI), without isotope, was used for second strand synthesis (Figure 5.3B), according to manufactures suggestions. Streptavidin MagneSphere Paramagnetic Particles (Promega Co., Madison,WI), were used to isolate the biotinylated pieces from the rest of the cellular DNA, according to Promega protocol.

7. *BpmI* digest

To generate the final cellular DNA fragment, that was to be used in analysis, we digested with 10U *BpmI* (New England Biolabs) type II restriction endonuclease (Figure 5.3C). Protocol includes: Beads+DNA+ Buffer(up to 94ul), 1xBSA, 10U *BpmI,* and incubattion at 37°C for 1 hour, gently mixing intermittently. It was then important to

inactivate at 65°C for 20 minutes, wash 3x in .1xSSC (~300ul), and resuspend in 67ul premixed 1xLigase buffer.

8. Linker Ligation

The DNA sample fragments were in two pools. One pool was ligated to a *NotI-EcoRI* linker (N-E), the other a *SacII-AatII* linker(S-A) (Table 5.3). Using two different linkers guards against the possibility of the cellular DNA containing one of the utilized restriction enzyme sites (Figure 5.3D). Step one: dilute shorter half of linker (linkerA) to 350ng/ul (33ug/94ul), and dilute longer half of linker (linkerB) to 200ng/ul (33ug/165ul). Step two - Kinase the 5'to3' linker (A): linker A(11ul), 10x kinase buffer(2ul), 10mM ATP (2ul), 10U T4Kinase, H20(4ul), incubate at 37°C for 30 minutes, and inactivate at 65°C for 10 minutes. Step three - Anneal linkers A&B: LinkerA(20ul of kinase mix, final concentration=200ng/ul), LinkerB(9ul), heat at 95°C for 2 minutes, incubate at 65°C for 10 minutes, incubate at 37°C for 10 minutes, incubate at room temperature for 20 minutes. Step four – Ligation: BeadDNA+buffer(67ul), Annealed linkers (29ul), 20U T4Ligase, incubate at 20°C for 2 hours, inactivate at 65°C for 10 minutes, wash 3x in .1xSSC (~500ul), wash 1x in 1xNEB Buffer #1, and resuspend beads in 95ul premixed 1x NEBbuffer #1 + 2ul BSA.

9. *KpnI* Digestion and Purification

To purify the fragments from the biotin-streptavidin complex, we utilized the incorporated *KpnI* site (from step 5) to digest the connection site. Using standard digestion procedures, we also tried this step with Tsp451(New England Biolabs), targeting a site within the HIV LTR. With magnets, the supernatant containing the DNA

fragments, called tags, were separated from the biotin-streptavidin complex. Tags were purified with Mermaid-geneclean Kit (Bio 101) for smaller DNA pieces.

10. Formation of Diatags and Concatamers

Theoretically, step 9 should have created *KpnI* sticky-ends that easily ligate to one another (Figure 5.3E). The ligated tag dimers, called ditags, would then undergo PCR, with biotinylated linker-primers, amplifying the ditags (Table 5.3). The probability of any two tags binding together and then being overly represented in the final pool, would be easily identified and disregarded [65]. Testing different aliquots of ditags, the following protocol was used:

Table 5.5: Protocol for Formation of Diatags and Concatamers

	[stock]	[final]	1X(ul)		Cycles	Temp °C	Time
template		420ng			1	95	2min
Buffer	10X	1X	5				
Primer	20uM	1uM	2.5			95	30s
DNTP	4000uM	200uM	2.5		30	54	30s
			3			72	30s
Pfu Poly	2.5U/ul		1				
H2O			Up to50		1	72	10min

Samples were visualized on a gel, purified, and concentration was determined by spectrophotometry.

By using the incorporated restriction enzyme sites on the linkers (*NotI* or *EcoRI* and *SacII* or *AatII*), we could theoretically again create sticky-ends that should easily ligate together into a concatenated strand. Concatamation includes as many ditags as possible for sequencing (Figure 5.3F).

Table 5.6: Protocol for Concatamation

	Not I	*EcoRI*	*SacII*	*AatII*
Template	N-E linker + DNA – 80ul	N-E linker + DNA – 80ul	S-A linker + DNA – 80ul	S-A linker + DNA – 80ul
Buffer	#3 – 10ul	Any – 10ul	#4 – 10ul	#4 – 10ul
Enzyme	20U – 2ul	10U – 1ul	10U – 1ul	10U – 1ul
BSA	1ul	0	0	0
H20	7ul	9ul	9ul	9ul

Samples were incubated for 1 hour at 37°C. Once again, by using the Streptavidin MagneSphere Paramagnetic Particles (Promega Co.), the biotinylated pieces were purified from the rest of the cellular DNA, according to the following protocol:

i) Prewash 2 tubes of PMPs 3x with .5x SSC (600ul per wash). Resuspend in 800ul of .5xSSC. Make 2mL of .5xSSC + 20 ul 100x BSA.
ii) Mix well and aliquot 200ul into 8 tubes (2 for each of 4 purification cycle). Label the tubes(A1,B1,C1,D1,A2,B2,C2,D2)for each linker set.
iii) To the completed digest, add 400ul .5x SSC and 4ul 100x BSA. Using a magnet, remove the wash buffer from the "A" tubes. Aliquot the digest mix to each "A" tube. Mix on a rotator, at room temperature, for 15 minutes.
iv) Place on magnet for 2 minutes. Meanwhile, remove wash buffer from "B" tubes. Transfer supernatant from "A" tubes to "B."
v) Immediately add 200ul of .5x SSC + BSA to "A" tubes. Pipet back and forth several times to remove residual ditags. Mix on rotator "A" and "B" tubes for 15 minutes at room temperature.
vi) Repeat this process for all the tubes. Tubes should be rinsed, incubated and collected twice each. Consolidate supernatants for both sets of tubes (~550ul).

Concatamers were purified (Mermaid-geneclean Kit; Bio 101), ligated overnight with T4 ligase, and cloned with Topo (Invitrogen, Carlsbad, CA) for sequencing.

11. Analysis

Each tag was estimated to contain 11 to 13 bp of host DNA. Past studies have shown, that at a random tag site, 9bp includes enough information to distinguish up to 262,144 transcripts [65].

SASI Results

The preliminary work focused on optimizing or finding an efficient site-directed mutagenesis protocol to insert the *BpmI* enzyme site in the HIV LTR region, as part of the SASI protocol. Due to the size of HIV-1 (9.7 kb), an optimal and efficient site-directed mutagenesis strategy was challenging. The HIV-1 plasmid, p89.6 (figure 5.4), contains the full length HIV-1 strain along with the cloning vector (12.1 kb). p89.6 was generated from a highly macrophage-tropic strain of HIV-1, noted for efficient replication in macrophages and highly cytopathic in primary lymphocytes [71]. p89.6 is also documented to form syncytia in monocyte derived macrophages. Using a similar technique as SAGE, a previous study introduced a new *BpmI* site into the U5 LTR of the moloney murine leukemia virus, resulting in similar integration and replication kinetics as the wildtype[72]. Various strategies and approaches were designed to insert the *BpmI* site as outlined below.

