MULTIPLE DETERMINANTS WITHIN HIV-1 GAG-VIF ARE RESPONSIBLE FOR A NOVEL POST-ENTRY RESTRICTION IN MACROPHAGES.

By

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To my family; Lowell, Suzanne, and Katie for their love and support.

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AIDS	Acquired immune deficiency syndrome
CA	Capsid
CCR5	CC chemokine receptor 5
CXCR4	CXC chemokine receptor 4
CypA	Cyclophilin A
D	Dual
HAD	HIV-associated dementia
HIV	Human immunodeficiency virus
IN	Integrase
L	Lymphocyte
LTR	Long terminal repeats
Lv1	Lentivirus susceptibility factor 1
M	Macrophage
M-CSF	Macrophage colony-stimulating factor
MA	Matrix
MDM	Monocyte-derived macrophages
MVB	Multi-vesicular bodies
NC	Nucleocapsid
NSI	Non-syncytium inducing
p.i.	Post infection
PBMC	Peripheral blood mononuclear cells
PIC	Pre-integration complex
PMA	Phorbol 12-myristate 13-acetate
PR	Protease

R5	CCR5-using
R5X4	CCR5-using & CXCR4-using
Ref1	Restriction factor 1
RT	Reverse transcriptase
SI	Syncytium inducing
T	T cell line
TRIM5α	Tripartite motif protein 5α
X4	CXCR4-using

Abstract of Dissertation Presented to the Graduate School
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MULTIPLE DETERMINANTS WITHIN HIV-1 GAG-VIF ARE RESPONSIBLE FOR A NOVEL POST-ENTRY RESTRICTION IN MACROPHAGES

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As of 2007, an estimated 33 million individuals are living with HIV/AIDS. While there is currently no vaccine or cure for HIV, drug therapies exist that reduce the onset of the disease while lengthening the lives of infected individuals. The current drug therapies are successful at eradicating virus produced from short-lived activated cells, but fail to remove virus from long-lived, non-dividing, and quiescent cells such as macrophages. Macrophages serve as both targets for HIV infection and as reservoirs for viral progeny. However, maintenance of HIV within macrophages is poorly understood. The objective of this study was to identify post-entry, HIV-1 genetic determinants associated with macrophage tropism. By creating genetic recombinants between macrophage-tropic and non-macrophage-tropic strains of HIV-1 and testing their ability to replicate in macrophages, we identified unique macrophage determinants, or HIV-1 genomic regions, necessary for efficient macrophage infection. Specifically, macrophage tropism of HIV-1 maps to multiple, independent determinants within the Gag-Vif genes. Moreover, the macrophage-restricted genotype is cell-type specific and does not restrict replication in peripheral blood mononuclear cells (PBMC). Temporally, the restriction occurs after first round replication and only blocks viral spread from

macrophage to macrophage, but not macrophage to PBMC. These multiple determinants work in concordance and confer additive effects towards either fitness or restriction in macrophages. Identification of these multiple HIV-1 genetic determinants provides a basis for investigation of novel macrophage specific factors that support replication and retention of virus, and may identify new targets for future drug therapies.

CHAPTER 1
BACKGROUND

HIV/AIDS Epidemic

From the 2008 UNAIDS/WHO global summary of the AIDS epidemic, the number of individuals living with the human immunodeficiency virus (HIV) as of December 2007 was 33 million (Figure 1-1) (1). The report continues to break down the number of infected individuals by numerous factors including age, sex and location, as well as highlights the number of newly acquired infections, 2.7 million (Figure 1-2), and the number of AIDS deaths, 2.0 million (Figure 1-3) and reports that over 25 million people have died due to HIV/AIDS. While the number of newly acquired infections has continued to decline since 1993 (Figure 1-4) given the advancements to antiviral drugs, the total number of infected individuals continues to increase. These statistics provide a basis for over $2.9 billion dollars of NIH sponsored research on HIV/AIDS in 2008 alone (2). While the distributions of funds are broken down into different fields of research from behavioral and social science to vaccines and microbicides, understanding how HIV interacts with its host on a molecular basis is essential for understanding its pathogenesis.

HIV Replication

HIV is the causative agent of AIDS. HIV infects CD4+ immune cells and acts to cause disease by reducing the number of CD4+ T cells from within an infected individual (8, 26, 48). By reducing the number of CD4+ T cells, individuals are left with an impaired immune system that leaves them susceptible to opportunistic infections, lymphadenopathies and cancers. HIV is a retrovirus of the *Lentivirus* genus and comes in two types, aptly named HIV type 1 (HIV-1) (Figure 1-5) and HIV type 2 (HIV-2). While

both types are genetically similar, HIV-2 has been observed to be less pathogenic as well as being immunologically different from HIV-1 (18). Like all retroviruses, the HIV-1 transcribes three major polyproteins; *gag*, the group antigen or structural proteins; matrix (MA), capsid (CA), nucleocapsid (NC), and p6, *pol*, the polymerase or enzymatic proteins; protease (PR), reverse transcriptase (RT), and integrase (IN), and *env*, the envelope or surface proteins; gp120 and gp41 (Figure 1-5).

HIV-1 infects target cells by recognition of Env to host cell CD4 and co-receptor. After infecting a target cell, HIV uses its virally encoded RT that is present within the virion to convert its RNA genome in to cDNA (Figure 1-6) (70) and forming a pre-integration complex (PIC). The PIC is then shuttled into the nucleus of the cell with the help of virally encoded Vpr (51), where the viral cDNA integrates into the host chromosome by its virally encoded IN. After integration, transcription of HIV-1 is controlled by the long terminal repeat (LTR) found at the 5' end of the genome. Using alternatively spliced transcripts to produce its regulatory proteins, Tat and Rev, HIV-1 is able to increase its production of transcripts, via Tat binding to the LTR and promoting increased transcription (20), and to increase the number of non-spliced transcript variants via Rev (52). While HIV-1 accessory proteins, Vif, Vpr, Vpu, Nef, and surface protein Env are translated from alternatively spliced transcripts (Figure 1-7), Gag and Pol are translated from unspliced transcripts. Gag, Pol, and Env proteins are transcribed into polyproteins that are cleaved into fully functioning mature proteins by either viral protease (PR), or host cell protease furin for Env. Gag and Pol proteins are found in different reading frames and thus ribosomal frameshifting occurs about one in twenty translations to produce the Pol proteins. Gag and Pol processing occurs during or

immediately after budding from an infected cell and occurs in a highly ordered process. Observing the cleaving of Gag and Pol into their fully functioning mature form is one way to assess virion maturation and is essential for infectivity of the progeny virions. After budding from a cell and cleavage of the Gag and Pol polyproteins, virions form a cylindrical electron dense core that is shown diagrammatically in Figure 1-8. This virion is now fully infectious for a new target cell.

HIV-1 Entry

HIV-1 adsorption and entry is mediated by Env (71). The Env surface protein is made of trimeric forms of two non-covalently linked proteins named gp120 and gp41. As entry into a cell is a necessary step for propagation of viral progeny, HIV-1 Env is a major viral determinant for infection. HIV-1 adsorbs to target cells by attachment of gp120 to host cell CD4. Recognition of CD4 induces a conformational change in gp120 and promotes its binding to a co-receptor, most notably CC chemokine receptor 5 (CCR5) (3, 22, 24, 82) or CXC chemokine receptor 4 (CXCR4) (25). A major determinant of co-receptor use is the overall charge of the amino acid residues of the hypervariable domain V3, with high charge indicative of CXCR4 use (12). Once bound to the target cell, Env gp41 mediates the fusion between the viral lipid membrane and the host lipid membrane allowing for the transfer of the viral core. The efficiency of these proteins to allow entry into target cells directly contributes to the fitness of a virus.

HIV-1 is not limited to infecting CD4+ T cells. HIV-1 can infect many different cell types that express CD4 and either CCR5 and/or CXCR4, including monocytes, dendritic cells, and macrophages. By identifying the type of cells HIV-1 is able to infect, or cell tropism, and through which co-receptor, one is able to define a phenotype of a specific virus. Table 1-1 defines the common phenotypes identified for HIV-1. The common

phenotypes; lymphocyte (L)-tropic, macrophage (M)-tropic, dual (D)-tropic, and T cell line (T)-tropic, describe cell tropism in context of only two co-receptors and are not intrinsically linked, thus providing evidence for post-entry determinants of cell tropism.

HIV-1 Post-Entry Determinants And Host Cell Factors

Viral fitness is defined as how efficiently a virus is able to replicate within a target cell and can be determined at both an entry-level and a post-entry level. While entry determinants, defined by attachment and penetration of a virus, can be attributed to Env, post-entry determinants are not completely understood. Table 1-2 shows a list of post-entry viral determinants and their host factors of interaction that are necessary for efficient replication in target cells. Some host cell factors are inherently restrictive for the virus and others are necessary to facilitate viral infection. Identifying and understanding the interactions between post-entry viral determinants and their interacting host factors may identify new targets for disruption by therapeutic agents.

Post-entry genetic determinant of retroviruses were identified in mice and led to the identification of host cell restriction factors. These murine restriction factors termed lentivirus susceptibility factor 1 (Lv1) and restriction factor 1 (Ref1) were observed in human cells and later determined to be the same protein, tripartite motif protein 5α (TRIM5α) (10, 37, 41, 62). TRIM5α is one of more than 70 human TRIM genes. TRIM5α, through multimerization and E3 ubiquitin ligase activity, demonstrates restriction between different retroviruses based on their ability to replicate within target cells of different species (9, 46, 50, 87, 88). TRIM5α acts in a species-specific manner to interact with the HIV-1 capsid (CA) protein with the proposed mechanism of prematurely disassembling or degrading the virion core before reverse transcription can

occur (79, 86, 89). At least three other innate restriction factors have been identified in human cells. Cyclophilin A (CypA) is another species-specific protein that interacts with HIV-1 CA and has been shown to be necessary for efficient replication (10, 36, 69). CypA is a peptidyl-prolyl cis-trans isomerase that acts to promote uncoating of incoming virions (40). A recent publication determines CypA as the necessary component in TRIM5α mediated resistance to HIV-1 in Old World monkey cells (10). Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like protein 3G (APOBEC3G) is another well-characterized host restriction factor. APOBEC3G has two known mechanisms of action, one, by creating hypermutations in the cDNA of a newly synthesized HIV-1 transcript within an infected cell, and two, by interfering with the strand transfer during the reverse transcription process (refer to Figure 1-6) (14). The viral protein Vif counters the anti-viral activity of APOBEC3G by directly binding to APOBEC3G and targeting it for ubiquitination and subsequent degradation through the proteosome (54, 75). The third characterized restriction factor is Tetherin/Bone marrow stromal cell antigen 2 (BST-2)/CD317. Tetherin acts on HIV-1 after budding by tethering new virions to the surface of the producer cell (53, 61). The mechanism of Tetherin is currently unknown, but the accessory protein Vpu blocks its antiviral effect by specifically targeting Tetherin for degradation (59, 68). Inspection of the NIAID HIV-1, Human Protein Interaction Database shows a large number of host cell factors that may play a role in host cell restriction and viral fitness. Identifying the determinants of HIV-1 and understanding their ability to subvert these innate host cell restrictions will provide insight into post-entry effects on viral fitness.

HIV-1 Infection In Macrophages

HIV-1 cell tropism can have a significant impact in disease progression as infection of cells in the brain can lead specifically to HIV-associated dementia (HAD) (32). Cell tropism of HIV-1 can be controlled at two different stages of infection, entry and post-entry. Identifying post-entry determinants of HIV-1 infection will give insight into its mechanisms involved in replication. While all HIV-1 is able to infect T lymphocytes, not all HIV-1 is able to infect macrophages (Table 1-1) (83). The environment within a macrophage is distinctly different from a T cell, with macrophages being terminally differentiated and having limited replication potential. Although some CCR5-using (R5) viruses are cytopathic, infections in macrophages show little to no cytopathic effects (58). Pathogenic effects from R5 viruses are distinctly different from X4 viruses (34, 35). HIV-1-infected macrophages can be isolated from bone marrow, blood, brain, lung, lymph nodes, and skin of infected individuals (13, 28, 29, 43, 73). Macrophages are likely to be the first cell type to be infected by HIV-1 (58, 84). Early categorical schemes were devised to differentiate viruses that possessed the ability to infect macrophages (M-tropism) and those with the ability to infect T cell lines (T-tropism). The relationships identified were non-syncytium inducing (NSI) R5 envelopes with M-tropism and syncytium inducing (SI) X4 envelopes with T-tropism (17, 23, 31, 73, 76). The discovery of viruses that were able to infect both cell types through the use of either co-receptor (dual tropic, D-tropism) and their uncoupled effect of co-receptor use and cell tropism showed the necessity to create a need for new phenotypic definitions (30, 32, 33, 60, 77, 81). While viruses may enter a target cell by recognizing CD4 and these co-receptors on a variety of cells, there are post entry determinants that play major roles in their ability to replicate.

Macrophages Are A Viral Reservoir

T cells are the major targets of HIV-1 infection. Long-lived memory T cells can harbor infection and act as a viral reservoir, while activated T cells are short lived upon infection (55, 65). Macrophages can live for weeks or months while harboring infection and can continue to spread infections to T cells *in trans* (5, 74). Macrophages are long-lived hematopoietic cells of the innate immune system that play a role in fighting infection by phagocytosis and destruction of foreign material. Macrophages are mobile cells that can be found in most tissues of the body and are also involved in removing dead cellular debris. After infection from an invading organism, monocytes from the blood can extravasate at the site of injury and differentiate into macrophages. For HIV-1 infection, macrophages can act as a vector for dissemination of virus into different tissues as well as from person to person (58). Experiments show that macrophages represent an important long-living reservoir for viral production that is maintained within a host (11, 16, 31, 38). Moreover, macrophages can produce significant amounts of HIV-1 with viral stores observed within multivesicluar bodies (MVB) (5, 66). Different signal transduction pathways exist between macrophages and lymphocytes; these pathways can be altered by HIV-1 and affect their susceptibility to infection (6). The replication life cycle of HIV-1 takes longer to complete in macrophages than in lymphocytes (63).

M-tropism And Disease

Over the course of infection, HIV-1 expansion of co-receptor use, from R5 to X4, is observed in ~50% of infected individuals; the use of X4 envelopes is associated with rapid advancement of disease progression and immune suppression (42, 67, 73, 80). However, the ~50% of the infected individuals who do not harbor viruses with expanded

co-receptor use, still progress to AIDS, arguing that switch to CXCR4 use is not a necessary component for disease progression (21, 78). HIV-1 R5 viruses isolated from individuals with AIDS have enhanced M-tropism related to CCR5 efficiency and cause increased CD4+ T cell death compared with HIV-1 R5 viruses isolated from asymptomatic individuals or individuals in early disease stages (33, 44, 49, 81). HIV-1 viruses that replicate in macrophages are usually associated with the phenotype of NSI and R5 envelopes (15, 49, 82). Recent experiments have shown that viruses isolated early in infection are not always M-tropic and that M-tropism is not necessary linked to CCR5 use, thus confounding the effect of what elements are important for M-tropism (32, 33). These findings indicated replication in macrophages might be independent of CCR5 use.

Existence Of D-tropic Viruses

D-tropic viruses have the ability to replicate efficiently in both macrophages and T cell lines. Phenotypically, D-tropic viruses use either CXCR4 alone, or in combination with CCR5 as co-receptors to enter target cells (19). D-tropic viruses have been isolated from the brain, CNS, lymph nodes, and thymus (32, 90). The presence of D-tropic viruses is associated with, and has a causal relationship towards, advancement of disease (57, 81). HIV-1 infection by X4 or R5 viruses can have separate signaling events. Some groups show signaling through CCR5 or CXCR4 may control a differential capacity for infection in different cell types and contribute to viral pathogenesis (6, 31). Yet, Amara *et al*. (4) have shown that efficient infection of macrophages is not dependent on CCR5 signaling. Also, macrophages have different profiles of expression between CCR5 and CXCR4. The existence of D-tropic viruses has confounded the ability to strictly associate co-receptor use and viral tropism.

Significance And Specific Aim

Macrophages play a pivotal role in HIV-1 pathogenesis with respects to two significant areas: macrophages are a significant viral reservoir contributing to the persistence of HIV-1 infection within an individual and increased M-tropism is associated with advanced disease. R5 viruses develop different replication strategies and phenotypes over advancement of disease state and understanding these changes is important in developing strategies to cure or reduce viral pathogenesis. The hypothesis for the following research is that HIV-1 has determinants that map outside of gp120 and play a role in M-tropism. Identifying these determinants and their impact on macrophage-specific restriction could lead to novel drug therapeutics and vaccine designs to address this important viral reservoir.

The specific aim of this project is to identify mechanism of restriction within non-M-tropic viruses with R5 envelopes. HIV-1 strain LAI is a X4 virus. Its inability to infect macrophages was originally thought to be restricted at the level of entry, but when combined with an R5 envelope, infections produced a unique restricted phenotype in macrophages, but not T lymphocytes. The new understanding is LAI has a novel cell-type specific restriction outside of Env that restricts spread of virus from macrophage to macrophage. The restriction may play a role in removing infection from macrophages. The specific goals of this dissertation are to map the viral genetic determinants of restriction by creating chimeric viruses between regions of an M-tropic virus AD8 and the restricted T-tropic virus LAI, to identify where in the replication cycle macrophage restriction occurs, and to identify the viral protein processing phenotype.

Table 1-1. HIV-1 viral phenotype is associated with co-receptor use and cell tropism.

Phenotype	Co-receptor use	Ability to infect lymphocytes	Ability to infect macrophages	Ability to infect T cell lines
L-R5	CCR5	Yes	No	No
M-R5	CCR5	Yes	Yes	No
D-R5X4	CCR5 and CXCR4	Yes	Yes	Yes
D-X4	CXCR4	Yes	Yes	Yes
T-X4	CXCR4	Yes	No	Yes

Table 1-2. HIV-1 post-entry determinants interact with known host cell factors.