Transformer Site-Directed Mutagenesis Kit (Clontech Laboratories, Palo Alto, CA)

The theory behind this approach is to introduce two defined mutations into a target plasmid of known sequence. This method (Figure 5.5) is considered a "non-PCR" technique because it does not use polymerase chain reaction to multiply the mutated plasmid. Two primers were annealed to one strand of a denatured plasmid to produce the experimenter's desired mutation and a selection mutation (Table 5.7 in Appendix). The selection mutation theoretically changes a known restriction enzyme site into a unique restriction enzyme site on one strand. Primary selection is supposed to take place using

the restriction enzyme for the original unmutated selection site. Those plasmids that do not incorporate the selection mutation primer, statistically should not have incorporated the target mutation primer; and should therefore, contain the unmutated parental strands susceptible to selection restriction digest. Linear (cut) plasmids do not transform efficiently. The mutated plasmids are uncut, and should then transform into MutS E. Coli cell for clonal expansion. MutS cells clone without DNA repair. The following plasmid extraction would contain two populations: unmutated parental plasmids and double stranded mutated plasmids. Secondary selection should have taken place using the selection restriction enzyme, which linearizes the parental plasmids. The second transformation ideally results in the cloning of plasmids with both a mutated selection sites and a mutated target sequences. This strategy was unsuccessful, most likely due to the fact that the 12.1kb p89.6 plasmid was too large for this protocol.

Segmental PCR Mutagenesis

The idea behind this technique is to utilize known restriction digest sites and bring the total manipulated segment of DNA down to a manageable size less than 1kb. This technique (Figure 5.6) used 4 primers (primers A, B, C, and D), 2 of which overlapped and contained the mutation sequence (primers B and C). Primers A and D contained the sequence of known restriction digest sites (Table 5.7). Two sets of forward and reverse primers were used in separate PCR reactions. PCR reaction #1 utilized primers A and B. PCR reaction #2 used primers C and D. PCR reaction #1 contained a reverse mutation primer (primer B), while PCR reaction #2 contained a forward mutation primer (primer C). The mutation primers B and C overlapped. The PCR reactions resulted in two

segments of DNA: segment AB, and segment CD. After PCR amplification, the majority of DNA segments contained the mutated sequence in primers B and C. In PCR reaction #3 only primers A and D were used with equal aliquots of the DNA segments AB and CD. The resulting DNA segment AD should contain the desired mutation in the middle of the segment. This DNA segment was then ligated back into the original plasmid with the corresponding unmutated AD segment excised. This strategy based on segmental PCR proved to be unreliable due to the similarity in size of the restriction digest segments, and the possibility of mispriming.

Single-PCR Mutagenesis

The single-PCR mutagenesis protocol for large plasmids is featured in BioTechniques 29:976-978 (November 2000) [73]. This technique (Figure 5.7) also utilizes two overlapping forward and reverse mutagenesis primers that are complementary to opposite sides of the plasmid (Table 5.7). The recommended *Pfu* Turbo DNA polymerase is designed to polymerize vector targets up to 15kb in length and will not displace the mutant primer. A small amount (~10ng) of denatured parental plasmid template was used to anneal the mutation primers, which were then extended by *Pfu* Turbo polymerase in a single (16 cycle) PCR reaction. Cosolvents, such as DMSO, were included in the PCR reaction mix in order to improve denaturation and help reduce secondary structures in the primers and plasmid template. The resulting PCR product was then incubated with *DpnI* restriction enzyme to eliminate wild-type DNA. Parental template plasmids grown and isolated from bacterial cells were methylated, and therefore recognized and digested by *DpnI*; *DpnI* could not digest newly polymerized

complementary strands because they were unmethylated. An aliquot of the pool of digested parental and undigested complementary DNA was then transformed into competent *E.coli* cells for cloning. Ideally the isolated end product would have been full-length mutated plasmids. This approach to optimize a PCR-based method for large plasmid site-directed mutagenesis led to more encouraging data but was not consistent because of polymerase mistakes.

QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA)

This particular strategy is an optimized version of the Single-PCR mutagenesis protocol for large plasmids. There are two main differences between the protocols, however, the concept of a mutagenesis primer PCR reaction followed by a *DpnI* digestion is the same (Figure 5.7). One difference is the use of a "QuikChange solution", instead of other cosolvents, like DMSO, in the PCR reaction to facilitate replication of large plasmids. The other difference is the use of special "XL10-Gold ultra competent cells" for the transformation of large plasmids. XL10-Gold cells contain the Hte phenotype that increases the transformation efficiency of large DNA plasmids. After plasmid purification, the end products were full-length large plasmids that contained the desired site-directed mutation. This approach successfully inserted the required point mutation, resulting in a *BpmI* type II restriction enzyme recognition sequence in the U5 LTR of HIV-1.

Post Mutation

From here, the next step was to transfect a human myelogenous leukemia cell

line, and primary PBMCs with the mutated HIV-1. The goal was to find a suitable host to grow mutated virus, for future harvest and infection of fresh cells. PBMCs were determined to be too fragile for this purpose, because they would repeatedly die after transfection (Figure 5.8). We also tested two human myelogenous leukemia cell line, K562 and 28SC, to perform as viral hosts. The K562 cell line was chosen as the optimal host to produce mutated virus, because this cell line was proven to be robust and produced relatively high amounts of p24 HIV-1 protein (Figure 5.9).

After growing and harvesting virus, both mutated and normal, we then tried to infect K562 and 28SC cells. As proven with the transfection experiments, both cell lines were believed to be hardy and easy to grow. Unfortunately, all attempts at infection, with and without the additives Dextran and Polybrene, were unsuccessful (Figures 5.10 & 5.11). We then decided to use primary monocyte-derived-macrophages (MDMs) as our hosts for infection and integration. We had no problems infecting the MDMs (Figure 5.12).

All eleven steps of the SASI protocol were completed two times with the same negative results. Each time approximately 20 "concatamer" plasmids were sequenced. All sequences were found to either be cloning vector, HIV-1, or non-human contamination.

In order to determine why the SASI protocol was not working, aliquots were taken for sequencing whenever possible throughout the protocol. After extraction from MDMs, the DNA used for the rest of the SASI steps was always high quality human DNA. After the restriction digest, in step 5, samples taken for sequencing revealed human DNA. However, samples taken for sequencing after step 7, and later, always

resulted in, either cloning vector, HIV-1, or non-human contamination.

SASI Discussion

In summary, the Transformer Site –Directed Mutagenesis Kit, Segmental PCR Mutagenesis, and Single-PCR Mutagenesis experimental procedures all resulted in variations of the HIV plasmid, p89.6, ranging from 3kb to 10kb. The complete p89.6 plasmid containing the full length HIV-1 sequence is 12.1kb in length. Therefore, none of the resultant smaller plasmids were tested for the mutation in sequencing, because they were too short. The QuikChange XL Site-Directed Mutagenesis approach resulted in 1 of 64 total experimental samples, demonstrating the required 12.1kb in length, as seen on a 0.8% TBE Ethidium Bromide gel (Figure 5.13). We were able to successfully mutate two basepairs at the end of the U5 LTR in the HIV-1 LTR. This sample was sequenced, and found to contain the desired restriction enzyme *BpmI* recognition sequence (Figure 5.14). The sample was also cut with *BpmI*, and found to contain the correct number and sizes of fragments. Previously, Roe et al. (1997) introduced a new *BpmI* site into the U5 LTR of the Moloney murine leukemia virus without detecting changes in replication kinetics [72]. *BpmI* cuts 14 and 16 nucleotides downstream of its recognition sequence. By inserting the *BpmI* site into the U5 LTR of HIV-1, we should have been able to isolate short segments of cellular DNA. While the LTR of HIV-1 has been difficult to selectively point mutate, the QuikChange XL Site-Directed Mutagenesis protocol was proven successful.

Due to the extended period of elongation and the superstructure obstacles, large plasmids are extremely hard to successfully undergo site-directed mutagenesis. Also,

viral LTR's are notoriously hard to mutate because of the repeated sequences with multiple possible homologous primer sites. While, the 1.6% success rate of the QuikChange XL Site-Directed Mutagenesis Kit is lower than expected, one mutated sequence was adequate to proceed with the project. It is unclear as to whether the "QuikChange solution" and the "XL10-Gold ultracompetent cells" were both responsible for the successful mutation and length of the large plasmid, or perhaps one or the other was the key ingredient.

Interestingly, the mutated plasmid (m89.6) repeatedly (data not shown) expressed more p24 HIV-1 protein than the experimental control, p89.6 (figure 5.9). Replication is known to be effected by changes in the LTR [46], however, since infection experiments of K562 were unsuccessful (Figure 5.10 & 5.11), it is unlikely that integration took place, and therefore, unlikely that replication happens. Perhaps changes in the LTR affect transcription as well?

Unfortunately, all attempts at infection of the myeloid progenitor cell lines, with and without the additives Dextran and Polybrene, were unsuccessful (Figures 5.10 & 5.11). Since primary cultures of myeloid progenitor cells can be infected by HIV, it is probable that the "immortality" of the cell-line cells has effected receptor expression. Therefore, it is likely that the cell lines did not contain the proper receptors for HIV-1 entry. The successful infections of MDMs, allowed us to continue with the protocol. The kinetics of HIV-1 infection and establishing virus production and integration is different in macrophages compared to T-cells [74]. DNA was extracted from HIV-1 infected MDMs 72 hours after infection, which may explain some of the results we obtained. Thus the number of targets available for analysis from the SASI protocol was

likely limited.

All the various sequencing samples reviewed, in an attempt to discern the root of the problems with SASI, has led to the conclusion that complicated protocols, with this many steps, and not enough controls, are unwise. Sequencing samples taken for steps 7 and later, always revealed negative results. In retrospect, it is likely that biotinylated segments of DNA, in steps 7 and later, could have interfered with the cloning process, and resulted in false sequencing information, because of the bulk of the biotin molecule. It is also believable that using magnetic particles, for purification of small pieces of DNA, was a mistake, due to the possibility of rust interference. The concept of being able to "wash" out very small segments of DNA from the grasps of metallic, charged, magnetic particles, seems inefficient, because of DNA's charged nature, and its proven attraction to glass. It is also probable that restriction enzymes would be unable to perform their normal duties under the charged, magnetic conditions. It is possible that the magnetic particles served as obstacles to the enzymes, and the rust particles prevented them from releasing the desired DNA fragments. Thus, the enormous loss in yield, after each magnetic particle DNA purification, could be interpreted as proof for these theories. In conclusion, because the protocol was so long and complicated, there were too many variables to decipher the exact reason for failure. The experiences rendered from the SASI protocol, led to the conclusion that a much simpler protocol was needed to accomplish the goal to analyze integration sites.

Further attempts to try to elucidate integration sites led to a wide variety of imaginative PCR techniques. Unfortunately, trying to manipulate small segments and small quantities of DNA, through several rounds of PCR cycling, resulted in what was

essentially competitive PCR. With HIV-1 specific primers, but unknown, non-clonal genomic sequence, the milieu of different products would not amplify sufficiently for visualization. So, in retrospect, it is possible that some of these PCR attempts did work, however, because we relied on visualization for verification we did not proceed to sequencing. However, the cumulative experience, regardless of failure, lead to more successful endeavors.

Table 5.3. Primers and Linkers used in Proposed Methods.

Type of Primer or Linker	Sequence of Primer 5' ⇒ 3'
KpnI-Biotin Primer	5Bio/c t t a a a g g t a c c t g a c c t c t g a c t g g
Degenerate EcoR1 Linker	_ _ g c g g c c g c t t t t t g a a t t c g NNc g c c g g c g a a a a a c t t a a g c
Degenerate AatII Linker	_ _ t t t c c g c g g t t t g a c g t c t c NNaaag g c g c c a a a c t g c a g a g
EcoR1 Linker-Primer	c g a a t t c a a a a a g c g g c c g c
AatII Linker-Primer	g a g a c g t c a a a c c g c g g a a a

Table 5.7. Primers used in Preliminary Results.

Type of Primer	Sequence of Primer 5' ⇒ 3'
Clontech's Selection Primer	g t g c c a c c t g a t a t c t a a g a a a c c
Clontech's Mutagenesis Primer	g t t a g t g t a g a a a a t c t g g a g c a g g g
Segmental PCR Primer A	a t c t g a g c c t g g g a g c t c
Segmental PCR Primer B	c c c t g c t c c a g a t t t t c t a c a c t a a c t a g
Segmental PCR Primer C	c t a g t t a g t g t a g a a a a t c t g g a g c a g g g
Segmental PCR Primer D	c a g t g a g c g a g g a a g c g g
Single PCR & QC's MutF Primer	g t t a g t g t a g a a a a t c t g g a g c a g g g c g g g c a g c a a g c
Single PCR & QC's MutR Primer	g c t t g c t g c c c g c c c t g c t c c a g a t t t t c t a c a c t a a c
Sequencing Primer p89.6F	g c t t a a c t a r g c g g c a t c
Sequencing Primer p89.6R	a c a c a g g a a a c a g c t a t g a c

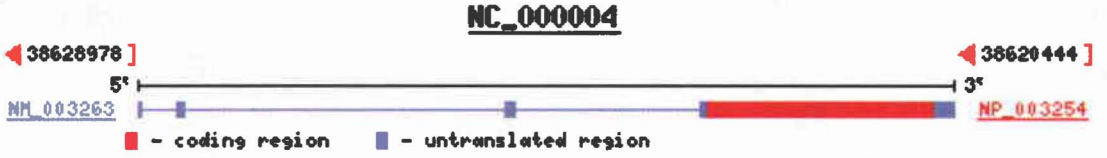
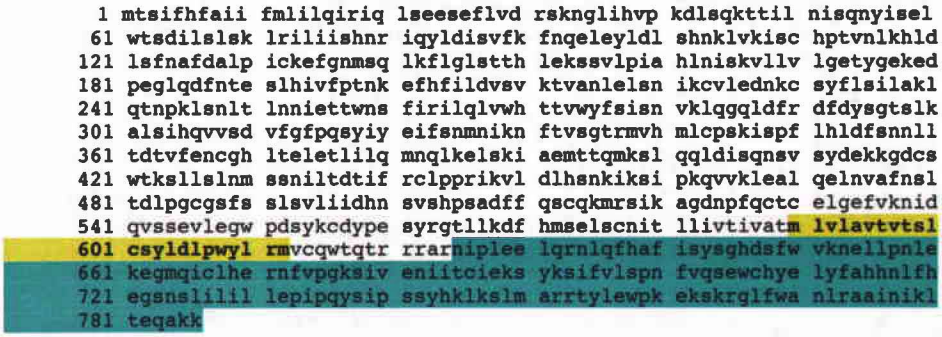


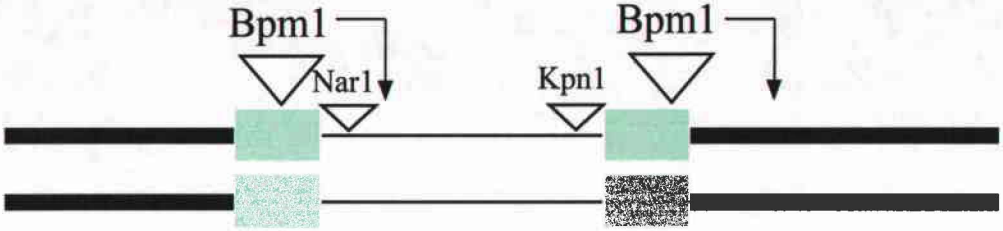
Figure 5.1. The LP-PCR resulted in the identification of an HIV-1 integration site within the Toll-like receptor 1's coding region (red). The integration was toward the 3' end of the coding region.