Viral protein	Host cell factor(s)	Host cell factors attribute for infection
Vif	APOBEC3G	Restrict
	APOBEC3F	Restrict
	Cul5 ubiquitin ligase complex	Restrict
Vpr	Importins	Facilitate
	Nucleoporin	Facilitate
Vpu	BST-2 (Tethrin)	Restrict
Nef	CD4	Facilitate
	MHC-1	Facilitate
Rev	Importins	Facilitate
	Exportins	Facilitate
RT	dNTPs	Facilitate
CA	TRIM5α	Restrict
	CypA	Facilitate
Gag	AnxA2	Facilitate

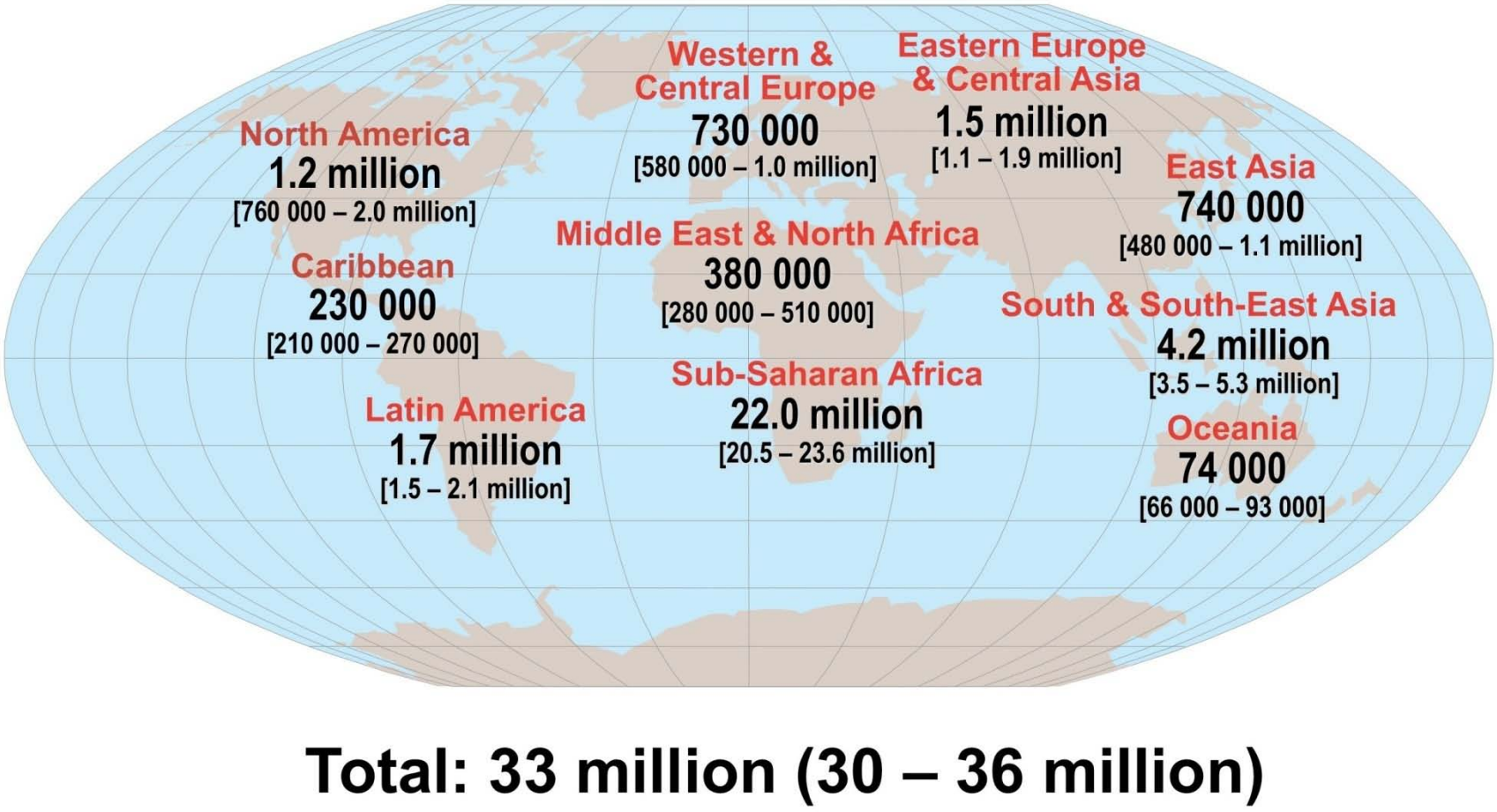

Figure 1-1. Estimated individuals currently living with HIV/AIDS as of 2007 (1).

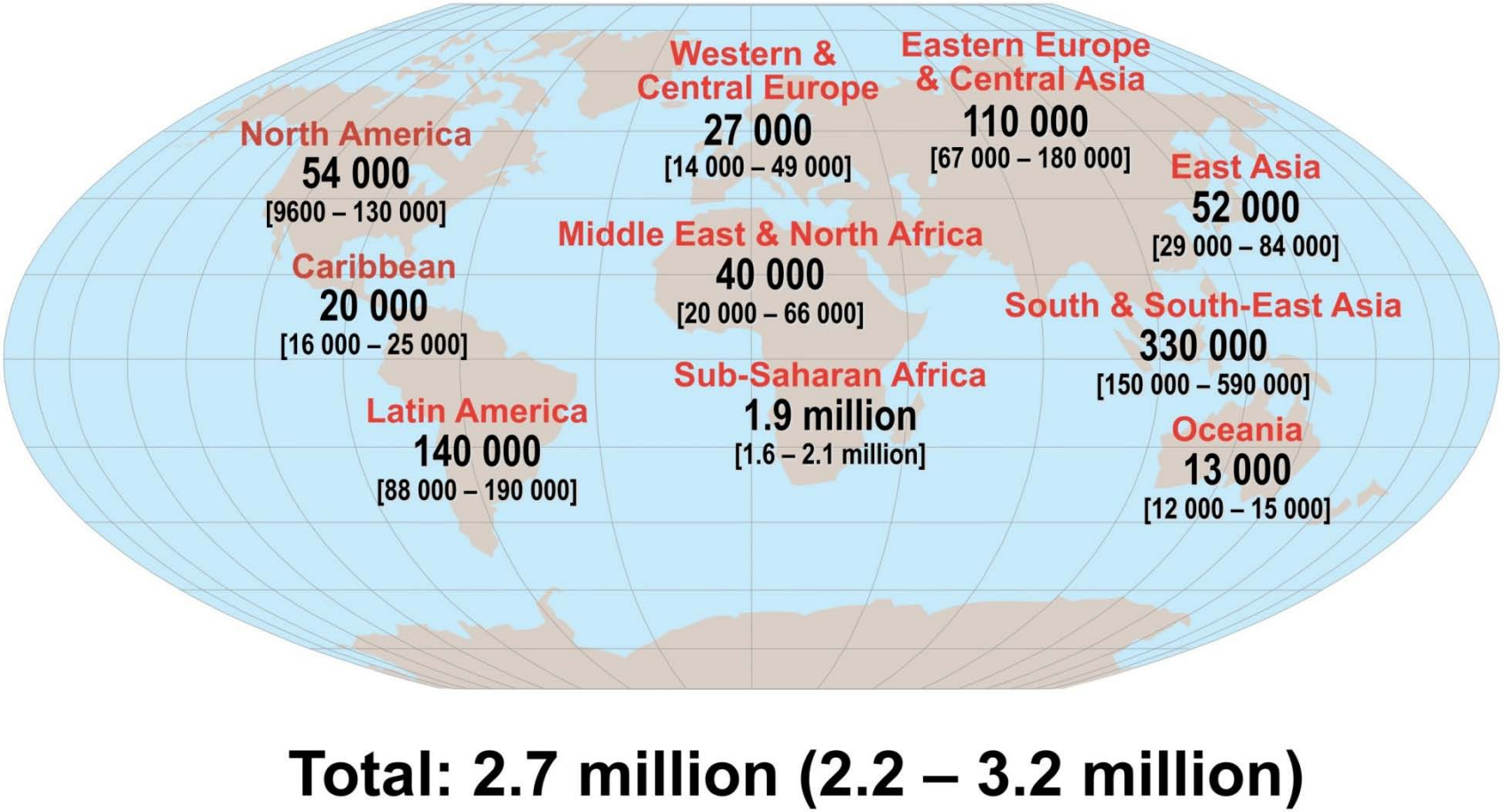

Figure 1-2. Estimated number of new HIV infections in 2007 (1).

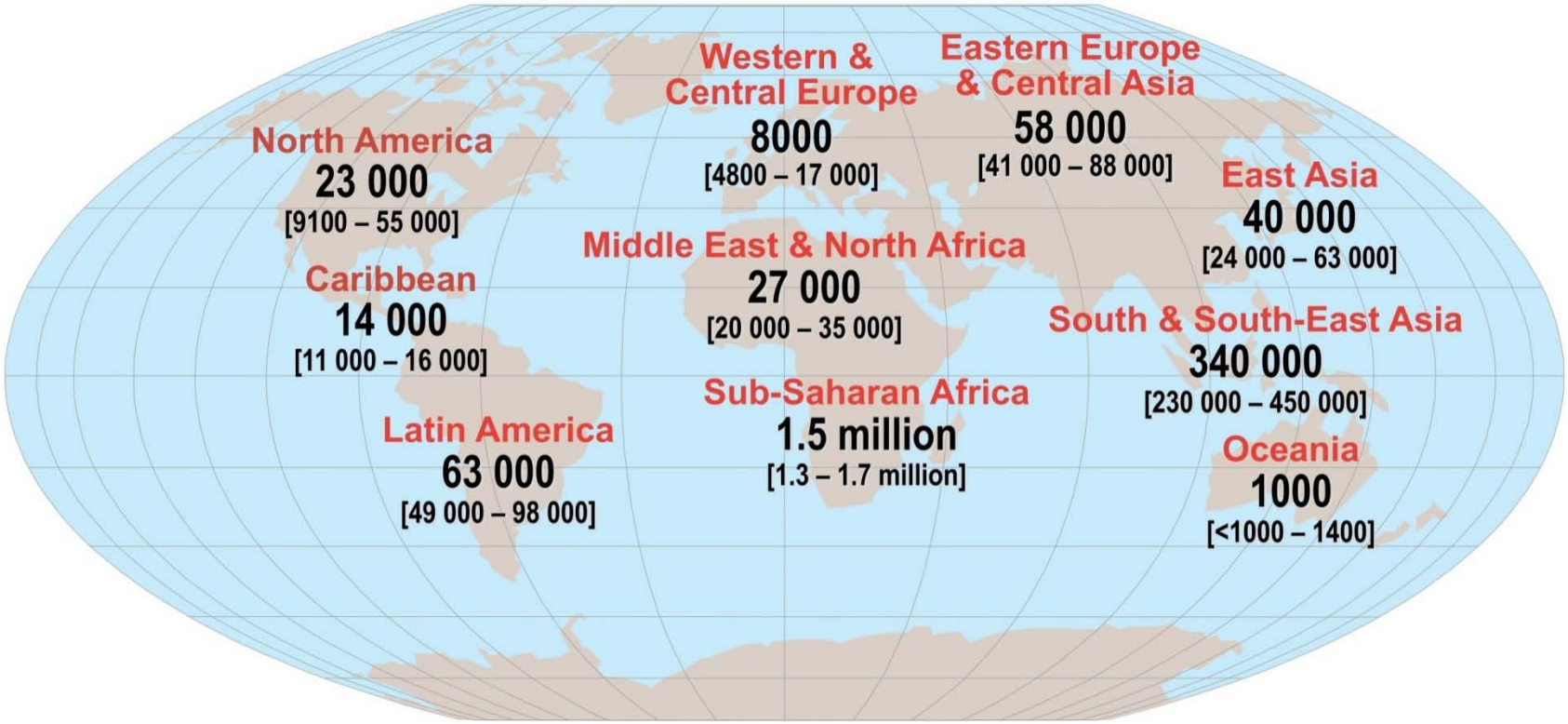

Figure 1-3. Estimated mumber of HIV/AIDS related deaths in 2007 (1).

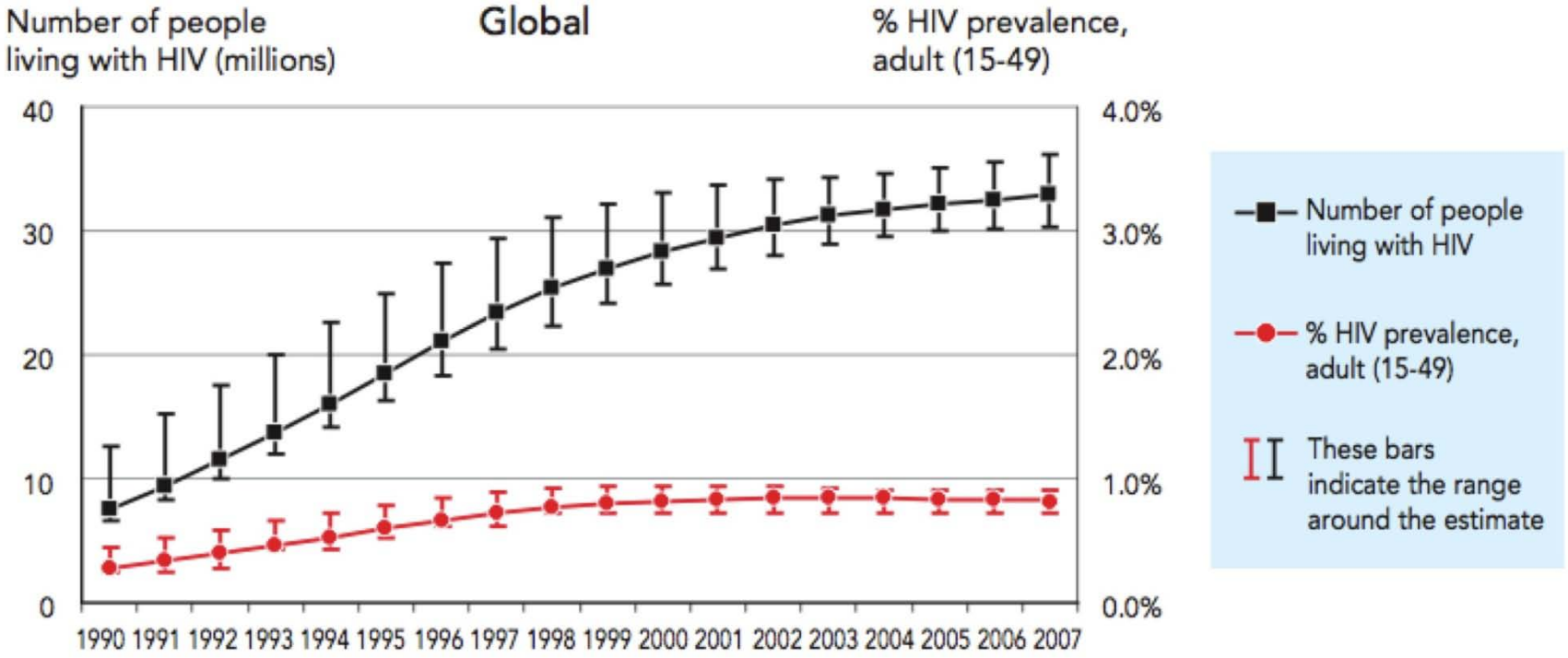

Figure 1-4. The number of individuals living with HIV per year and the percentage of total adult population 15-49 year of age with HIV (1).

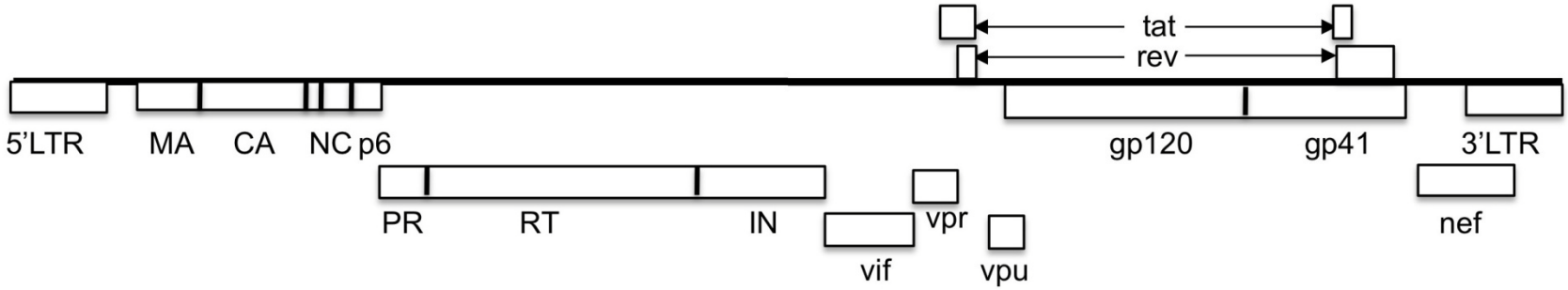

Figure 1-5. The HIV-1 genome. Horizontal bars indicate cleavage sites of viral or host proteases.

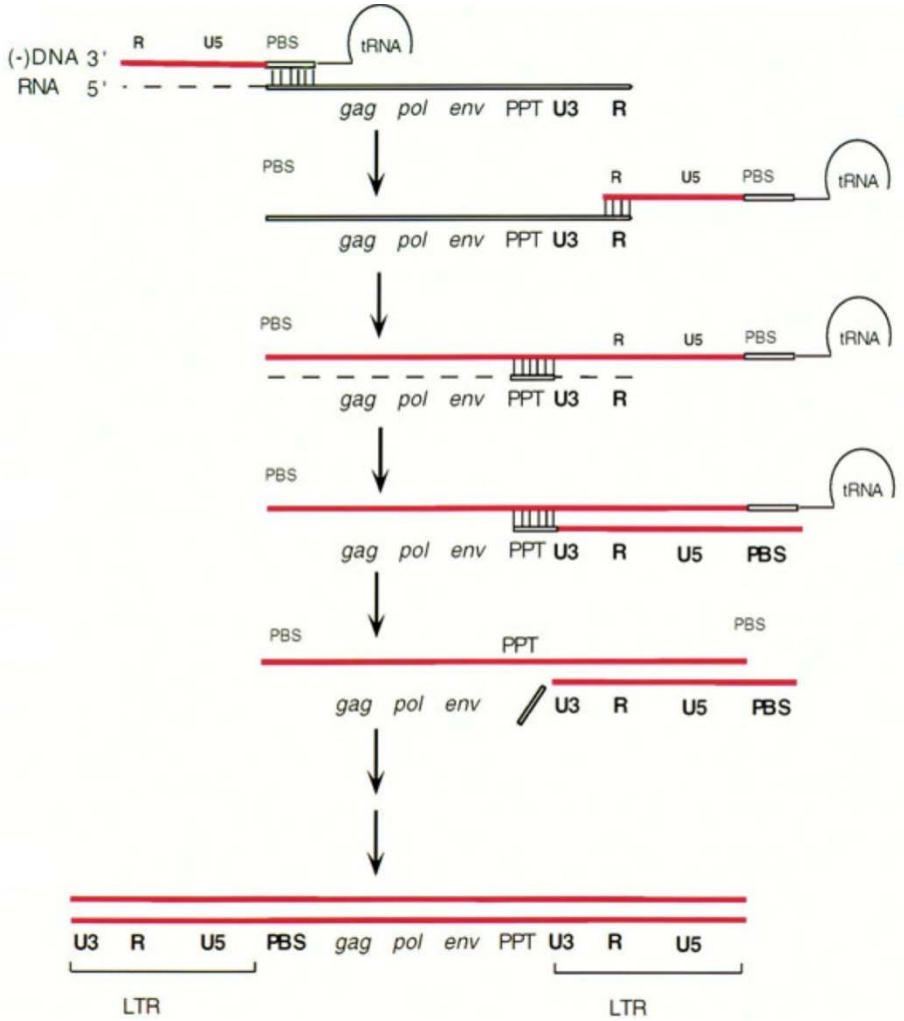

Figure 1-6. Steps showing reverse transcription of HIV RNA into cDNA. Dark lines indicate DNA. Gray lines indicate RNA. Open boxes indicate RNA primers (70).

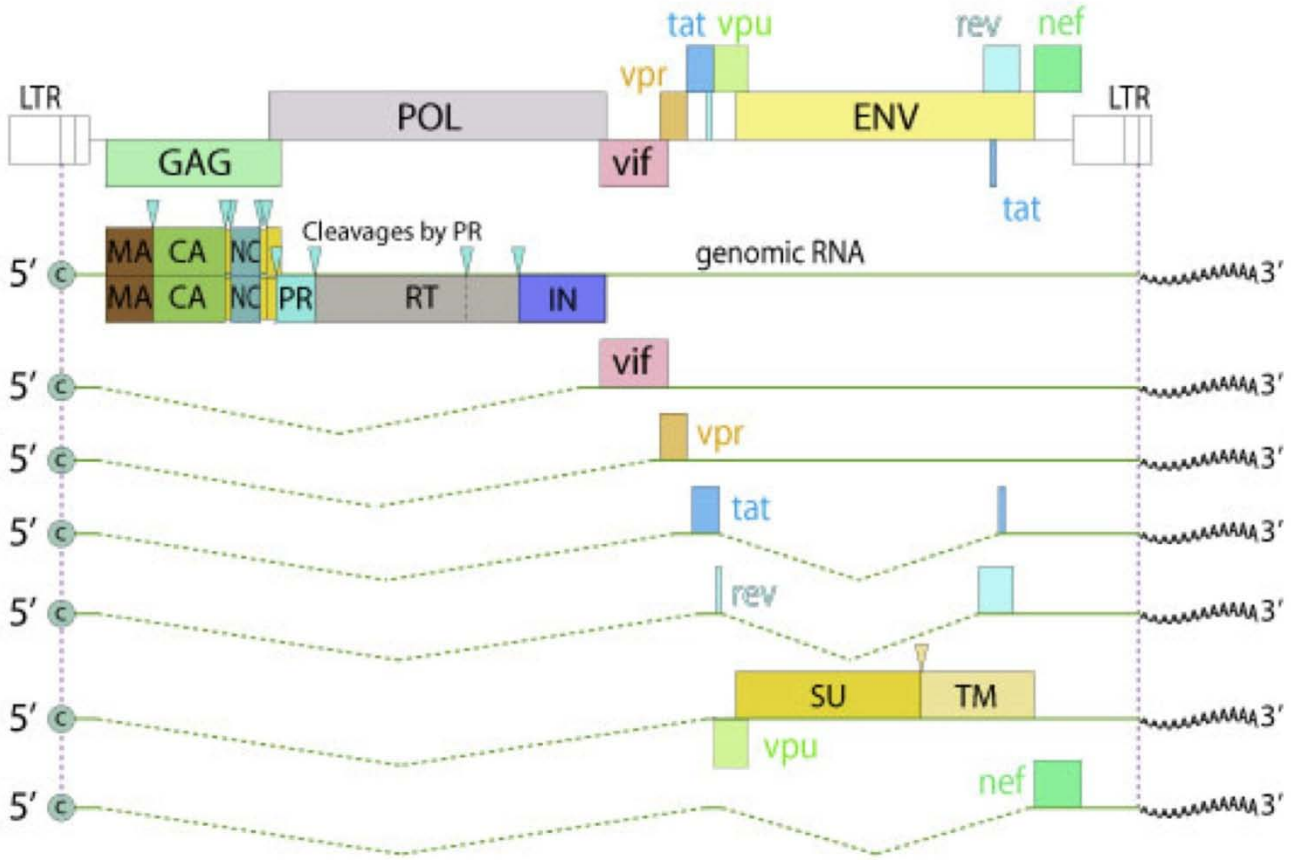

Figure 1-7. Transcription and splicing of HIV RNA leads to multiple transcripts and the pathways used by HIV to produce its various proteins (Source: http://ftp.expasy.org/viralzone/all_by_species/7.html).

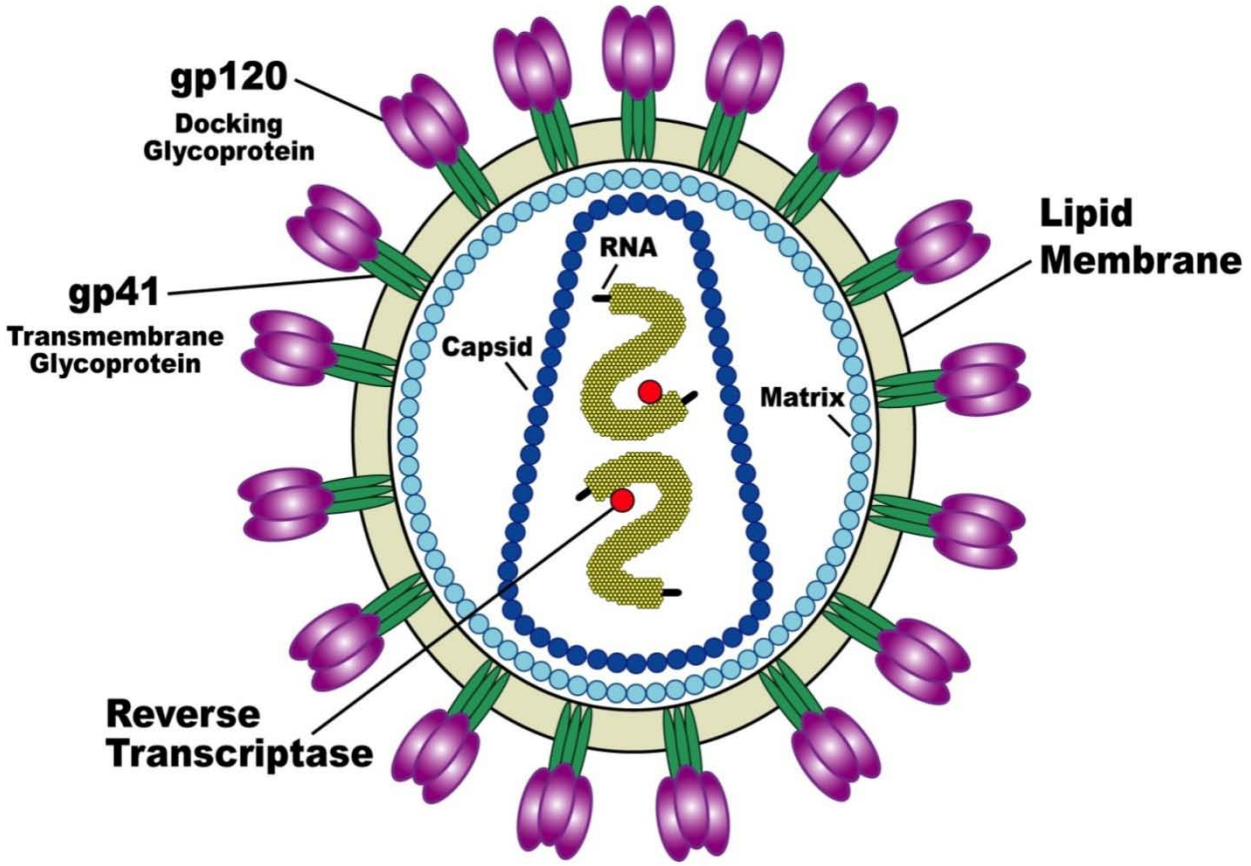

Figure 1-8. Fully mature HIV-1 virion (Source, NIH).

CHAPTER 2
HIV-1 MAPPING OF POST-ENTRY RESTRICTION IN MACROPHAGES

Introduction

HIV-1 infects multiple CD4+ cell types of both the lymphocyte and monocyte lineages including non-dividing macrophages. Macrophages play multiple roles in HIV-1 infection that are advantageous for both the virus as well as for HIV pathogenesis. Macrophages can act as long-lived reservoirs harboring the continuous production of infectious virus (5), provide hot spots for recombination (47), as well as maintain infection in the presence of antiretroviral therapy (ART) (45, 56, 91). Macrophages are non-replicating cells that are not killed by direct infection of HIV-1. They also display few cytopathic effects and can contribute significantly to disease progression. For example, in a rhesus macaque model, macrophages were the principal reservoir for sustaining high viral loads after rapid depletion of CD4+ T cells (39). Macrophages can act as indicator of disease as observed in studies showing enhanced viral M-tropism is associated with advanced disease progression and act as mediator of disease by direct transmission of infectious particles to CD4+ T cells (74). In a hu-SCID mouse model, as few as 500 HIV-exposed macrophages were able to induce the rapid depletion of millions of CD4+ T cells (27).

A body of publications has identified Env gp120 as a major determinant of M-tropism, correlating entry of virus into macrophages as an indicator of cell infection and tropism. By replacing the entire gp120 or the V1-V3 regions of Env gp120 of HIV-$1_{NL4.3}$ with a CCR5-using Env from either HIV-1_{AD8} or HIV-1_{BaL}, respectively, confers the ability of HIV-$1_{NL4.3}$ to infect and spread in macrophages (85). Thus, the restriction of wt HIV-$1_{NL4.3}$ is restricted at entry. Other studies identifying Env as the determinate of M-

tropism have observed infection of macrophages through the use of CXCR4, thus highlighting that CCR5 use is not strictly related to M-tropism (30, 72).

Evidence from previous research has indicated that M-tropism can be confer through CCR5 use, but research in our lab has provided a unique phenotype of a T-X4 molecular that does not confer M-tropism with CCR5 use. This observation led to the hypothesis that viral determinants outside of Env gp120 play major roles in M-tropism. To test this hypothesis chimeric viruses were created between the M-tropic molecular clone HIV-1_{AD8} and the T-tropic molecular clone of HIV-1_{LAI}. Although studies have shown that HIV-1_{LAI} can enter into macrophages via CXCR4 (72), albeit poorly, HIV-1_{LAI} was modified by replacing its Env gp120 with the CCR5-using Env gp120 of HIV-1_{AD8}. This technique provides a model of efficient entry for HIV-1_{LAI} and provides a template for gain of function studies identifying viral determinants of M-tropism. The following study describes a unique phenotype of HIV-1_{LAI} that identifies multiple regions in Gag-Pol that are responsible for its restriction in macrophages and that these determinants play important roles for M-tropism.

Results

Restriction Of LAI(AD8) In Macrophages.

Three HIV-1 molecular clones were used to identify viral determinants of macrophage tropism: an M-tropic, CCR5-using clone (AD8), a T-tropic, CXCR4-using clone (LAI) and a constructed chimeric clone which includes the V1-V5 hypervariable domains of pAD8 gp120 within the LAI backbone (LAI(AD8)) (Figure 2-1). Viruses were derived by transfection of 293T cells were titrated and used to infect PBMC to determine a baseline of overall viral fitness. Figure 2-2 shows the growth kinetics of the viruses AD8, LAI, and LAI(AD8) from a representative PBMC infection sampling supernatant

p24 every two days for eight days post-infection (p.i.). These data show all three viruses have similar growth kinetics with CA/p24 protein (p24) levels peaking around day eight p.i.

To determine the growth kinetics of AD8, LAI, and LAI(AD8) in macrophages, MDM were infected and cultures were sampled every three days for 16 days p.i. Infection with AD8 led to an M-tropic viral phenotype and infection with LAI led to a restricted T-tropic viral phenotype due to block at the level of entry (Figure 2-3). Infection with LAI(AD8) led to partial M-tropic viral phenotype that produced detectable levels of p24, but displayed a block in spread that occurs early after infection and persist over the time course of infection. In Figure 2-4, infections among multiple PBMC donors show LAI(AD8) produced a median value of 61.6% of supernatant p24 compared to AD8 at day eight p.i., indicating LAI(AD8) maybe inherently less fit than AD8 for infection through CCR5; differences observed in peak productions of p24 between LAI and AD8 are most likely due to their co-receptor use and susceptible target populations. Infections among multiple macrophage donors show LAI(AD8) produced a median value of 2.3% of supernatant p24 compared to AD8 at day 16 p.i. Comparing supernatant p24 between LAI(AD8) versus AD8 among the different cell types show LAI(AD8) produces significantly less p24 ($p=0.0007$) from MDM indicating a specific phenotype within this cell population (Figure 2-4). These results support the hypothesis that LAI is restricted for growth that is not defined by its inability to use a functional M-tropic gp120.

LAI(AD8) Fails To Spread In Macrophage Cultures Although Virions Are Infectious.

As observed in Figure 2-3, LAI(AD8) produces detectible, albeit low amounts of supernatant p24 from infection of MDM. These low, persistent levels of p24 could indicate two possible hypotheses for LAI(AD8) restriction. One, infection of MDM by LAI(AD8) is inducing rapid cell death among the infected cell population where p24 levels of spreading virus is limited due to lack of virion production. Or two, infection of MDM by LAI(AD8) is restricted for spread after first round infection and steady-state production of virus has been achieved. To test these hypotheses real-time qPCR was used to detect the number of viral cDNA copies produced upon infection of MDM. A representative experiment in Figure 2-5 shows infection of AD8 resulting in an increasing number of cDNA copies per cell over time, whereas LAI(AD8) shows detectible levels early in infection, but fails to increase over time. The total number of cells as tracked by ApoB copies between infections of AD8 and LAI(AD8) were not statistically significant indicating cell death was not induced by one virus over another (data not shown). These results support the hypothesis that LAI(AD8) restriction occurs at the level of spread after first round infection.

After identifying LAI(AD8) is unable to spread in macrophage cultures, we hypothesized that the supernatant p24 and thus, virions being produced from these MDM were inherently deficient and non-infectious. To test this hypothesis, MDM were infected with AD8, LAI, or LAI(AD8) and supernatants were collected after four days p.i. Supernatants were subsequently used to infect heterologous PBMC and sampled every two days for a total of eight days for supernatant p24. Figure 2-6 shows that both MDM-produced AD8 and LAI(AD8) were able to infect and spread in PBMC cultures. As a

control LAI passaged on MDM was unable to spread in PBMC. These results identified that MDM-produced LAI(AD8) is infectious and not inherently deficient and that LAI(AD8) virions are specifically restricted for spread in MDM cultures. This phenotypic observation reinforces the idea that the restriction observed in LAI(AD8) is cell-type specific and that the determinant is located outside of Env gp120.

Processing Phenotype Of LAI(AD8)

With the observation that MDM-produced LAI(AD8) is restricted in MDM, but infectious for PBMC, the next goal was to identify the maturation state of virions of both AD8 and LAI(AD8). By using an antibody that recognizes capsid in its immature Gag/p55 state as well as its mature p24 state, one is able to determine if there are any differences in the processing of these proteins that may elucidate the differences in growth kinetic observed in Figure 2-3. Using an *in vitro* transcription and translation assay, AD8 Gag and LAI Gag display similar processing profiles of p55 (Figure 2-7). These results indicate the Gag regions of AD8 and LAI do not have any inherent differences in cleavage efficiency that would bias their p55 processing in culture. A western blot analysis of supernatants taken from day 8 infected PBMC shows AD8 and LAI(AD8) have similar amounts of p24, with an absence of p55 (Figure 2-8). Western blot analysis of supernatants from day 10 infected MDM shows AD8 and LAI(AD8) have discrepancies in the ratio of processed p24 compared to unprocessed p55, with AD8 producing a ratio of 1.60 p24 to p55 and LAI(AD8) producing a ratio of 0.13 of p24 to p55 (Figure 2-8). These results suggest that less processing of LAI(AD8) from immature p55 to mature p24 is contributing to its restriction in MDM.

These results identify inefficient processing of p55 as a possible mechanism for LAI(AD8) restriction in MDM. It should be noted that western blot is able to identify the

total ratio of p55 and its cleavage products in the supernatant, but is unable to describe the processing effects on individual virions. Thus, supernatant virions could be either all partially processed or a mixed population of fully and partially processed virions are produced. Although, from passaging LAI(AD8) from MDM to PBMC, there must be at least a minority of virions that are fully processed and infectious.

Construction Of Chimeric Viruses.

From the previous experiments, the processing of p55 maybe responsible for the restriction that is observed in MDM. Therefore, three molecular clones were constructed to map the genetic determinants of M-tropism outside of gp120 by swapping regions of AD8 into the backbone of LAI(AD8) (Figure 2-9). The rationale behind the design of these constructs was to indentify regions of HIV that would likely restore function of LAI(AD8) as well as harbor determinants of M-tropism. Given that LTR regions control the transcriptional regulation and replication of HIV, pLAL was constructed which is an AD8 clone containing both the 5'LTR and 3'LTR from LAI. The construct 5' was constructed with the Gag-Vif coding regions of AD8 in the backbone of LAI(AD8). The p5' construct was used as an analog for the construct NL4.3, as NL4.3 was constructed similarly with components of NY5 and LAI. NL4.3 is able to be complimented with a CCR5-using envelope and produce an M-tropic phenotype. The construct p3' was constructed containing the gp41-Nef coding regions from AD8 in the backbone of LAI(AD8). The p3' construct was created to determine if gp41 was playing a role in M-tropism. While infections of PBMC within a single donor with these viruses had similar growth kinetics as AD8 (Figure 2-10), infections in MDM by LAL and 5' produced a median value of 35.9% and 38.9% supernatant p24 relative to AD8, respectively. 3' produced a median value of 4.8% supernatant p24 relative to AD8 (Figure 2-11). These

results show that the Gag-Vif region of AD8 is responsible for overcoming the macrophage restriction seen by LAI(AD8) and that this restriction does not map to the regions between gp41-Nef.

These results indicate that the Gag-Vif region harbors unique determinants of M-tropism that act downstream of virion entry into a cell. While differences observed between LAL and AD8 point to the LTR region playing a significant role in macrophage fitness, our goal was to identify LTR independent regions that play a role in M-tropism. These constructs together rule out Vpr, Vpu, Nef, Tat, Rev, and gp41 as determinants of this restriction phenotype. While Gag-Vif of AD8 was used to overcome the restriction observed in LAI(AD8), this region was predicted to contain determinants given the ability of NL4.3 to acquire an M-tropic phenotype when complimented with a CCR5-using gp120. Together these data show the Gag-Vif region to be playing the major role in M-tropism and is a novel macrophage restriction phenotype.

Construction Of Chimeric Gag-Vif Viruses.

A second panel of constructs was created to localize the viral determinants that are responsible for M-tropism that map within the Gag-Vif region of AD8. After genome scanning of the region for amino acid differences, the greatest number of consecutive changes was found in the p6 region. A unique, but *in vivo* observed, PTAP amino acid sequence duplication was found in LAI, but missing in AD8 (Figure 2-12). As PTAP is a known late domain of HIV-1 and is necessary for the proper assembly and release of mature virions a 1-PTAP clone was created to remove the sequence duplication from LAI(AD8). Four other constructs were made with names describing the region of AD8 swapped into LAI(AD8): Gag-PR, AD8 Gag in context with its own PR, RT-Vif, the remaining region from the 5' construct, MA-CA, and CA-Vif, AD8 PR in the context with

its own Pol region (Figure 2-13). All constructs were tested for infectivity in PBMC (Figures 2-14, 2-15, 2-16) and used to infect multiple MDM donors.

Figure 2-17 shows the median values of supernatant p24 from the molecular clones compared to 5' in percent. CA-Vif plays the largest role in restoration of the 5' phenotype with a median value of 84.4% followed by Gag-PR with a median value of 29.5%. These results indicate that the duplication of the PTAP region in LAI does not play a role in LAI(AD8) macrophage restriction and observations from these infections also lead to the conclusion that there are multiple determinants within the Gag-Vif and more specifically the CA-Vif region that play a vital role in M-tropism and macrophage fitness.

Model Of LAI(AD8) Restriction In Macrophages.

The results above provide evidence for a novel macrophage restriction phenotype of LAI that maps to multiple determinates within the CA-Vif region. Figure 2-18 depicts a model of the restriction phenotype observed. LAI(AD8) produced from 293T is able to infect both MDM and PBMC, but virus is only allowed to spread in PBMC cultures and from MDM to PBMC. Two possible mechanisms exist for LAI(AD8) restriction: one, a host cell accomplice(s) that is specifically necessary for spread in MDM cultures is unable to interact with the CA-Vif region of LAI(AD8), or two, a host cell restriction factor that is specifically restricts spread in MDM cultures, but is overcome by an M-tropic CA-Vif or not restrictive within a permissive cell type. Given the body of literature on the subjects of host cell accomplices and host cell restriction factors it seems more likely that a host cell accomplice is playing a role in LAI(AD8) restriction in MDM, as will be discuss later, but the provided evidence does not rule out either possibility.