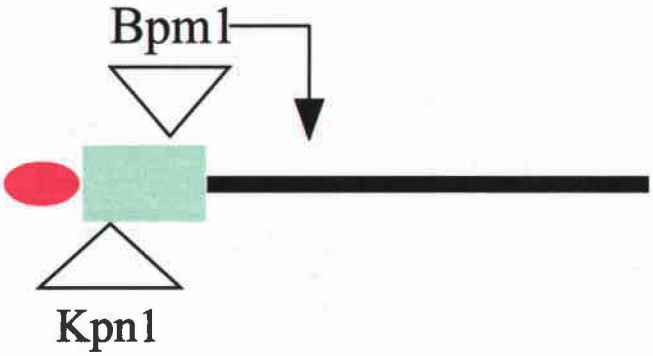
accession number: NP_003254

Figure5.2. The amino acid sequence of Toll-like receptor 1(TLR1). Yellow represents the translated region of TLR1 nucleotides that were identified in the LP-PCR HIV-1 integration sequence. The HIV-1 provirus inserted itself after, what is seen here, as amino acid # 612. Blue represents the TIR domain of the TLR1.

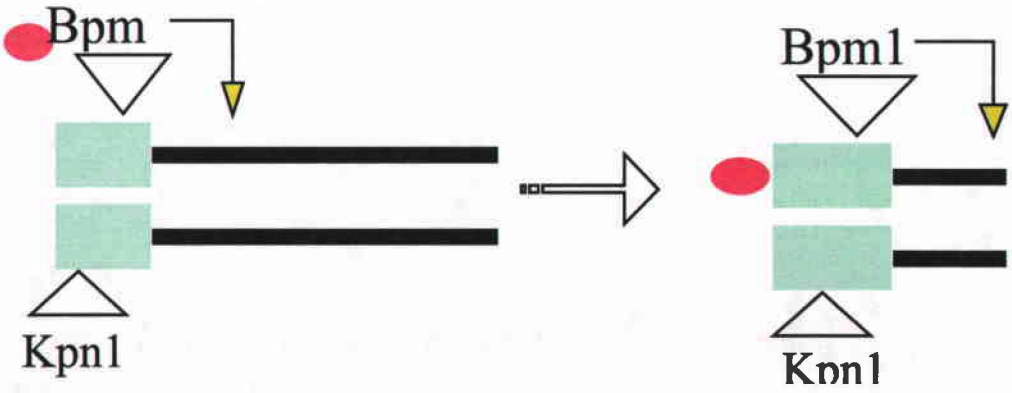
5.3A: Restriction Digest



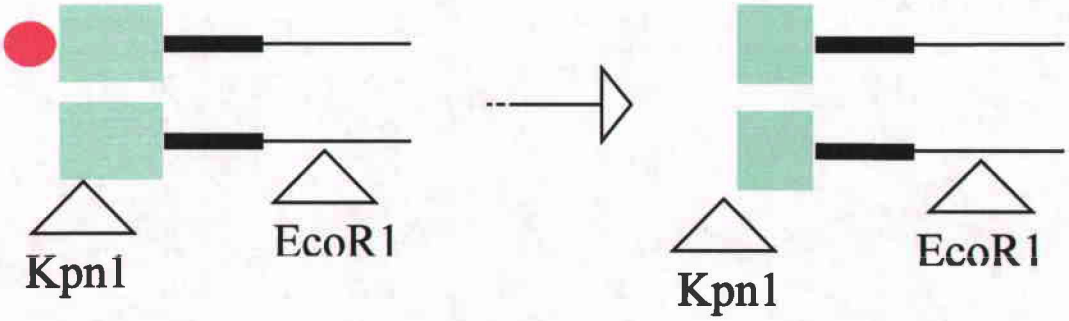
5.3B: LTR isolation



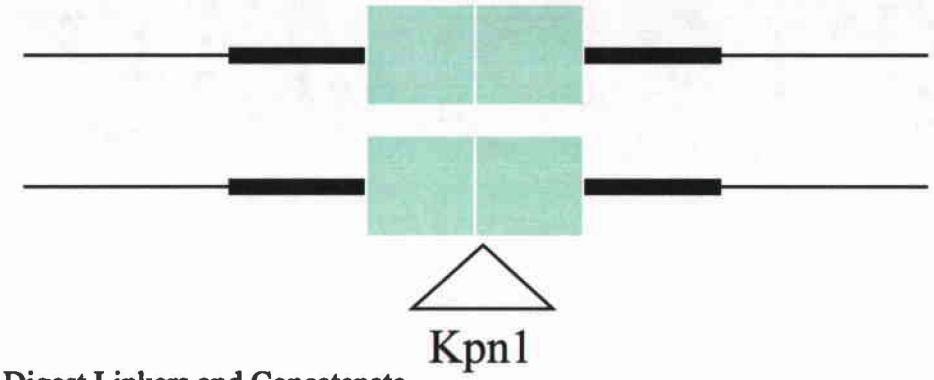
5.3C: BpmI digest



5.3D: Linker Ligation



5.3E: **Ligation**



5.3F: **Digest Linkers and Concatenate**

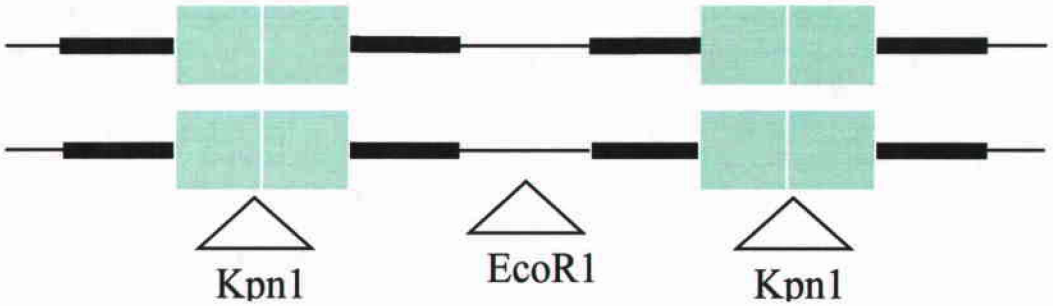


Figure 5.3. Diagram depicting the sequential steps of the SASI technique.

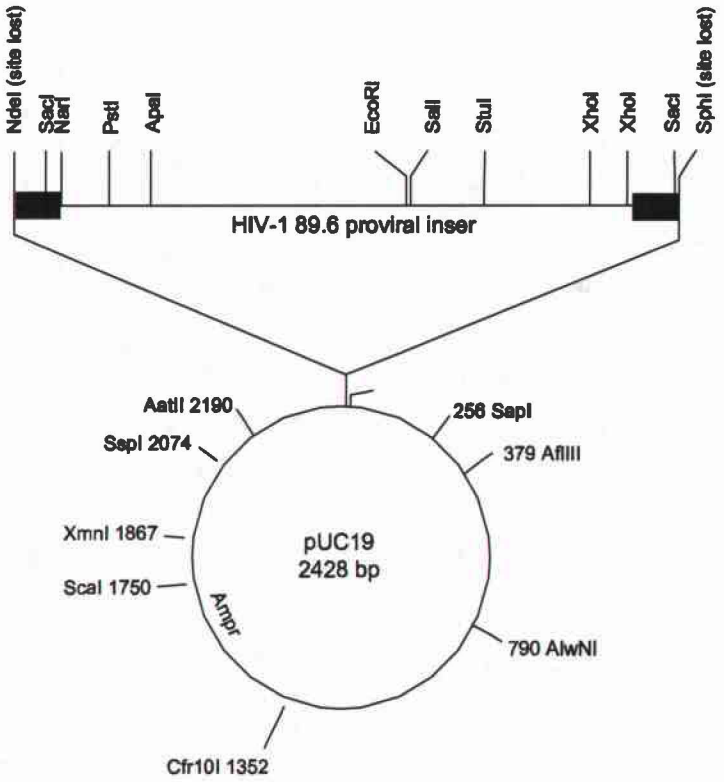


Figure 5.4. Diagram of the p89.6 plasmid made from the HIV-1 proviral insert in a pUC19 vector. www.aidsreagent.org

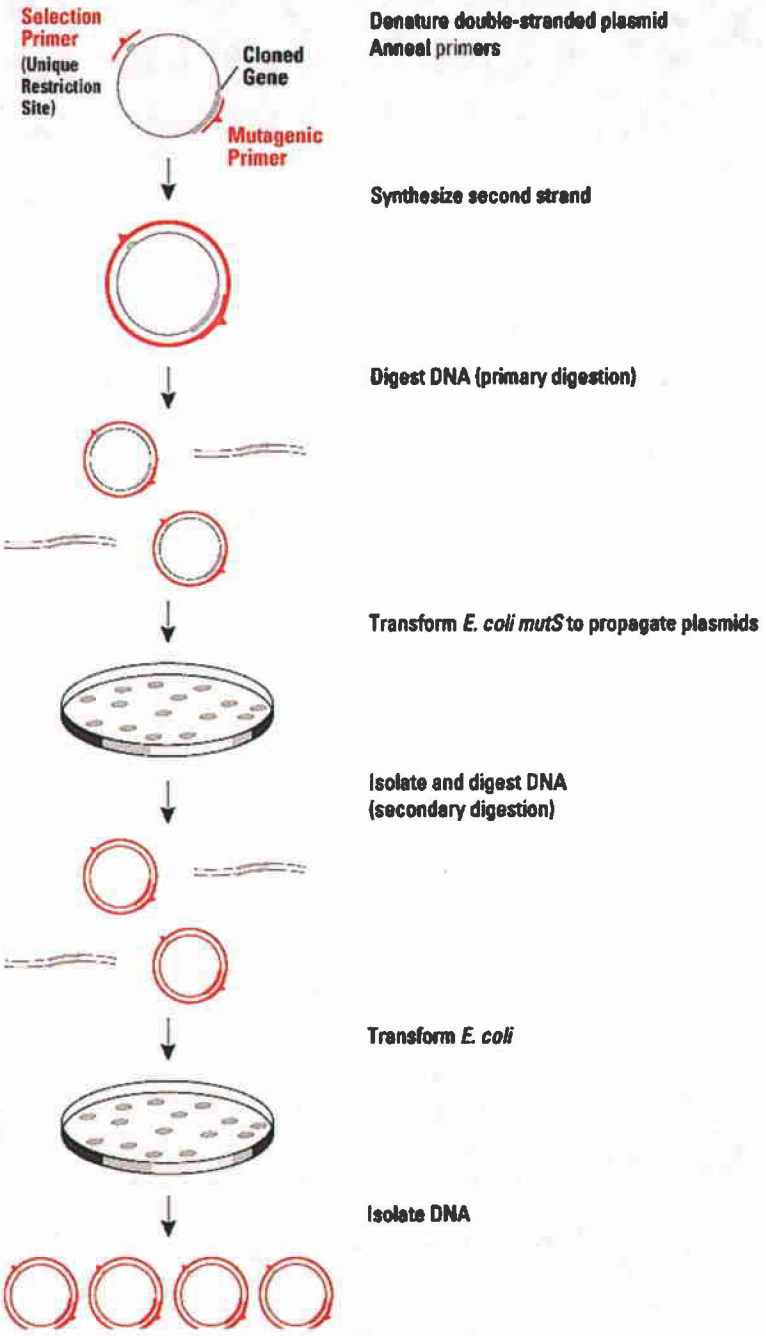


Figure 5.5. Diagram of the Transformer Site-Directed Mutagenesis Kit protocol (Clontech Laboratories Inc., Palo Alto, CA). www.clontech.com

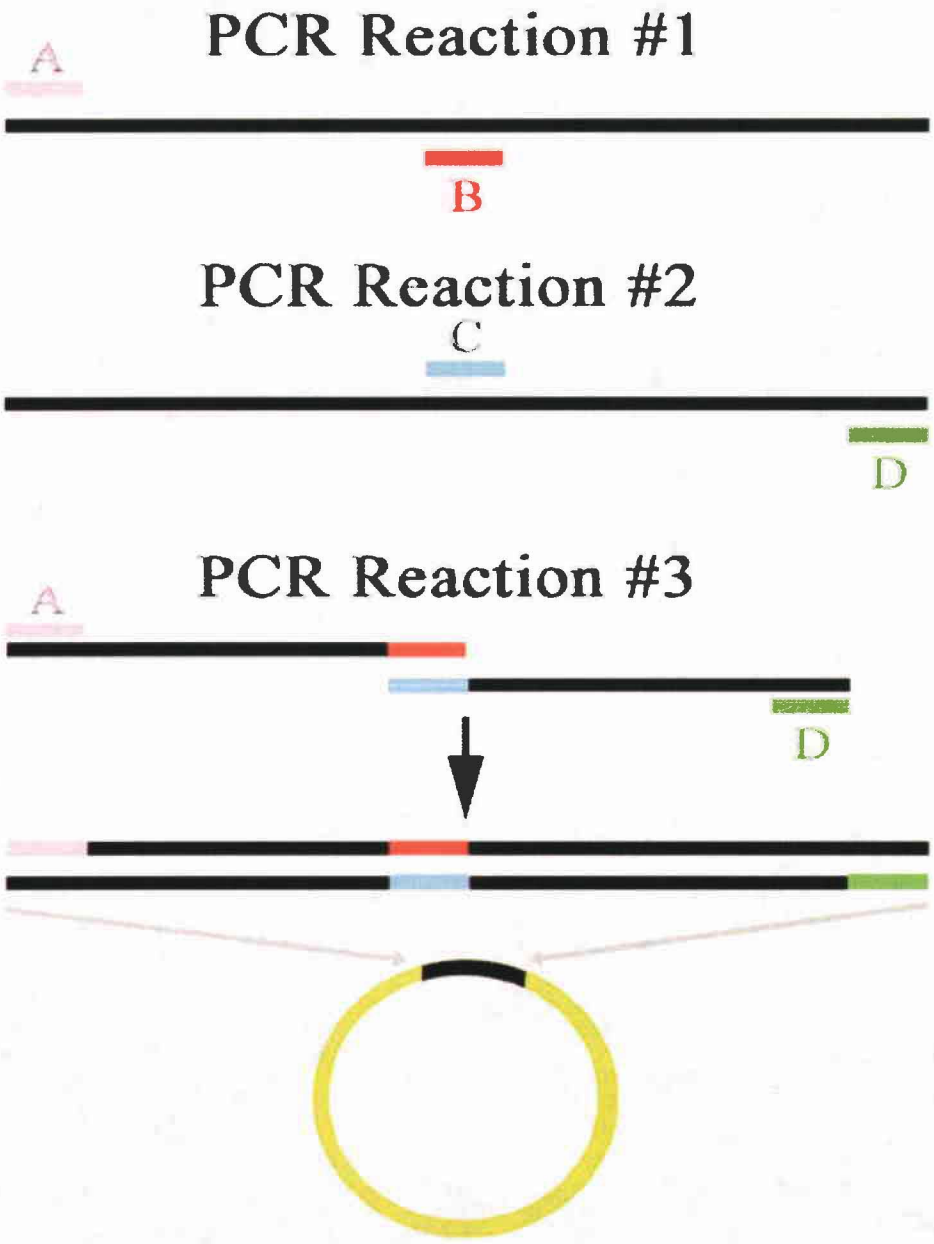


Figure 5.6. Diagram of the Segmental PCR Mutagenesis protocol.