Discussion

HIV-1_{LAI} contains a novel post-entry restriction that maps to multiple determinants in Gag-Vif that have an additive effect for M-tropism and viral fitness. Using a replication competent system involving a late stage T-X4 backbone complimented with a CCR5-using Env provides a model for unchaining entry events from post-entry events, thus yielding a system for identifying post-entry viral determinants and allowing for future identification of host cell factors. From Figure 2-3, LAI(AD8) infection shows a clear restriction in MDM compared to infection in PBMC, while infections of MDM with other complimented T-X4 viruses, specifically HIV-$1_{NL4.3}$, show no restriction for growth. These observations hint towards a post-entry restriction, and more specifically a post-entry restriction with a viral determinant that maps to Gag-Vif given the similarities between NL4.3 and LAI; NL4.3 being a constructed recombinant virus comprised of the 5'LTR-Vif region of the clone NY5 and the Vpr-3'LTR region of LAI.

Phenotyping of LAI(AD8) shows that the restriction in MDM is not a complete block in replication. Virions produced from transfected 293T are able to infect MDM by evidence of both supernatant p24 production as well as detection of early HIV-1 cDNA products. Also, virions produced from MDM are able to infect PBMC. These observations highlight that in MDM cultures, spread of virus is blocked and viral output is poor, but virions are still infectious for a permissive cell type. This indicates that the restriction is highly specific for macrophages and does not follow the trend of such identified restriction factors that restrict viruses for all cell types, such as APOBEC3G. While the current host cell factor(s) and mechanism of the observed restriction are unknown, the identification of this restriction provides evidence for post-entry

determinants that are required for stable maintenance of infection between macrophages and perhaps retentions of virus within macrophages reservoirs.

Creating genetic recombinants between AD8 and LAI(AD8) provides gain of function experiments that could be compared between multiple donors. While transcriptional regulation of HIV-1 is controlled by LTR function, it was not surprising that swapping the LTR region of LAI into AD8 would result in a change of viral fitness, as observed with LAL (Figure 2-11). Considering these results, it was surprising to find that the *gag-vif* region of AD8 is able to completely restore M-tropism for LAI(AD8) given that many of the accessory proteins, specifically Vpu and Nef, have been implicated in their necessity for efficient replication in macrophages. Further mapping this region led to the interpretation that having larger segments of *gag-vif* ultimately leads to enhanced fitness in macrophages. While the data show that removal of a 12 amino acid duplication in p6 containing a PTAP domain failed to restore M-tropism in LAI(AD8), the observation that creating chimeras of varying lengths of different regions has an additive effect on viral fitness with the CA-Vif virus largely restoring the 5' phenotype.

Post-entry determinants of M-tropism have been shown map to multiple regions with *gag-vif*. While first round infection, from entry to budding, is not blocked, second round infection is specifically blocked in MDM. From the genetic mapping results, these multiple determinants work in concordance to produce more fit virions. Western blots show that there is less processed p24 from the p55 precursor from virions collected from MDM infected with LAI(AD8) compared to AD8. This observation also supports the evidence that released virions from LAI(AD8) infections are properly mature when budding from MDM. The lower ratio of processed p24 is most likely derived from fully

functioning virions and not a mixture of partially processed p24 from every budding virus. This is evidenced by the fact that only fully processed, mature virions are infectious. While polymorphisms in *gag* can have dominant effects on protease processing in PBMC, little is known about processing effects of different proteases in MDM, but *in vitro* transcription and translation assays show that there are no inherent deficiencies in the Gag cleavage sites (Figure 2-7).

The restriction of LAI(AD8) in macrophages fits two proposed models. One, that LAI(AD8) is unable to interact with a host cell factor, or accomplice, that is necessary for its replication in macrophages, but is dispensable in PBMC. Or two, LAI(AD8) is restricted for growth by a host restriction factor that disables it specifically for spread in MDM. The data provided do not favor one model over another, and further characterization through a heterokaryon assay may prove most fruitful, but understanding how both restriction factors and host accomplices interact with replication, restriction by a failure to interact with a host accomplice maybe more likely. Most restriction factors work at the level of global virion production, like APOBEC3G and Tetherin, or at the level of uncoating, like TRIM5α. LAI(AD8) virions passaged through MDM are infectious for PBMC which does not fit the model of an APOBEC-like restriction; moreover, no other restriction factor has been described in the literature with this observed phenotype for HIV-1. As for TRIM5α, this restriction would be observable during first round infection. LAI(AD8) fits more of a CypA restriction phenotype, where interaction with CypA is necessary for efficient replication. While the restriction shown above is not the CypA restriction, as swapping the binding domains between AD8 and LAI(AD8) did not restore macrophage fitness, it mimics the phenotype of the CypA

restriction. Whether or not a CypA-like host accomplice – in phenotype, not mechanism – is in the producer cell or the target cell remains to be identified. The results of these experiments clearly show that there is a high degree of donor variability for HIV-1 infection and there is yet to be a reproducible cell culture system that would allow for the exploitation of the observed LAI(AD8) restriction. With these limitations further progress will be made to continue to identify the host cell factor, and to flesh out a working model of the observed restriction.

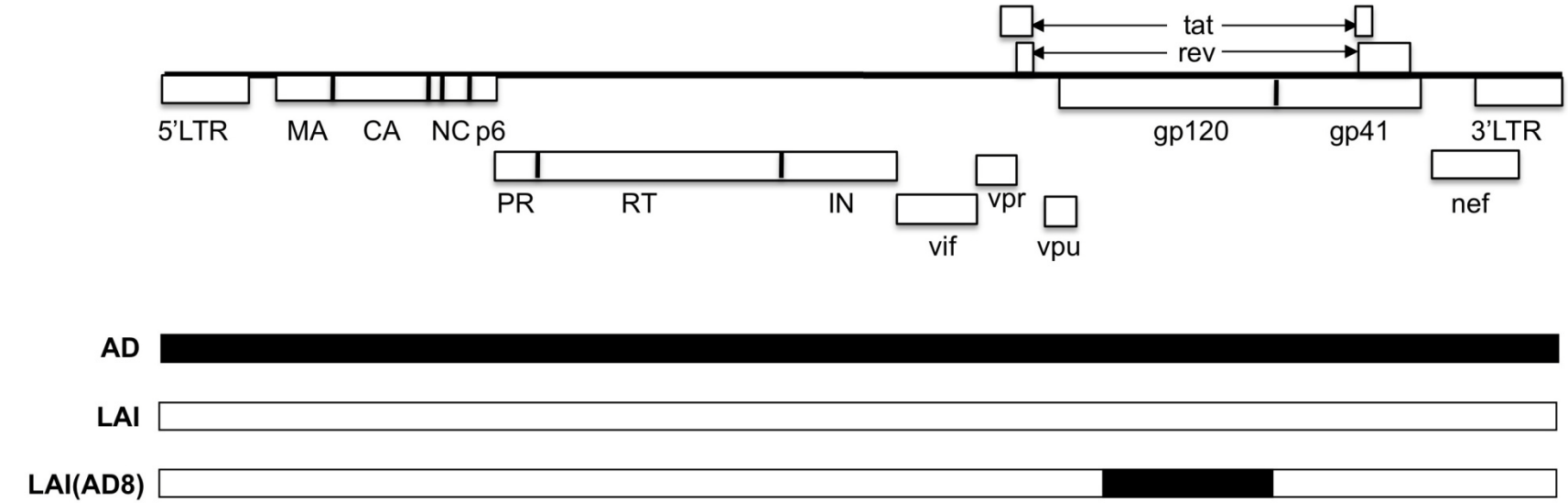

Figure 2-1. Graphical depiction of viral constructs, AD8, LAI, and LAI(AD8).

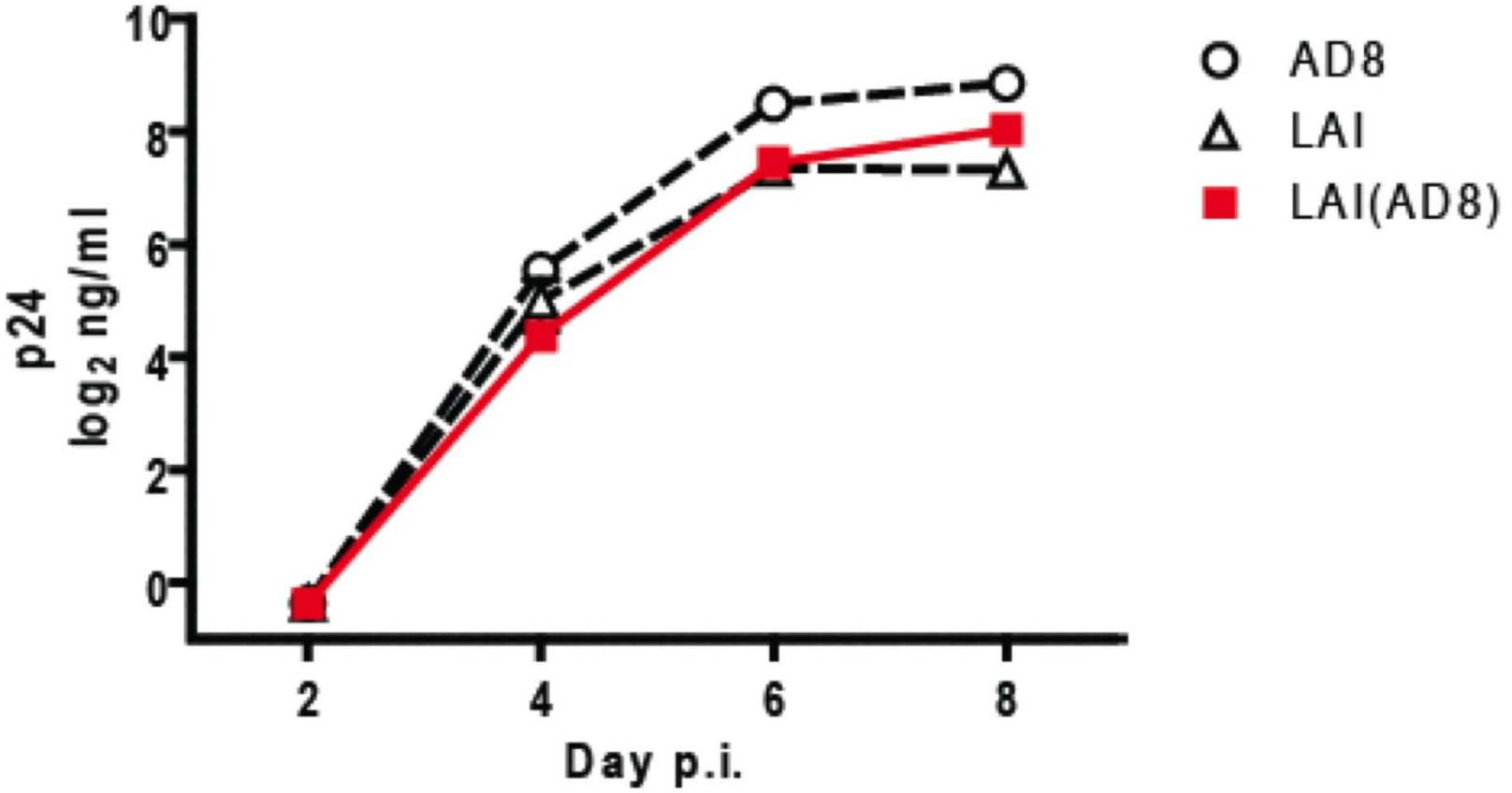

Figure 2-2. Replication kinetics of AD8, LAI, and LAI(AD8) infection in PBMC as measured by supernatant p24 over time. Infection were performed in triplicate.

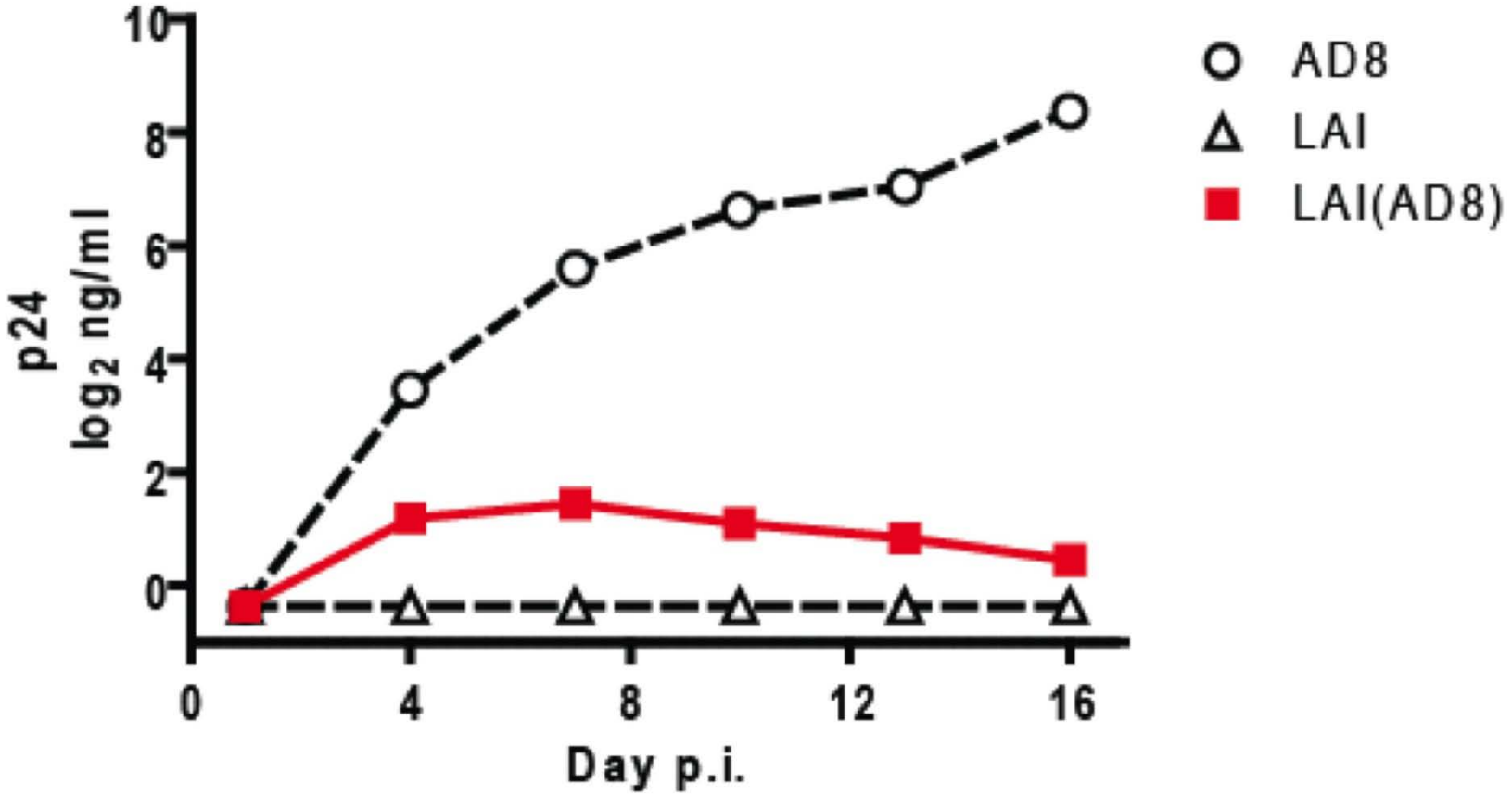

Figure 2-3. Replication kinetics of AD8, LAI, and LAI(AD8) infection in MDM as measured by supernatant p24 over time. Values are pooled triplicates.

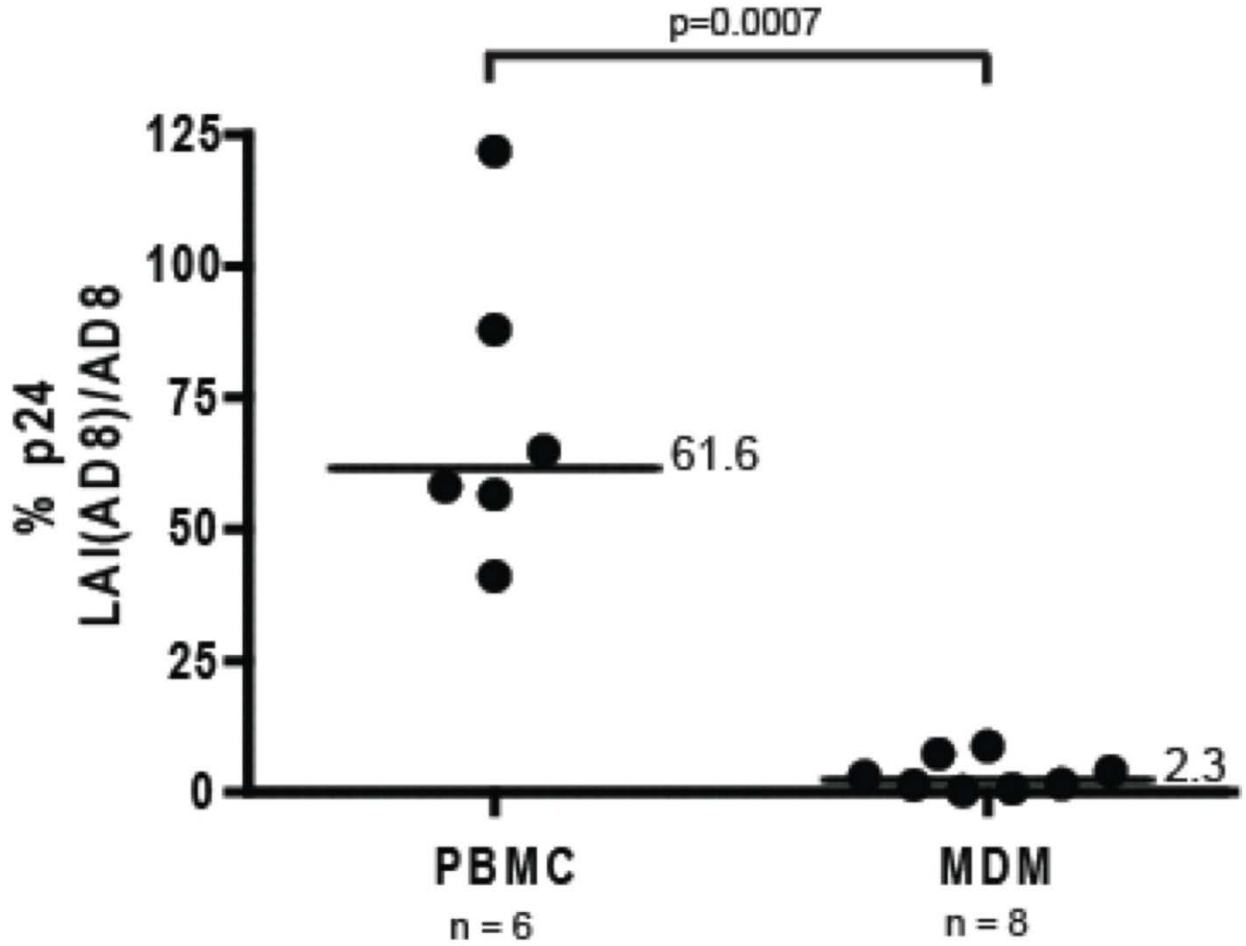

Figure 2-4. Relative supernatant p24 of LAI(AD8) and AD8 from independent donors for both PBMC and MDM infection with the number of donors listed below. Values were assessed 8 days p.i. for PBMC and 16 days p.i. for MDM.