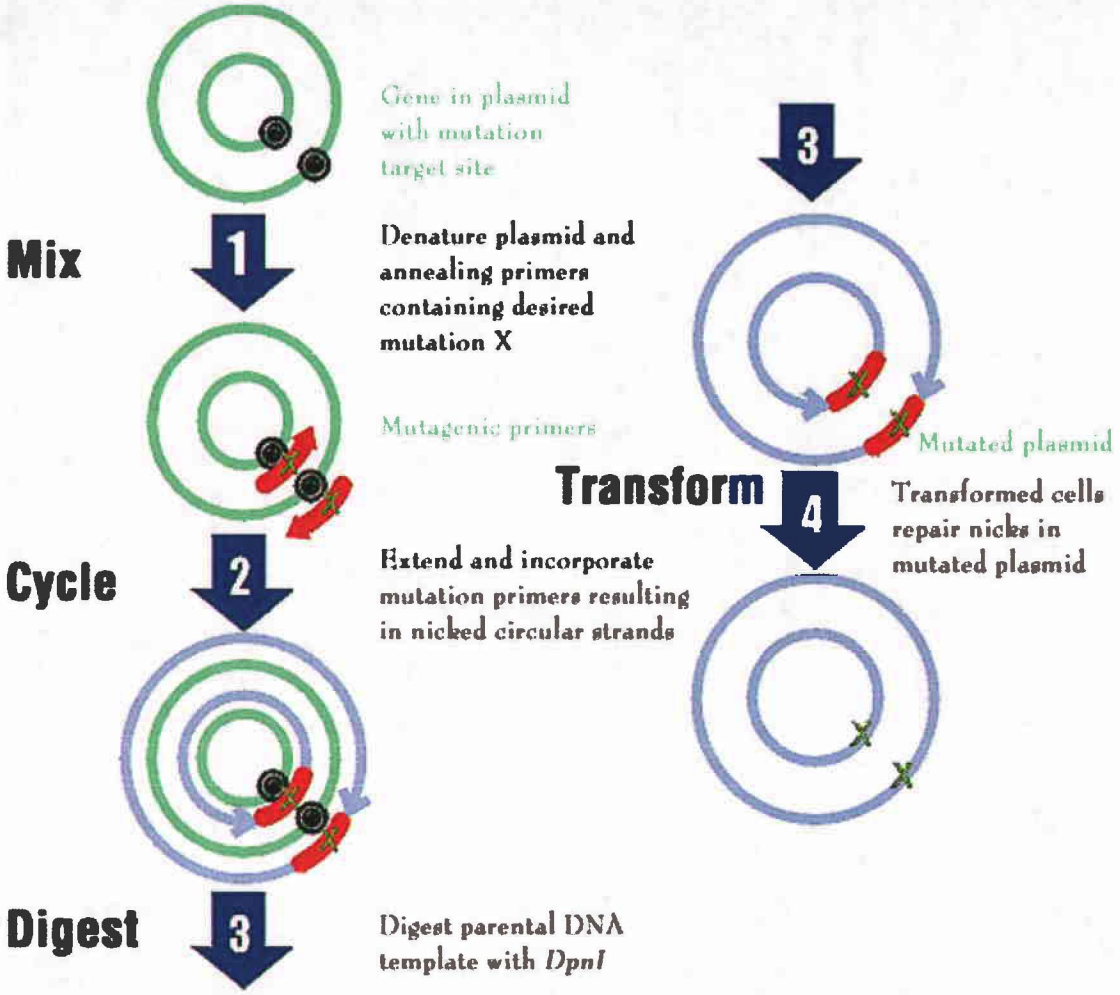


Figure 5.7. Diagram of both the Single-PCR Mutagenesis protocol and the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Modified from www.statagene.com

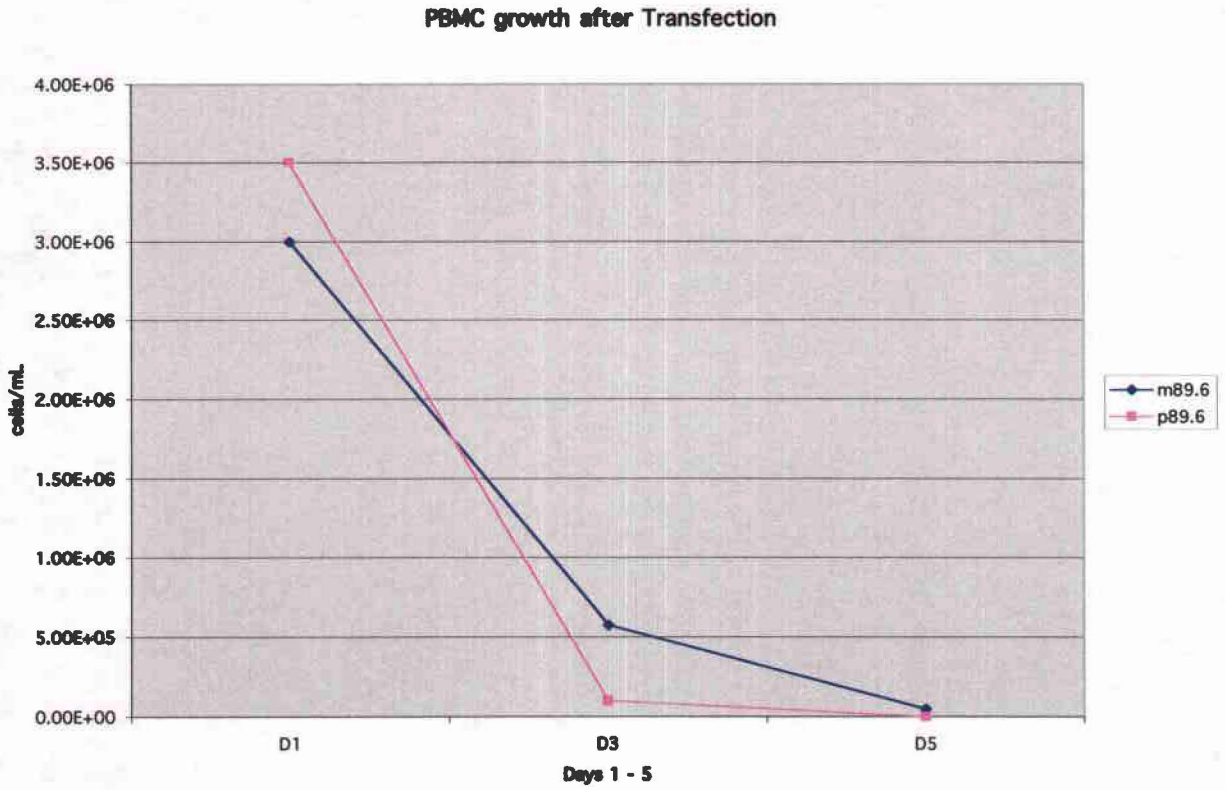


Figure 5.8. Chart created on 8/30/01 depicting the different PBMC cell growth levels after transfection. The experimental control, normal HIV-1, p89.6(pink), and the mutated HIV-1 LTR, m89.6 (blue), upon transfection into PBMCs, both cause cell death. PBMCs were determined not suitable for this transfection experiment.