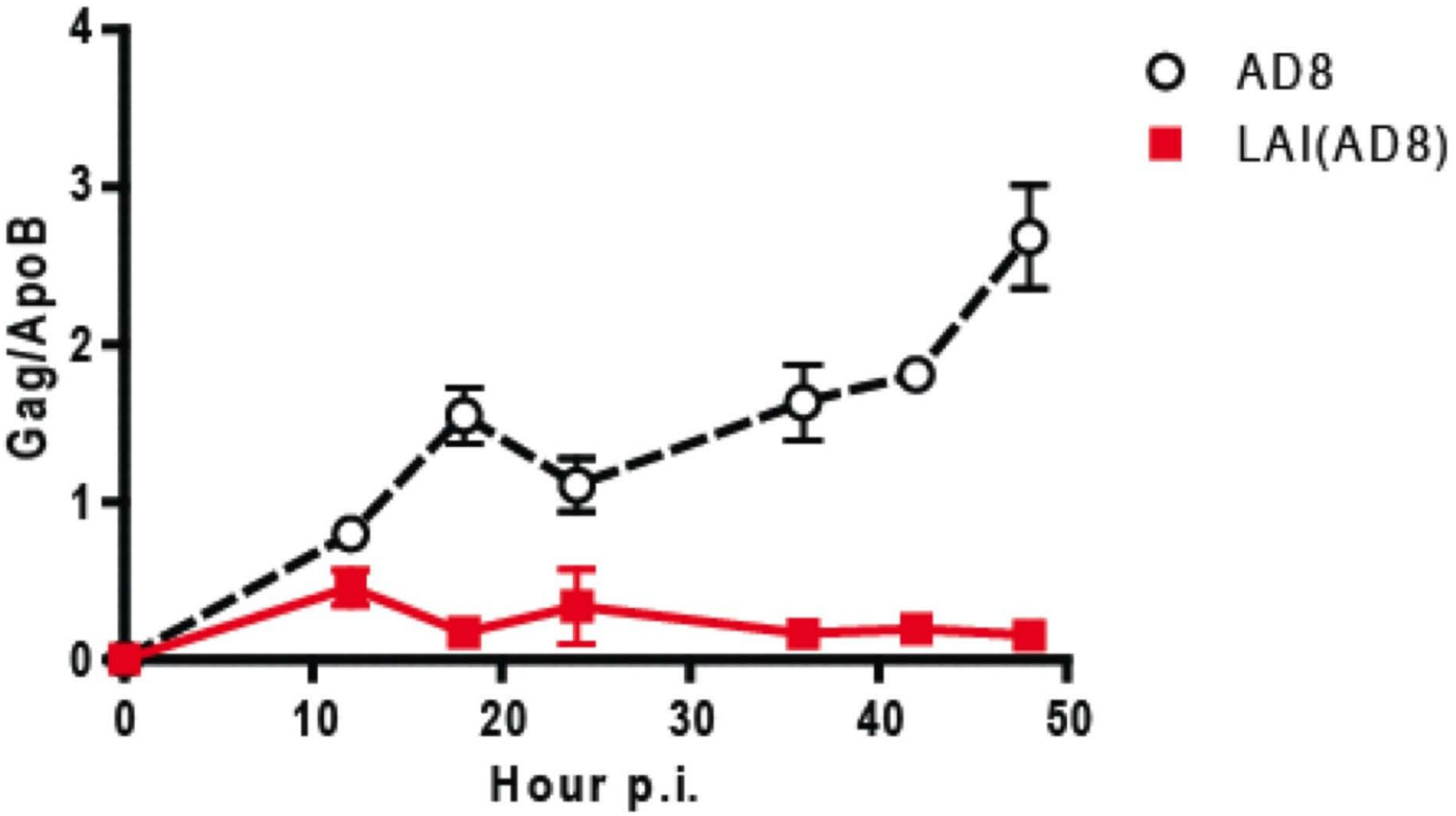

Figure 2-5. Relative copies of cDNA gag in MDM from infection of AD8 and LAI(AD8). Samples were assessed in triplicate.

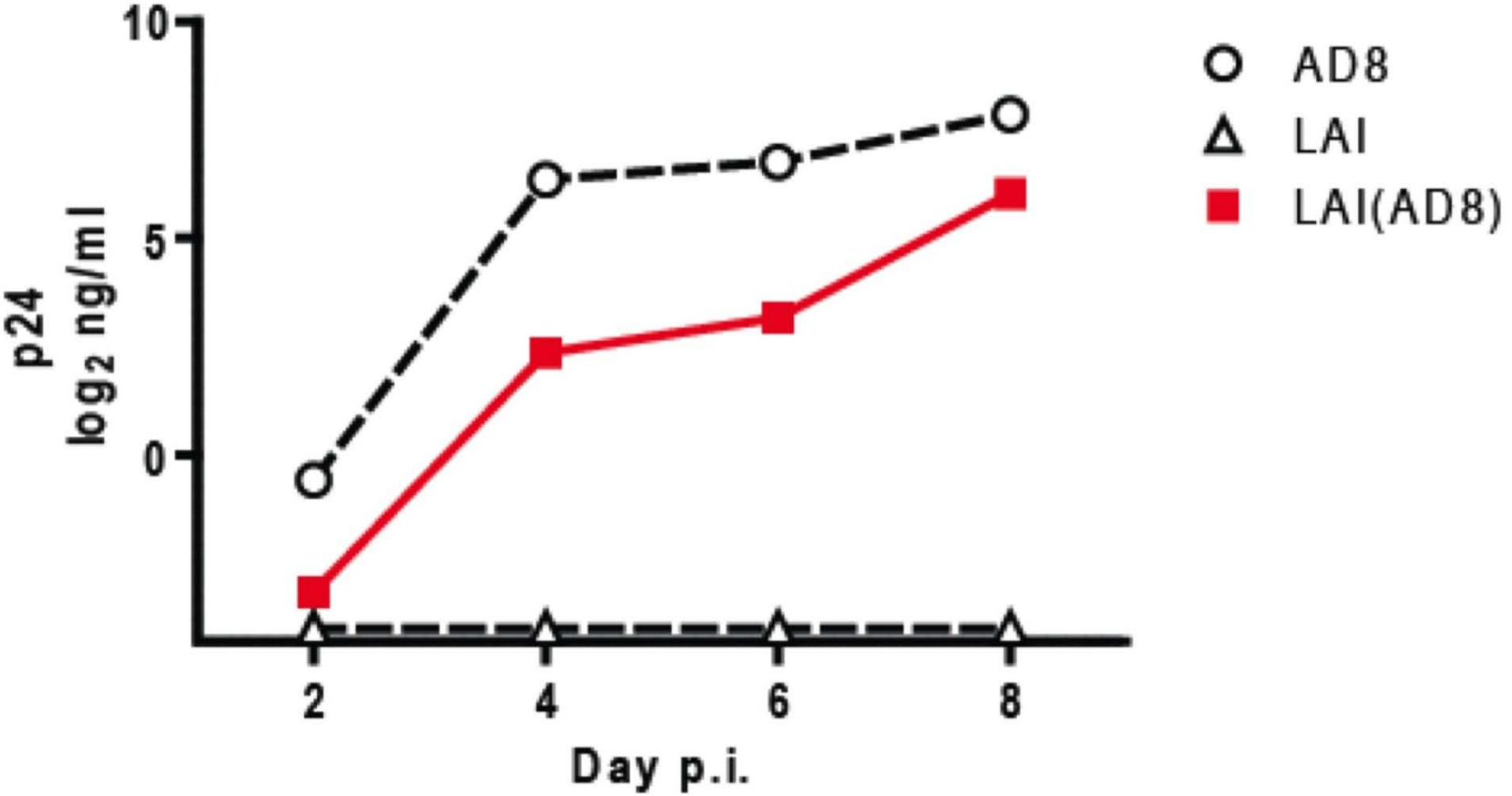

Figure 2-6. PBMC infection from viruses passaged on MDM for 3 days. Replication kinetics were assessed by supernatant p24 over time.

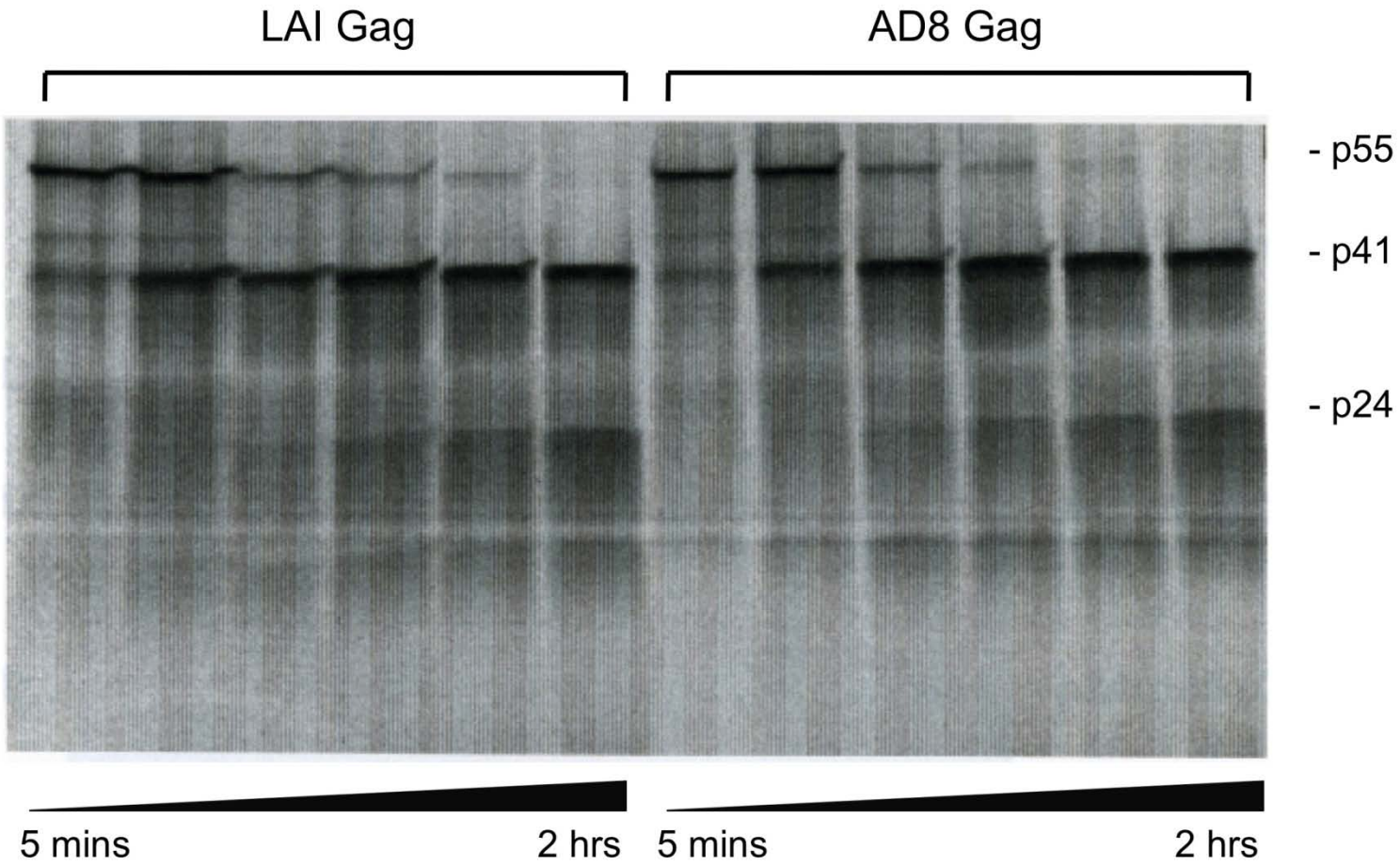

Figure 2-7. *In vitro* transcription translations assays of LAI and AD8 gag while adding exogenous PR. Cleavage profiles from 5 mins to 2 hours were assessed and resolved by SDS-PAGE.

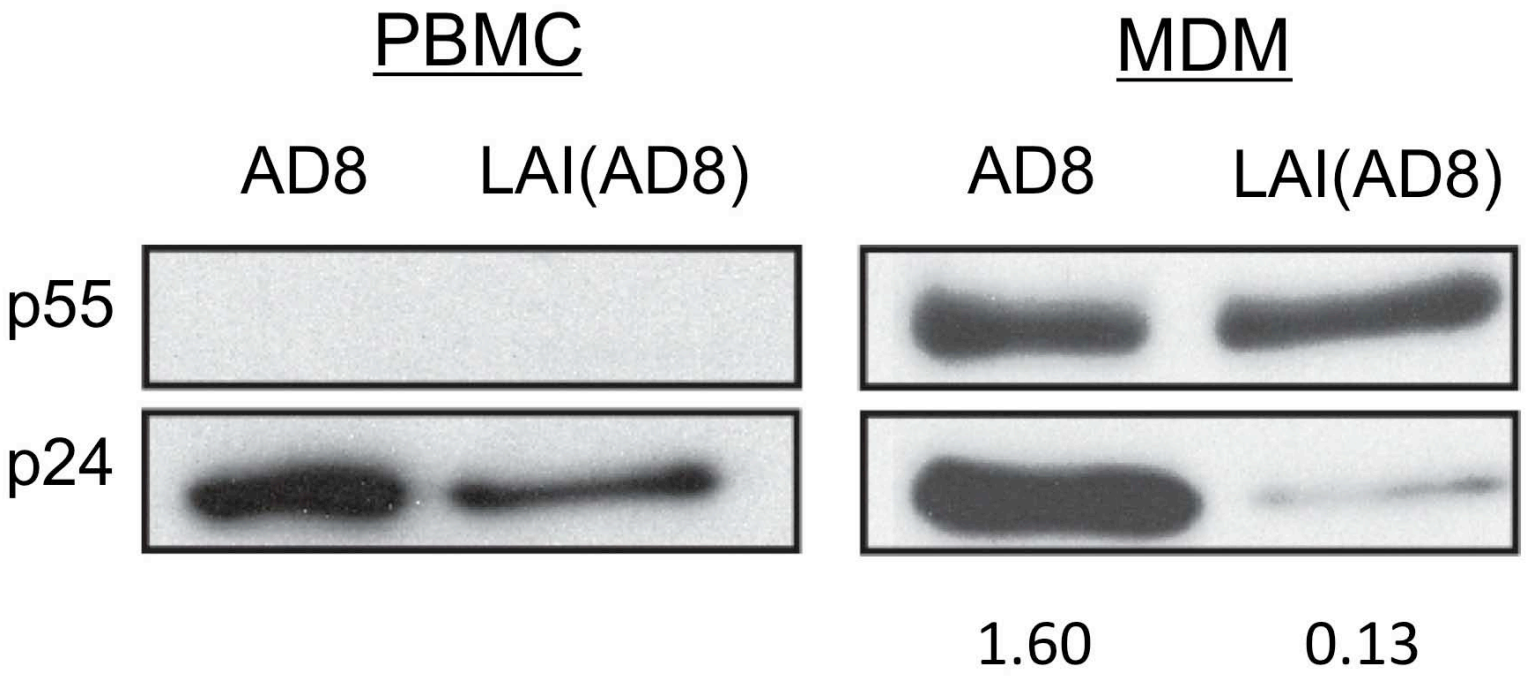

Figure 2-8. Western blot for detection of supernatant virus produced in PMBC and MDM.

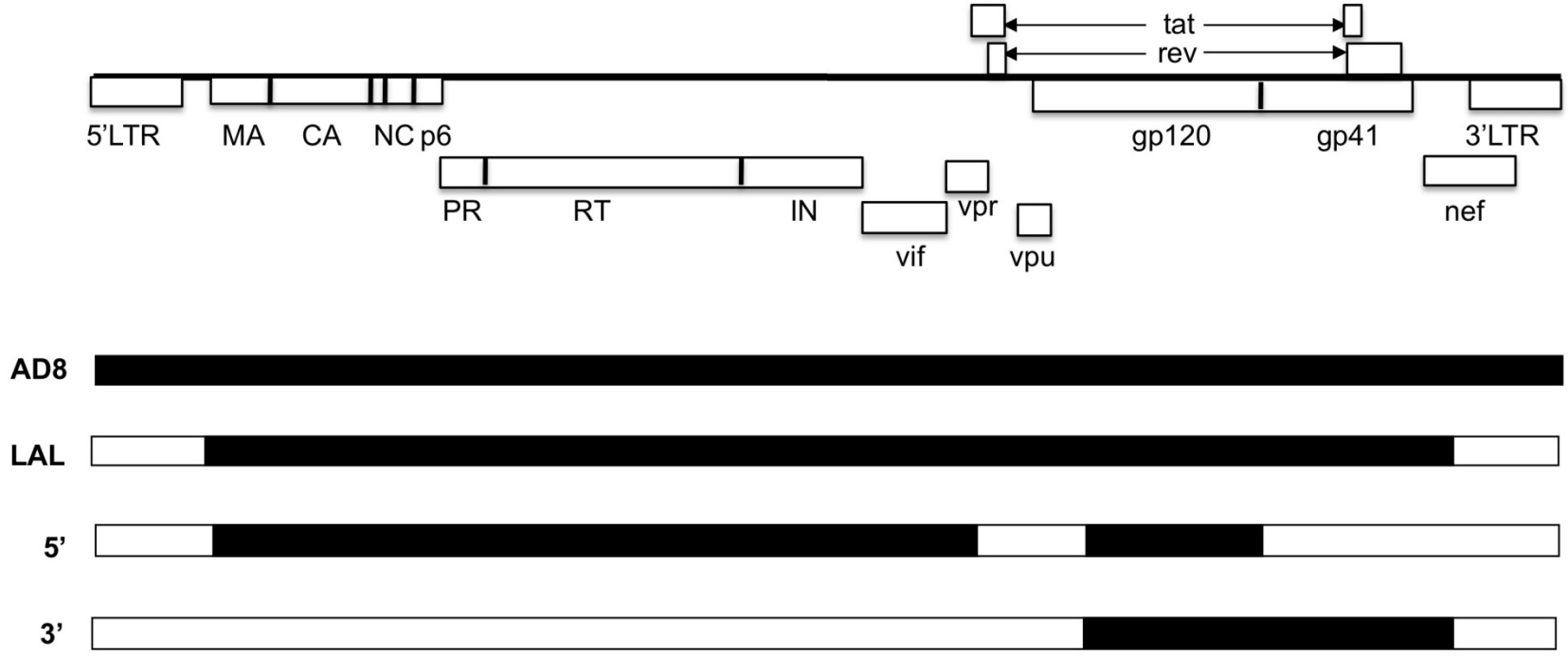

Figure 2-9. Graphical depiction of viral constructs.

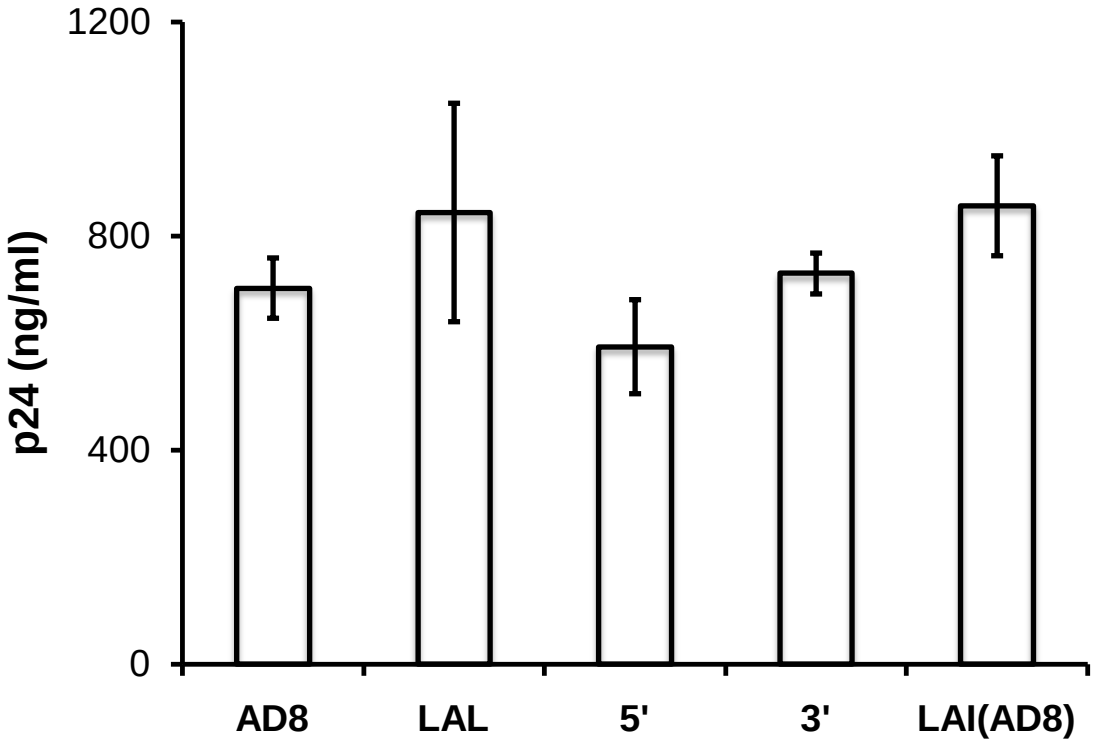

Figure 2-10. Supernatant p24 values from PBMC infections with HIV-1 molecular clones. Infections were performed in a single donor in triplicate.

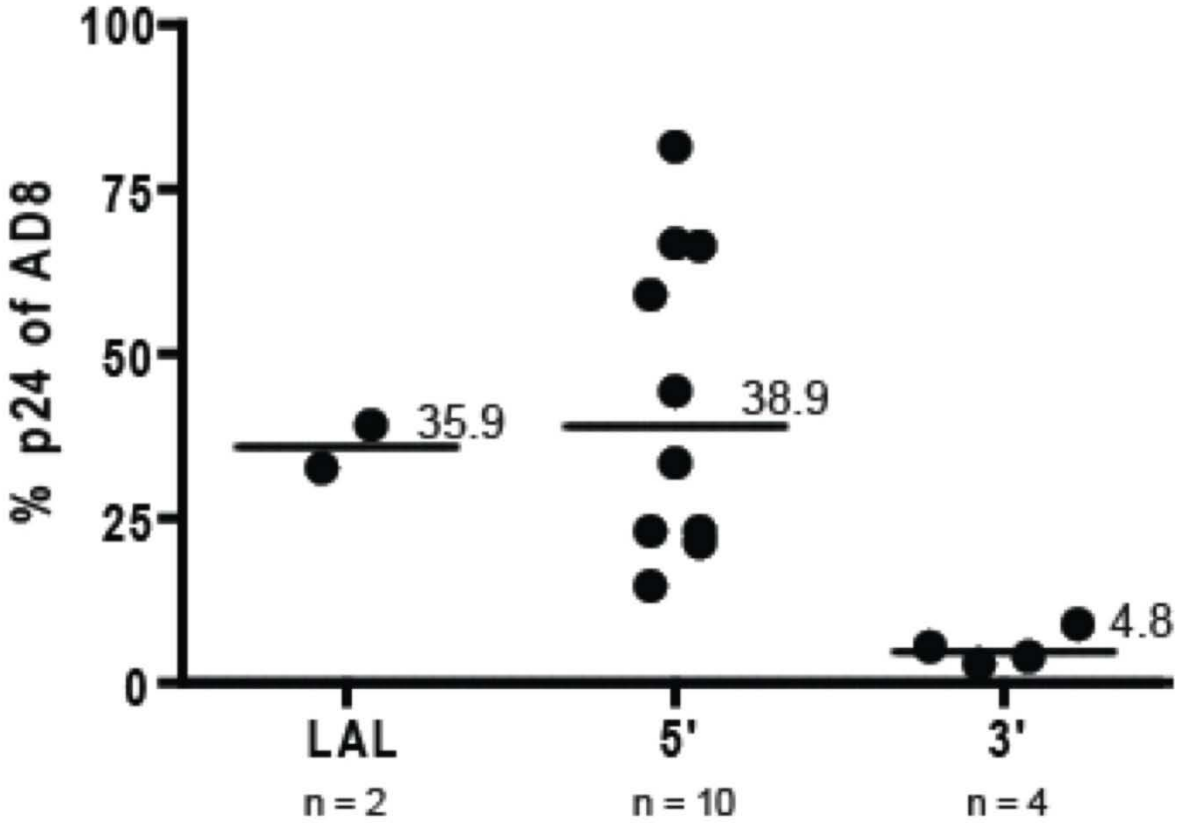

Figure 2-11. Relative supernatant p24 of LAL, 5', and 3' viruses to AD8 from infections of independent donors in MDM. The number of donors are listed below. Values were assessed at 16 days p.i.

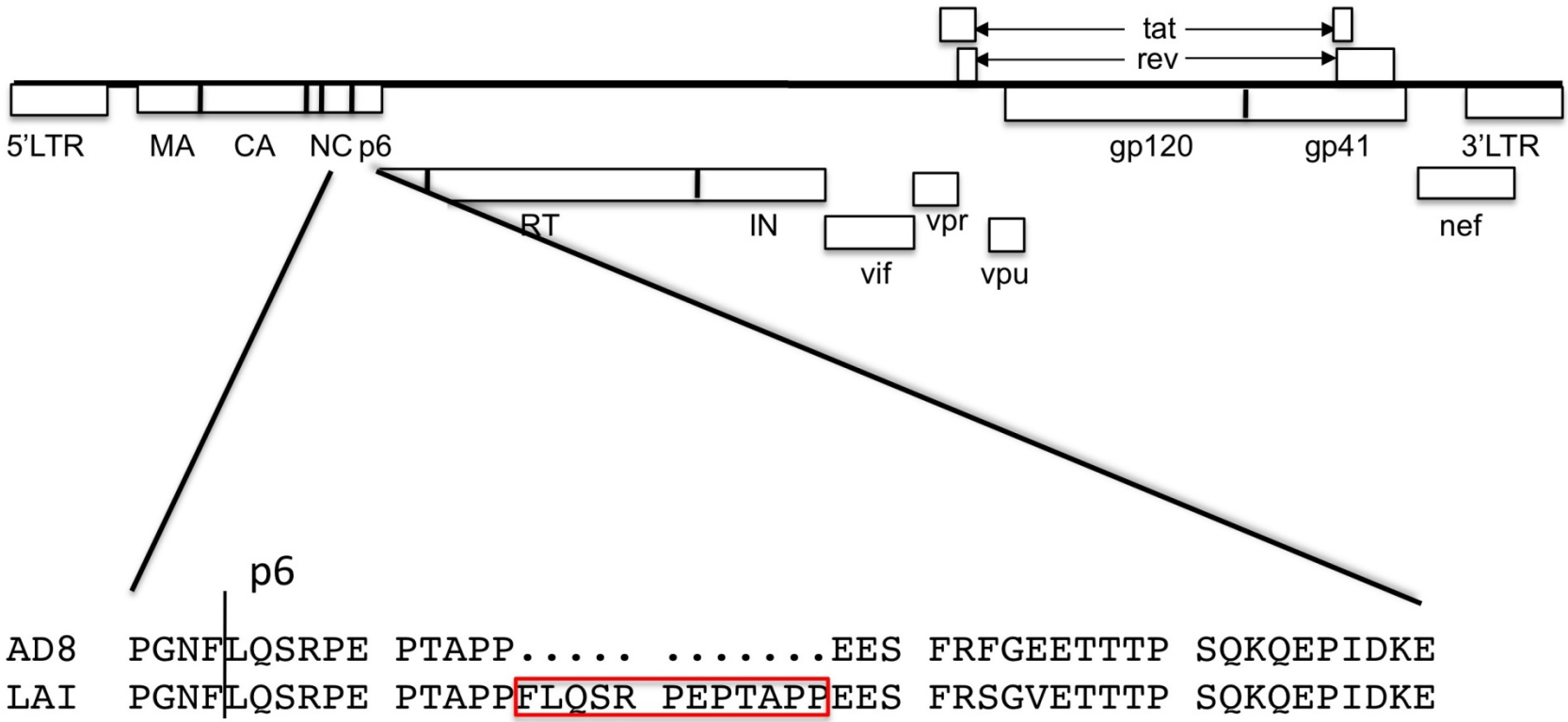

Figure 2-12. Map showing the 12 amino acid duplication in LAI highlighted in red.

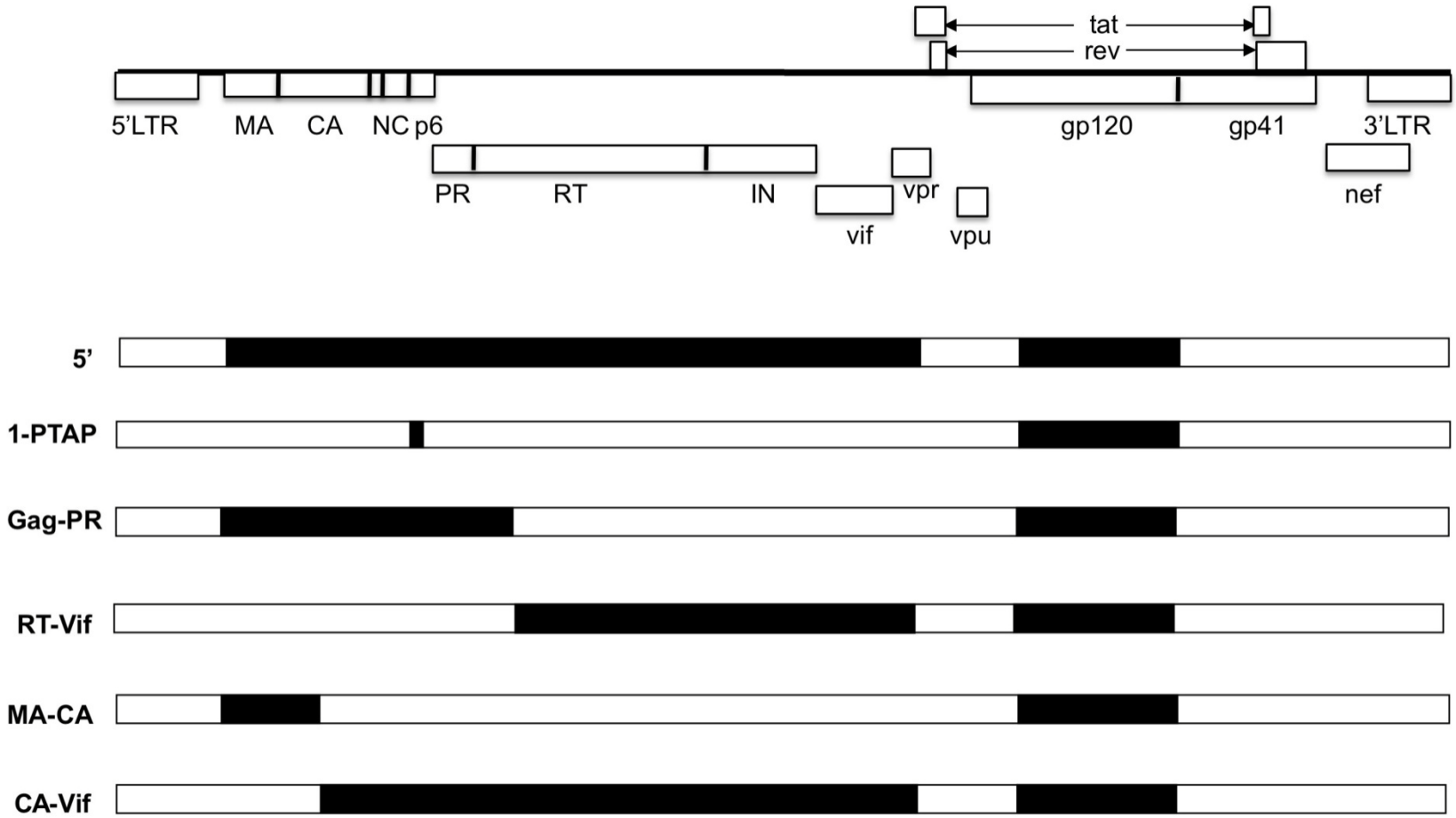

Figure 2-13. Graphical representation of viruses derived from different regions of gag-vif from AD8.

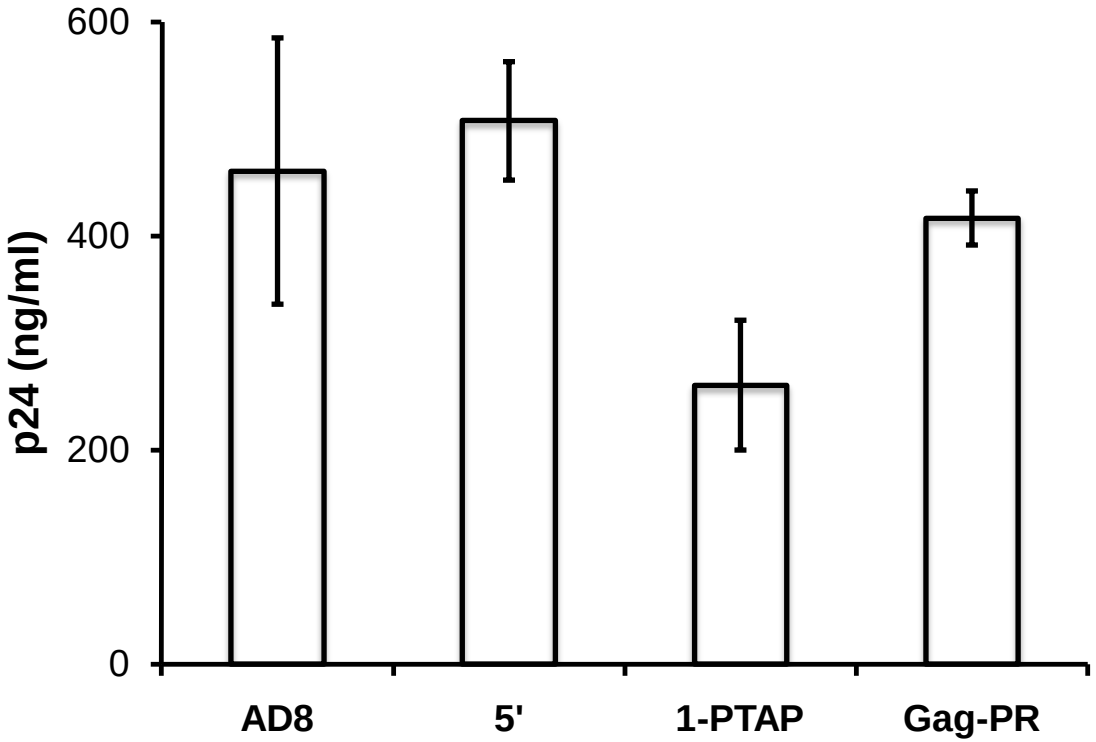

Figure 2-14. Supernatant p24 values from PBMC infections with HIV-1 molecular clones. Infections were performed in a single donor in triplicate.

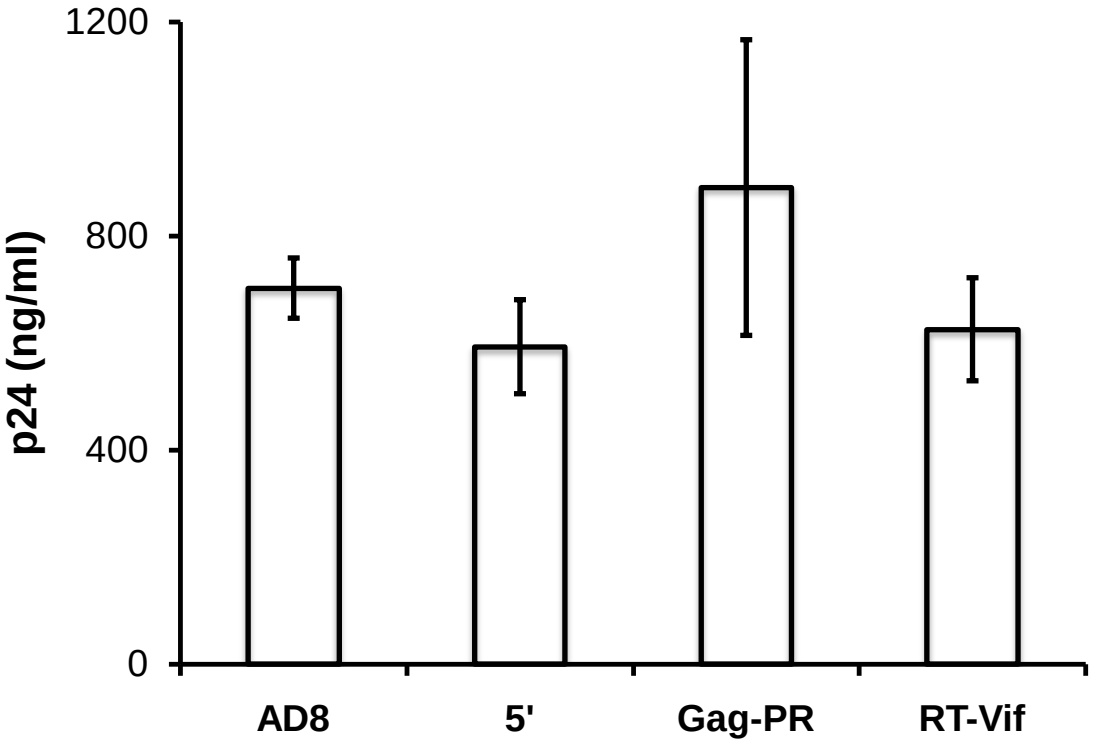

Figure 2-15. Supernatant p24 values from PBMC infections with HIV-1 molecular clones. Infections were performed in a single donor in triplicate.