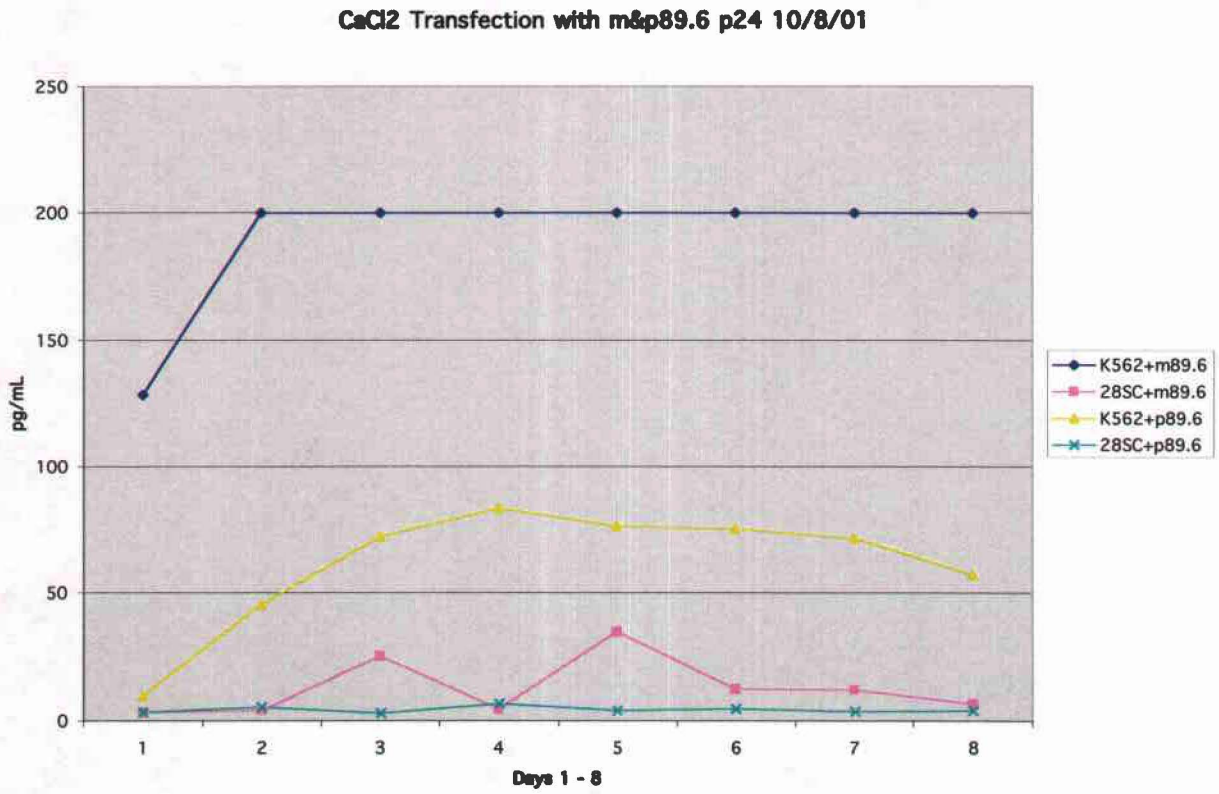


Figure 5.9. Chart created on 10/8/01 depicting the different p24 levels expressed after transfection of p89.6(experimental control: normal HIV-1) and m89.6 (mutated HIV-1 LTR with BpmI recognition sequence). Two human myelogenous leukemia cell lines, K-562 and 28SC, were transfected with p89.6 and m89.6. As seen here, K562 appears to express the p24 HIV-1 protein at higher levels.

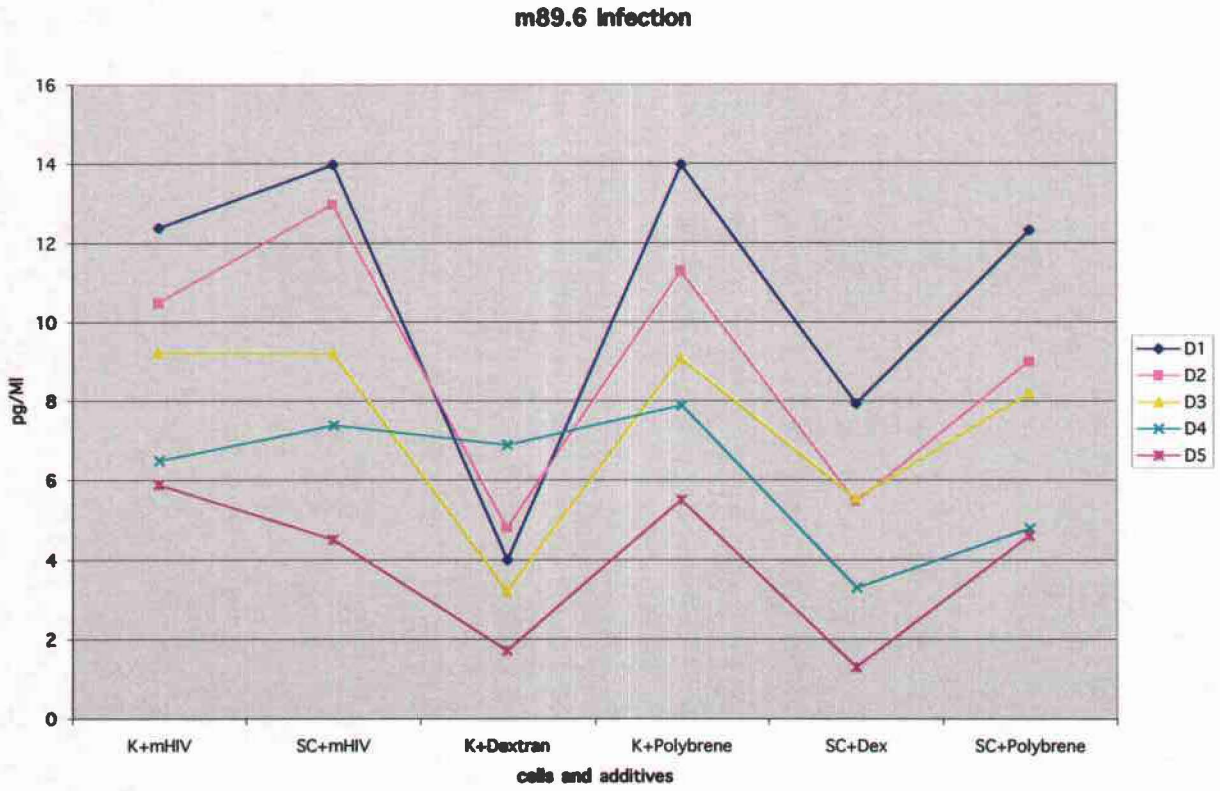


Figure 5.10. Attempted infection with harvested virus, over 5 days (D1-D5) of K562 (K) cells and 28SC (SC) cells, with mutated HIV-1, m89.6. Dextran and Polybrene were used to enhance infection. As depicted by the low p24 expression, infection was not successful in any condition shown.