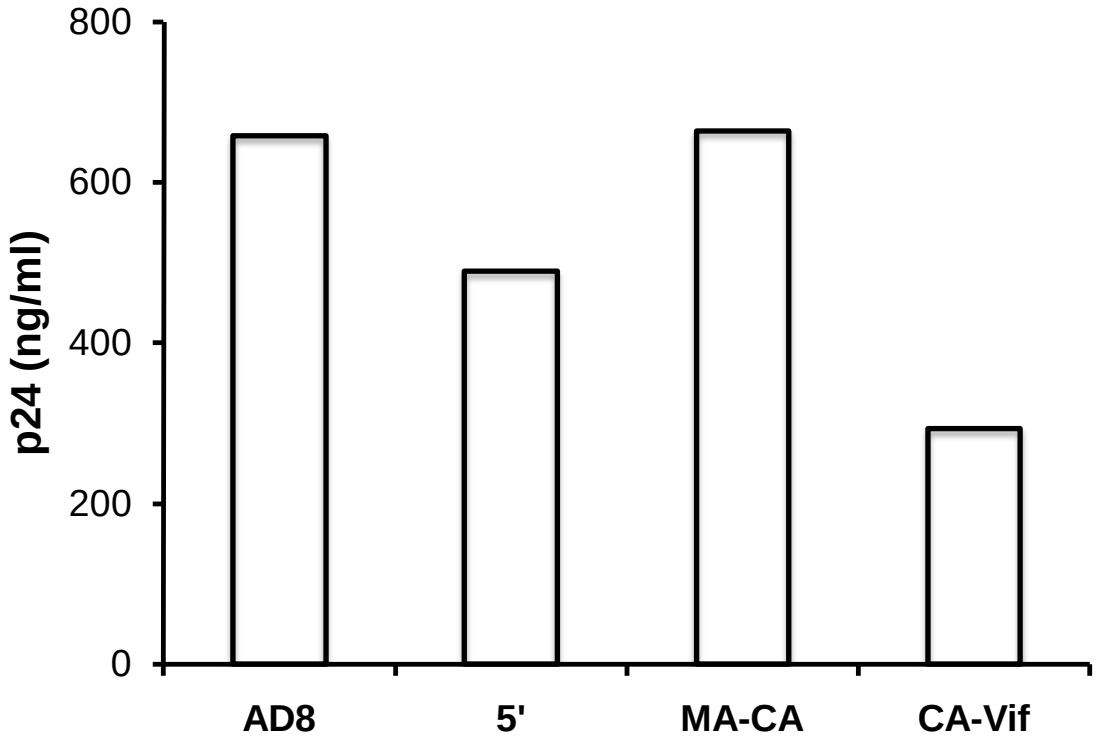

Figure 2-16. Supernatant p24 values from PBMC infections with HIV-1 molecular clones. Infections were performed in a single donor and values are pooled triplicates.

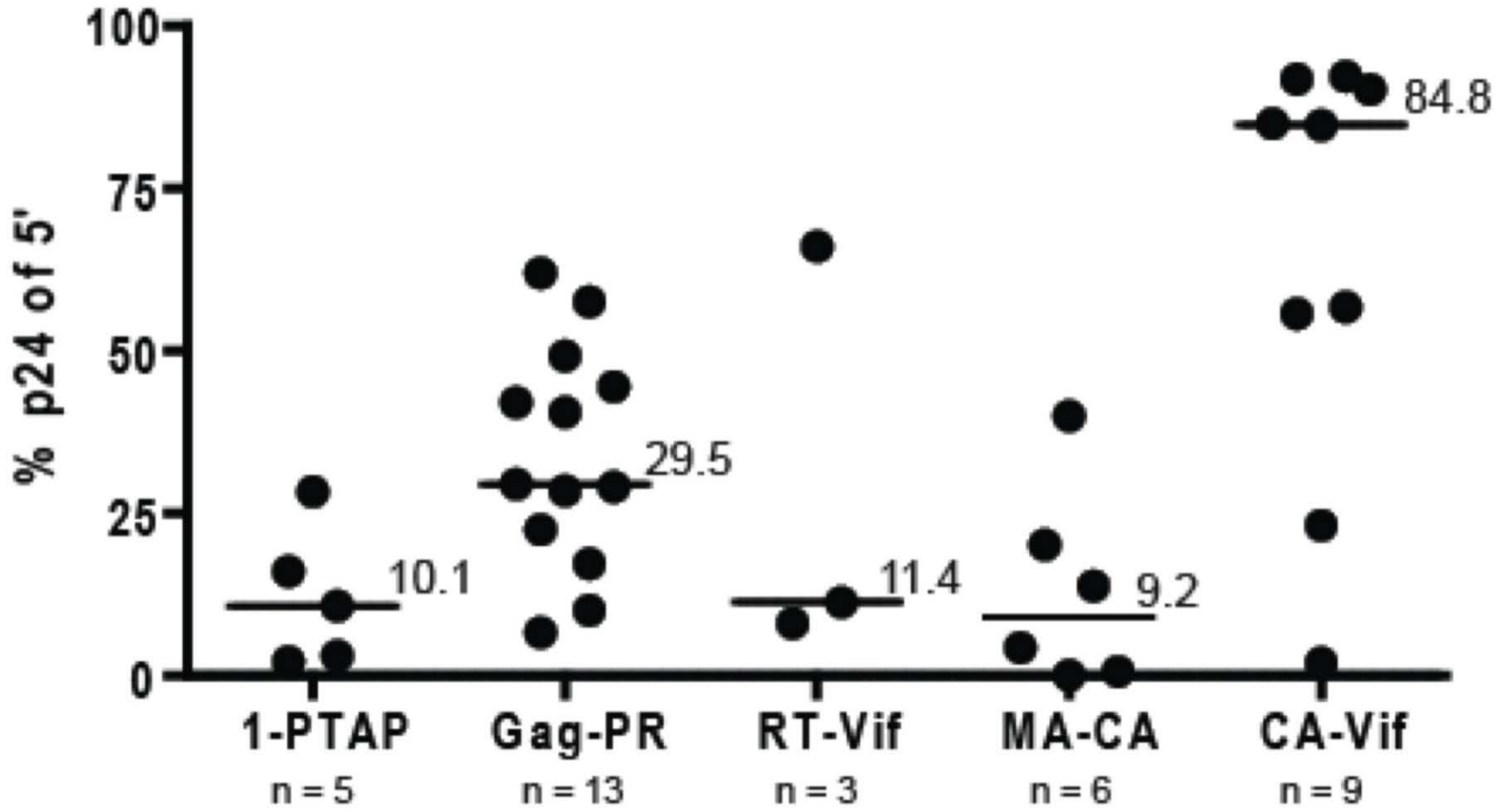

Figure 2-17. Relative supernatant p24 of 1-PTAP, Gag-PR, RT-Vif, MA-CA, and CA-Vif compared to AD8 from infection of independent donors in MDM. The number of donors are listed below. Values were assessed 16 days p.i.

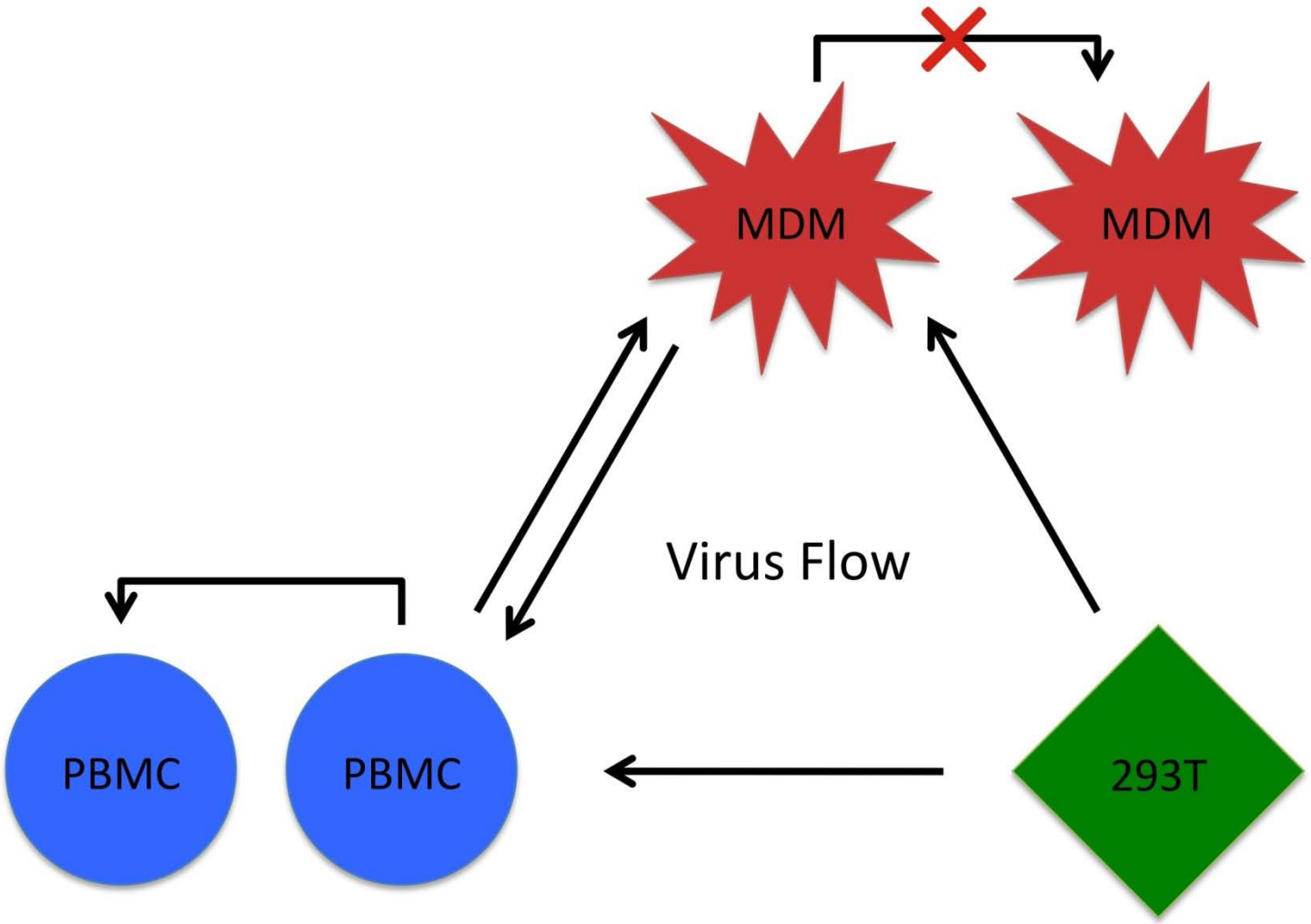

Figure 2-18. Diagram of the viral flow of LAI(AD8) and representation of restriction model.

CHAPTER 3
METHODS

Isolations Of Leukocytes From Whole Blood

Monocytes used for the purpose of studying immune activation and HIV infection have been isolated by two methods from whole blood. One method is by density centrifugation. The second method is by counterflow centrifugal elutriation (Gendelman 1988, Wahl 1984).

Density centrifugation involves the selection of peripheral blood mononuclear cells (PBMC) by density selection (Figure 3-1). Since mononuclear cells, such as monocytes and lymphocytes, have a lower buoyant density than other whole blood cells, such as erythrocytes and polymorphonuclear leukocytes, these cells can be selected over the polysaccharide gradient Lymphoprep (Axis-Shield, Oslo, Norway). To further enrich theses cells for monocytes the antibody cocktail RosetteSep (STEMCELL Technologies, Vancouver, BC, Canada) for monocyte enrichment is added to whole blood before centrifugation on Lymphoprep. Briefly, the antibody cocktail negatively selects for monocytes by linking non-monocytes to erythrocytes to form rosette complexes. In doing so, this increases their overall density allowing them to pass through the density gradient after applying centrifugation.

Counterflow centrifugal elutriation allows for the selection of monocytes by size discrimination (Figure 3-2). While centrifugal force is used to collect cells that migrate away from the rotor according to mass, a fluid buffer is pumped through in the counter-direction. By adjusting the flow rate of the fluid buffer, drag forces push smaller cells towards the rotor creating a column of cells. This column of cells can then be collected for further use and analysis. Both methods provide highly enriched pools of monocytes

that can be used for further differentiation of macrophages. For infection with PBMC, density centrifugation was used, for infections with MDM, counterflow centrifugal elutriation was used.

Monocytes were seeded into either 6-well or 48-well culture plates with a total of 3 million cells/well or 0.25 million cells/well, respectively. Monocytes were allowed to differentiate into macrophages for 5-7 days in the presence of 10% normal human serum, 1 μg/ml of macrophage colony-stimulating factor (M-CSF) and DMEM. Fifty percent media changes occur every 2-3 days. After 5-7 days of differentiation, macrophages were grown in the absence of M-CSF.

Construct Creation

The constructs pLAI, pAD8, and p3' were gifts from the lab of H. Gendelman. The construct pLAI(AD8) was created removing the large segment from *Sal*I through *Xho*I from LAI and placing it into a shuttle vector. The Env gp120 region of AD8 was removed by *Kpn*I and *Mfe*I and placing it into shuttle vector displacing the Env gp120 of LAI. The entire *Sal*I through *Bam*HI fragment containing AD8 Env gp120 was then placed back into the backbone of pLAI. The construct p5' was created by digestion of pAD8 with *Bss*HII and *Eco*RI and placed in the background of pLAI(AD8). pLAL was created by removal of the coding region of pAD8 from *Bss*HII to *Xho*I and placed in the backbone of p3'. pGag-PR was created by inserting the fragment *Bss*HII to *Bst*Z17I from pAD8 into pLAI, then adding back Env gp120 from AD8. pRT-Vif was created similarly by inserting fragment *Bst*Z17I to *Eco*RI from AD8 into pLAI, then adding back Env gp120 from pAD8. To create pMA-CA, the fragment *Bss*HII to *Sph*I from pAD8 was placed in the backbone of pLAI(AD8) and pCA-Vif was created from fragment *Sph*I to *Eco*RI from

pAD8 being placed into pLAI(AD8). For p1-PTAP, a shuttle vector containing the region from *Sph*I to *Sal*I of pLAI was created. Once in the shuttle vector, site-directed mutagenesis (Stratagene, La Jolla, CA, USA) was used creating redundant *Afl*II sites surrounding the PTAP sequence. After confirmation by sequencing, digestion of the shuttle vector with *Afl*II was performed followed by ligation. After deletion of the PTAP duplication was confirmed, site-directed mutagenesis was performed again to return the sequence to its original state and fragment was inserted into pLAI(AD8).

Transfection Of 293T And Virus Collection.

Human epithelial kidney 293T carcinoma cells were used for the transient transfection of replication competent HIV-1. All constructs were transfected using Superfect (Invitrogen, Carlsbad, CA, USA) with DNA to with a ratio of 1:8 v/v, respectively with a total amount of 10 μg of DNA. The Superfect:DNA mixture was then added dropwise to a tilted T-75 flask of 293T to directly expose the 293T to the mixture. The cells were then incubated for 2 hrs. at 37^{o}C. After two hours the cells are washed once with 1X PBS and replaced with 6 ml of complete DMEM. Forty-eight hours later, supernatants were collected and passed thought a 0.45 μm filter to remove cellular debris. Viruses were then aliquoted into cryovials and stored at -80^{o}C.

Titration Of Viruses

All constructs were assessed for viral infectivity on TZM.bl cells. TZM.bl are derived from HeLa cells that highly express CCR5, as well as both β-gal and luciferase reporter genes downstream of Tat-controlled promoter. Upon infection, HIV-1 Tat leads to the production of both β-gal and luciferase proteins which can be assayed for infectivity. While using either reporter has its advantages and disadvantages, the β-gal

assay providing a discreet number of infected cells directly leading to a number of infectious units per ml, and the luciferase assay being highly reproducible between individuals, the luciferase assay was chosen. In a 96-well plate, viruses were serially diluted from 4^2 through 4^{10} in quadruplicate. Ten thousand TZM.bl cells were then placed into each well in addition to a set of wells with no virus. After 48 hrs. all media and virus was removed and cells were lysed and assessed for luciferase protein production measured in relative light units (RLU). Individual wells were then scored based on a positive or negative compared to 2X the background RLU. Using the Spearman-Karber method, positive RLU wells were converted into 50% tissue culture infectious dose ($TCID_{50}$) per ml, thus providing the infectious concentration of virus.

Infections Of PBMC And MDM

PBMC were grown in RPMI 1640 plus 10% FBS and IL-2. PBMC were activated before infection for 3-5 day in the presence of phytohemagglutinin (PHA) that acts as a CD3 superantigen. One million PBMC per ml were infected at 0.001 MOI for 2-4 hrs. Afterward, PBMC were washed with 1X PBS to remove virus and plated in triplicate in a 96-well culture plate. Supernatant p24 samples were taken every two days for eight days to assess viral growth. MDM were infected at 0.01 MOI for 24 hrs. Afterwards, MDM were washed with 1X PBS to remove virus and media was replaced. Supernatant p24 is taken every three days for 16 days to assess viral growth. For passaging virus experiments, filtered virus, as explained previously, from day 3 or 4 p.i. MDM was placed on PBMC for 24 hrs.

qPCR Of Gag In MDM

To assess the presence of reverse transcribed cDNA products, quantitative real-time, taqman-based PCR was used. MDM were infected in 48-well culture plates. DNA

was isolated from samples in triplicate using Qiagen's QIAamp DNA mini and Blood mini kit (Valencia, CA, USA). Primers that specifically bind second strand synthesis of viral CA and primers that specifically bind host cell ApoB were used. Each cell contains only one copy of ApoB DNA, thus ratios can be used to compare the amount of accumulating viral CA cDNA to cells. Taqman-based real-time PCR tracks the amount of light produced from each round of PCR. Exploiting Taq polymerase's 5' to 3' exonuclease activity, a synthesized probe sits on the gene of interest and produced light as the probes fluorophore is released from its quencher (Figure 3-3). By tracking the amount of light produced over time, that assay can quantitate the relative amounts of PCR product.

Western Blot Of PBMC And MDM

For western blot analysis supernatant virions were concentrated by using ultra concentrators (Orbital Biosciences, Topsfield, MA, USA). Viral supernatants were first filtered using a 0.45 μm filter to remove cellular debris and then placed on an ultra concentrator for 15 min at 3500 g. After concentrated supernatants were then treated with triton X-100 to a final concentration of 0.5% v/v. Samples were then added to Laemmli sample buffer with fresh β-mercaptoethanol, sonicated, heat denatured, and resolved in 4-20% Tris-SDS gel electrophoresis. Using a semi-dry technique, proteins were transferred to pre-activated PVDF membranes for antibody probing. Using a rabbit polyclonal antibody from AIDS Research and Reference Reagent Program, membranes were exposed to antibody overnight at 4^{o}C. A secondary, HRP-conjugated goat anti-rabbit antibody was then exposed to the membrane for one hour at room temperature.

Membranes were then exposed to a luminol reagent (Santa Cruz Biotech, Santa Cruz, CA, USA) and exposed to X-ray film.

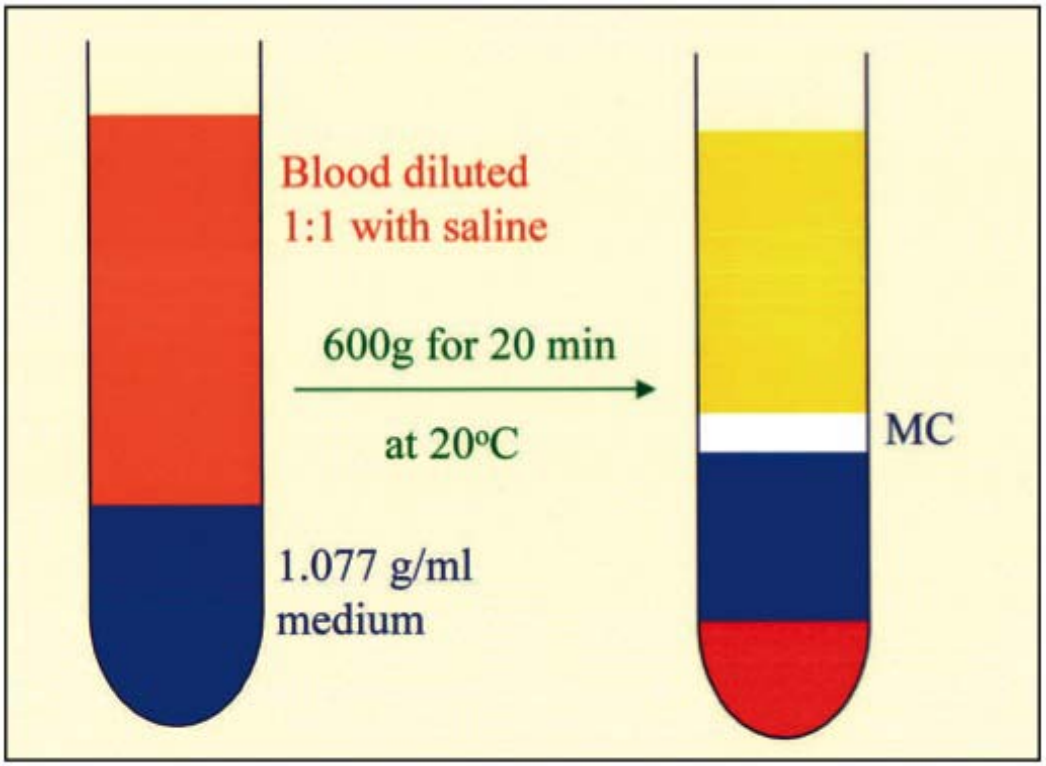

Figure 3-1. Density centrifugation over a specialized medium allows erythrocytes to pass though, but allow mononuclear cells (MC) to remain afloat (Source: Axis-Shield).

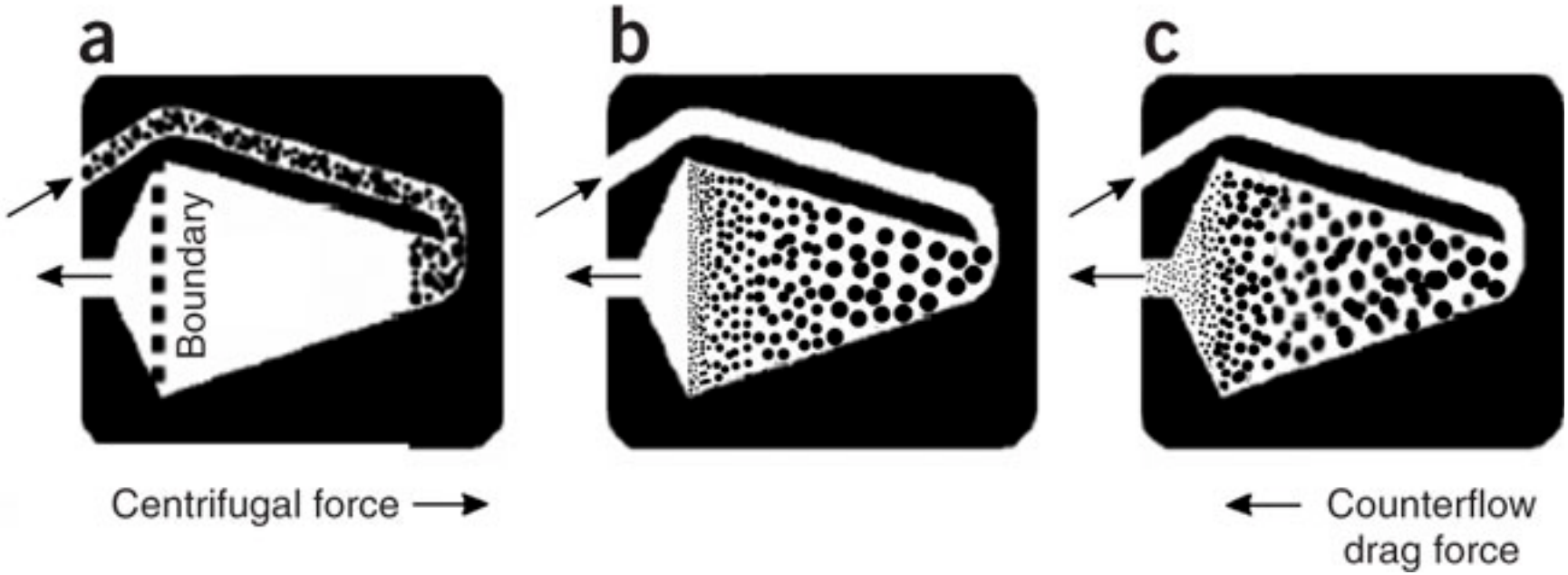

Figure 3-2. Counterflow centrifugal elutriation allows for the one-way passage of solution to counter the centrifugal force and separate cells by size. Adjusting the flow allows cells to be ejected and collected for subsequent use (7).

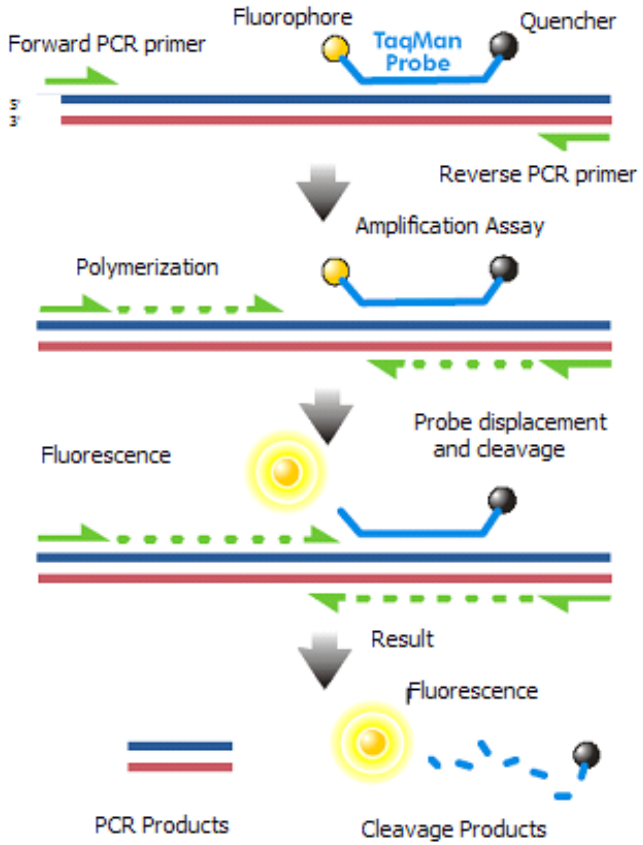

Figure 3-3. qPCR takes advantage of Taq polymerase ability to cleave DNA polymers downstream of its polymerase activity. In doing so Taq liberates the fluorophore from its quencher and can be detected spectroscopically.

CHAPTER 4
CONCLUSIONS

The goal of this project was to identify the unique mechanism of restriction observed by HIV-1_{LAI} upon infection of macrophages. While genetic mapping was the best approach for identifying a single viral determinant, the results show that this restriction is complex and requires multiple determinants. The restriction is also highly diverse between different donors and thus hampers one's ability to give weight to any single factor in the multiple determinant observations. The ideal system would make use of a cell line that could provide consistently reproducible results with the different chimeric viruses. The restriction was also identified as a post-entry phenomenon and affects only virions that target other macrophages as new hosts. Whether virions from infected macrophages are targeted for restriction before entering new macrophages, or occurs after entering new macrophages has yet to been observed. Finally, the inability of LAI(AD8) to efficiently process Gag/p55 may provide insight into the post-entry restriction observed. But the lack of processing may also due to an inherent inability of LAI PR to function efficiently in macrophages. Given the heterogeneity between macrophage donors, this alone may be the significant contributing factor in the multi-determinant restriction observed.

HIV-1 is able to setup and maintain infection within macrophages and contributes to the persistence of HIV-1 within infected individuals. Identifying these mechanism will provide insight into eradicate infection of these viral reservoirs that contribute significantly to the reemergence of virus after the cessation of current drug therapies, either voluntarily are due to a lack of adherence. Moreover, these results provide a larger picture of the complexities between invading viruses and host cell immunity.

While it would have been easier to make conclusions from a single determinant or single mutation that would have provided a clear answer for the restriction observed it is obvious that this interaction is multifactorial. Thus further studies between HIV-1 and macrophages will continue to elucidate these new post-entry interactions.

APPENDIX
DIFFERENTIATED THP-1 AS A MODEL FOR PRIMARY MACROPHAGES

The high variability of p24 production from infection of macrophages is a hindrance in the future identification of the host cell factors that may play role in this novel phenotype. The use of the cell line THP-1 was used to determine if they are able to recapitulate the LAI(AD8) phenotype seen in primary macrophages. THP-1 cells are a monocytic cell line that can be differentiated into macrophage-like cells with the treatment of a phorbol diester, specifically PMA. Treatment of THP-1 cells leads to the activation of the PKC pathway as PMA mimics a diacylglycerol. While many other labs use differentiated THP-1 as models for macrophage infection with HIV-1, my goal was to determine if differentiated THP-1 are able to reproducibly recapitulate the LAI(AD8) phenotype as well as determine if the level of PMA had any effect on THP-1.

Park *et al*. (64) showed that PMA optimization is necessary for differentiation of THP-1 for cell adhesion and response to stimuli. With this knowledge two different levels of PMA, 10 μg/ml and 100 μg/ml, were used. THP-1 were differentiated for 48 hrs, washed with PBS, incubated for 2 days, and then infected with either AD8, LAI(AD8), or LAI.

Figure A-1 shows the different morphology of differentiated THP-1 cells before infection. THP-1 cells treated with 100 μg/ml appear to be more differentiated and appear more macrophage-like then THP-1 cells treated with 10 μg/ml. Figure A-2 shows a time course infection of the three viruses in THP-1 cells differentiated with 100 μg/ml of PMA. AD8 is able to infect and spread rapidly where LAI is unable to produce detectible levels of p24, mostly like to its inability to enter. LAI(AD8) is able to infect differentiated THP-1 cells and has the similar restricted phenotype observed in primary

macrophages. Figure A-3 shows a second independent infection of THP-1 cells differentiated with 100 μg/ml of PMA. The phenotype appears to be reproducible even though the levels of p24 by AD8 at day 10 p.i. are approximately half that of AD8 from Figure A-2.

Figure A-3 shows a time course infection of THP-1 cells differentiated with 10 μg/ml of PMA. Both AD8 and LAI(AD8) display similar growth kinetics within these cells and LAI is unable to infect. Similar results are observed in a second independent experiment (Figure A-4). These cells are unable to recapitulate the LAI(AD8) restriction in primary macrophages.

Together, these results show that there is a minimum amount of PMA that is necessary to recapitulate the LAI(AD8) restriction observed in primary macrophages. The ability of LAI(AD8) to infect equally as well as AD8 in THP-1 cells differentiated with 10 μg/ml may provide a useful tool for identifying what host proteins are activated, or differentially expressed compared to THP-1 cells treated with 100 μg/ml.

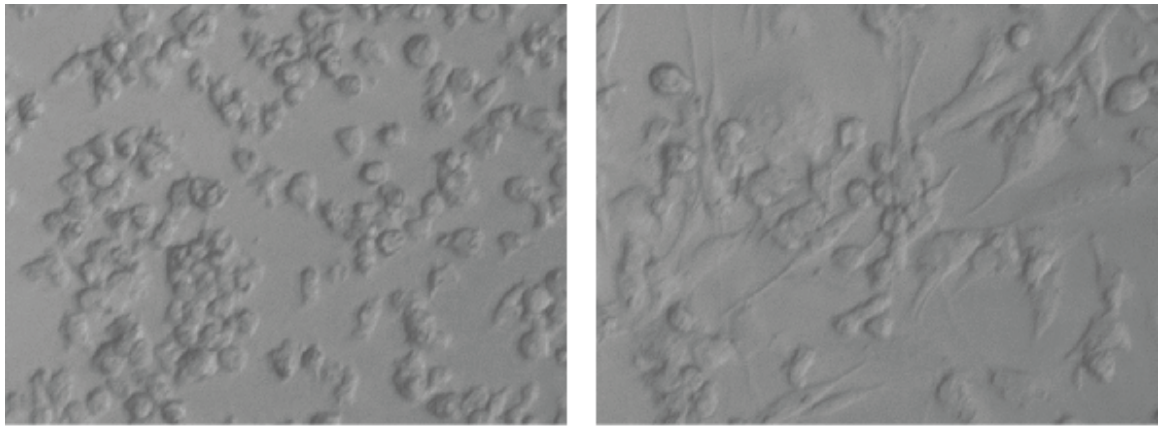

Figure A-1. THP-1 cells differentiated for 48 hours with different amounts of PMA.

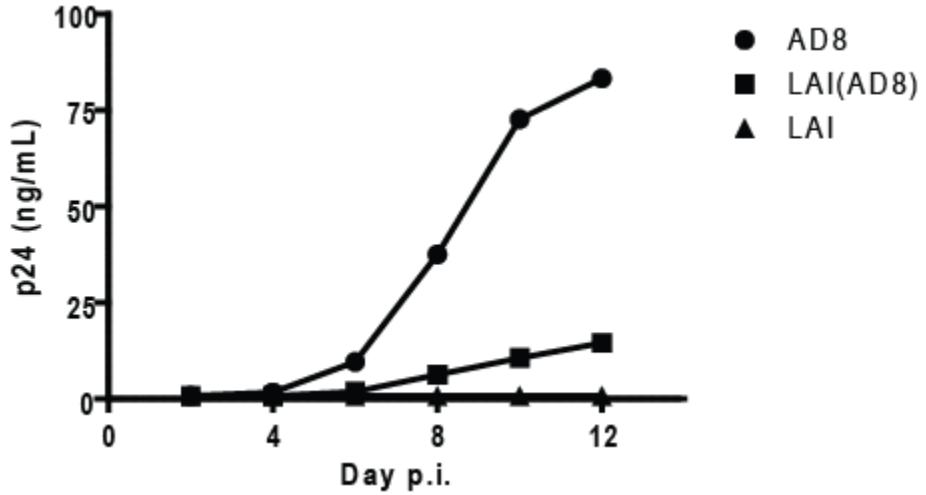

Figure A-2. THP-1 cells differentiated with 100 μg/ml of PMA recapitulate the growth kinetics for AD8, LAI(AD8), and LAI in macrophages. Infections were performed in triplicate and supernatants were pooled.

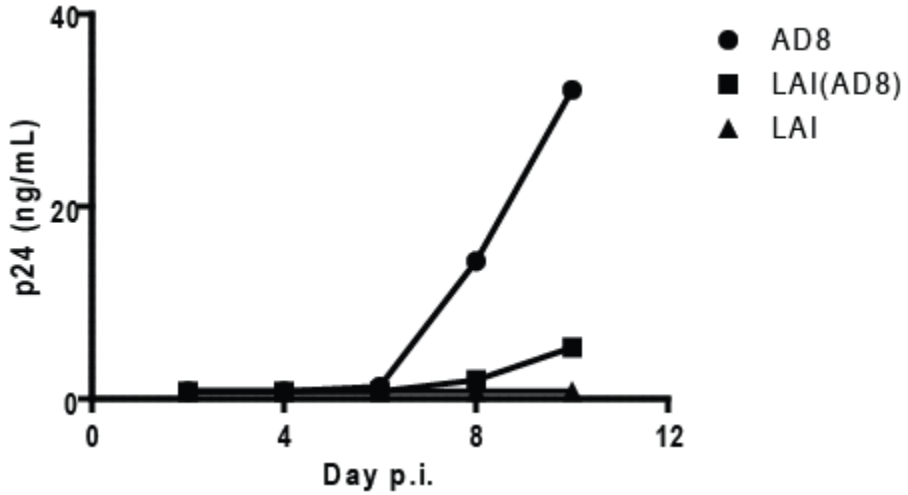

Figure A-3. A second independent infection of THP-1 cells differentiated with 100 μg/ml of PMA shows cells can provide a reproducible surrogate for macrophages. Infections were performed in triplicate and supernatants were pooled.

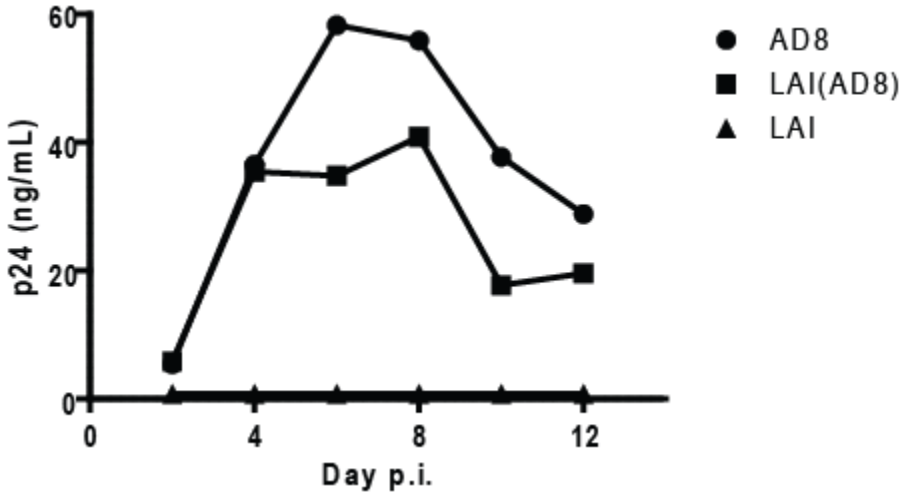

Figure A-4. THP-1 cells differentiated with 10 μg/ml of PMA provided similar growth kinetics for AD8 and LAI(AD8). Infections were performed in triplicate and supernatants were pooled.

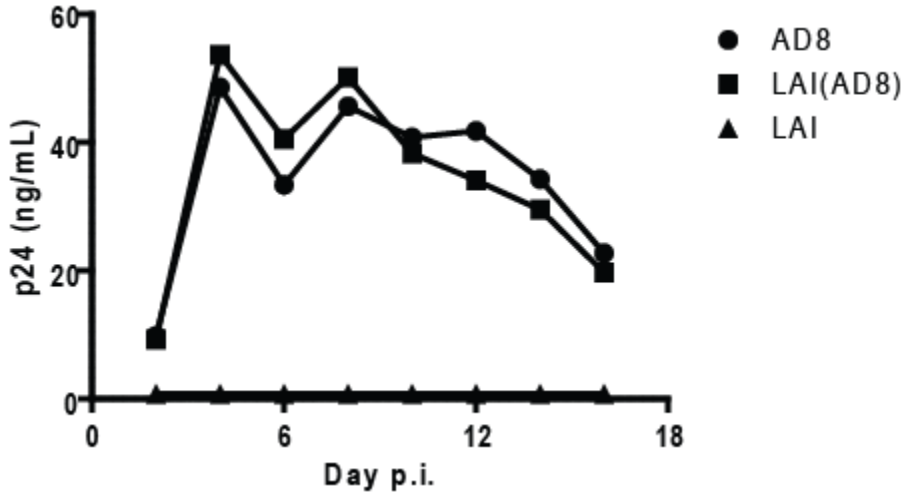

Figure A-5. A second independent infection of THP-1 cells differentiated with 10 μg/ml of PMA fail to recapitulate the growth kinetics of AD8 and LAI(AD8) in macrophages. Infections were performed in triplicate and supernatants were pooled.

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BIOGRAPHICAL SKETCH

Joshua C. Bunger was born in Seoul, South Korea, adopted by Lowell and Suzanne Bunger, and raised in Grinnell, IA, which to this day he still considers home. Upon commencement from Grinnell Community High School in 1999, Joshua attended Grinnell College and received a Bachelors degree in Biology in 2003. Later that year, he enrolled in the Interdisciplinary Program for the Biomedical Sciences at the University of Florida. Joshua later joined the lab of Dr. Maureen Goodenow in 2004 and obtained his Ph.D in 2009. After receiving his degree, he plans to start a post-doctoral fellowship with the Henry M. Jackson Foundation in Bethesda, MD to work in the laboratory of Dr. Allison O'Brien.