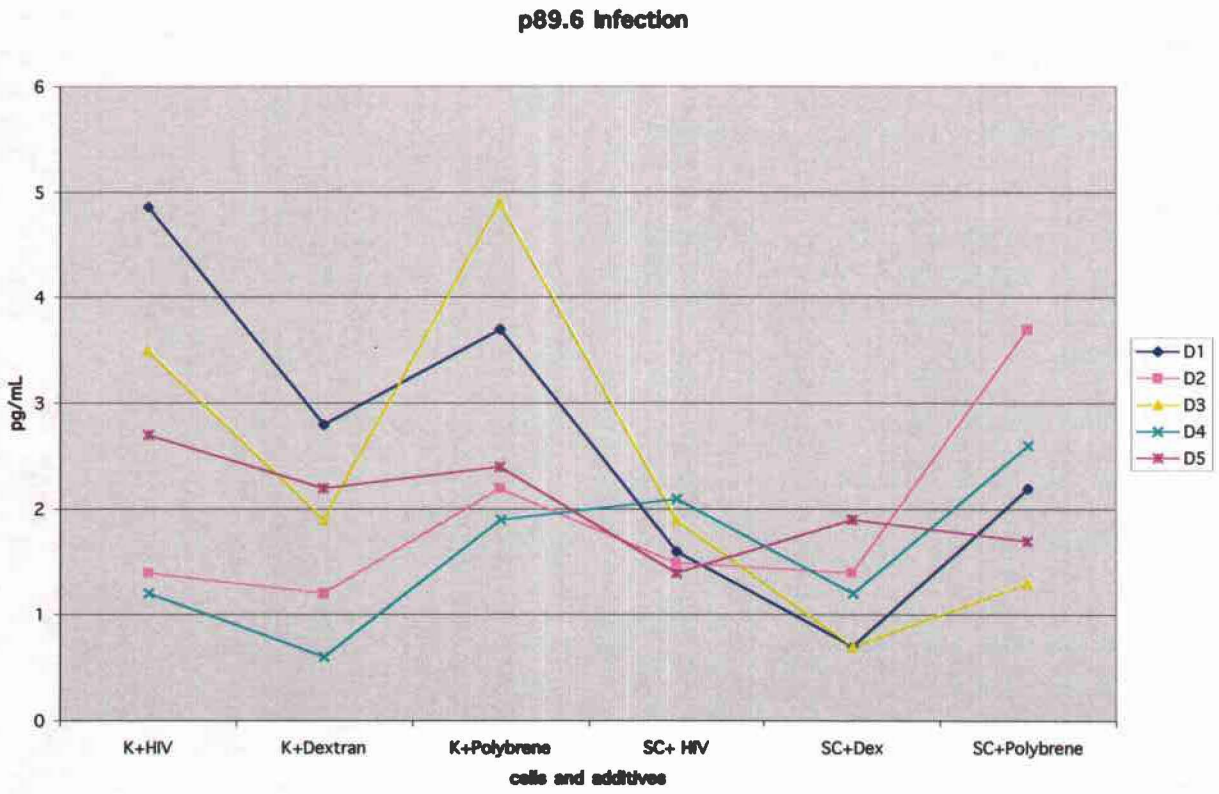


Figure 5.11. Attempted infection **with** harvested virus, **over 5** days (D1-D5) of K562 **(K)** cells and 28SC (SC) cells, with normal HIV-**1, p89**.6. Dextran and Polybrene **were** used to enhance **infection**. As depicted by the **low** p24 expression, infection was not successful **in any** condition shown.

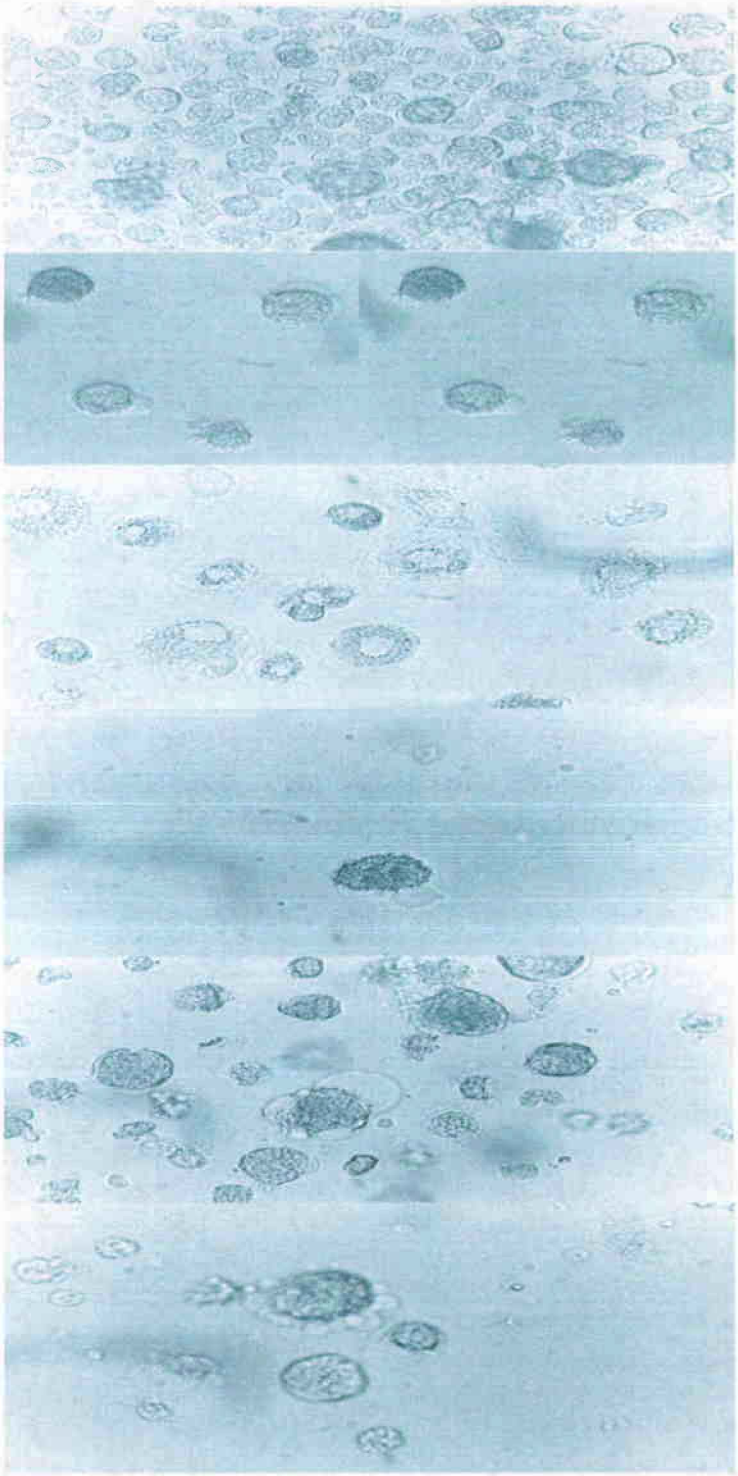


Figure 5.12. Successfully infected monocyte-derived-macrophages (MDMs). Shown here under a light microscope at 200X. Notice blebbing and syncytia formation.

Figure 5.13. 0.8% TBE Ethidium Bromide gel depicting (**L**) a Supercoiled DNA Ladder (Gibco BRL, Rockville, MD), (**C**) unmutated p89.6 as a control, (**Mut**) p89.6 sample containing the site-directed point mutation. Both, (**C**) and (**Mut**), are the required 12.1kb in length.

P89.6 1R
CCAGCTTGCATGCCCGCCCTGCTAGAGATTTTCTACACT
P89.6 2R
CCAGCTTGC.TGCCCGCCCTGCTAGAGATTTTCTACACT
QC-6AR
CCAGCTTGC.TGCCCGCCCTGCTCCAGATTTTCTACACT
QC-6BR
CCAGCTTGC.TGCCCGCCCTGCTCCAGATTTTCTACACT

Figure 5.14. Sequence alignment of wildtype p89.6 versus mutated p89.6. The top two lines are wildtype sequences and the bottom two lines are the mutated sequences. The mutated sequences are from the same sample, sequenced twice.

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