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Human Immunodeficiency Virus Type-1 Mutations and Associated Fitness Costs for Viral Replication

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Human Immunodeficiency Virus Type-1 Mutations and Associated Fitness Costs for Viral Replication

A dissertation submitted in partial satisfaction of the

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in Microbiology, Immunology, and Molecular Genetics

by

Justin Philip De La Cruz

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ABSTRACT OF THE DISSERTATION

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by

Justin Philip De La Cruz

Doctor of Philosophy in Microbiology, Immunology, and Molecular Genetics

University of California, Los Angeles, 2012

Professor Otto O. Yang, Chair

The $CD8^+$ cytotoxic T lymphocyte (CTL) is a critical component in the antiviral immune response to Human Immunodeficiency Virus type-1 (HIV-1). However, the efficacy of immune containment is limited due the high viral turnover, rapid rate of mutation, and remarkable sequence plasticity of HIV-1. Studies have shown that immune selection pressure exerted by CTL can also readily produce epitope escape variants. Although these virions have the capacity to elude immune detection, escape events occur only if the benefit of immune evasion outweighs the fitness cost of the mutation. The dynamics of escape and the effect of such mutations on viral replication, however, have been generally poorly understood.

Here, we measured the fitness costs of HIV-1 mutations across different viral proteins and evaluated their overall impact on replication. We examined the effect of specific mutations in the Envelope gp41 membrane-proximal tyrosine-based sorting signal on the susceptibility to CTL and developed a potential model for *in vivo* attenuation of Simian Immunodeficiency Virus (SIV) containing analogous mutations that confer subsequent protection from wild type challenge. We investigated pairing-disruptive synonymous mutations within a novel RNA secondary structure identified in the *pol* gene and their impact on HIV-1 reproductive success while, in parallel, used chemically-based procedures to confirm RNA structure modification. Finally, we analyzed the differential fitness costs of immune-mediated escape mutations identified in *gag-pol* in persons with acute or chronic HIV-1 infection and assessed the breadth of escape mutations with associated fitness impairments across individuals expressing class I human leukocyte antigen (HLA-I) types not implicated with superior control of viral replication.

The dissertation of Justin Philip De La Cruz is approved.

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2012

DEDICATION

I would like to dedicate this work to the most influential women in my life.

Without their love and support, none of this would be possible.

My grandmother: Inocencia Diaz Castro

My mother: Loreta De La Cruz

My sister: Janelle Dela Vina

My love, my everything: Michelle Yee

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Chapter 2 in this thesis is a version of De La Cruz *et al* (2009) –

> **De La Cruz, J**, Ali, A, Ng, HL, and Yang, OO. Disruption of Env Tyrosine-Dependent Sorting Signal Does Not Affect Susceptibility of HIV-1 to Cytotoxic T Lymphocytes. 2009. *AIDS 23:11, 1449-1451*

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> **De La Cruz, J**, Lewis, MJ, Ng, HL, and Yang, OO. Fitness Constraints on HIV-1 in Chronically Infected Persons Without Protective HLA Types. 2012. In preparation.

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CHAPTER 1:

Introduction

1.1 CD8+ cytotoxic T lymphocytes (CTLs)

When innate immunity is unable to suppress and eliminate infection, the adaptive immune system is induced to provide a directed response with greater specificity toward the invading pathogen. In contrast to the innate immune response, which relies on receptors that recognize molecular structure patterns common to many microorganisms, the adaptive immune response generates antigen-specific receptors that can identify antigen derived from an infected cell. Once a pathogen is cleared, the host develops immunological memory. This allows for a more rapid and effective response upon secondary exposure to the same pathogen and is the principle for which historically successful vaccines are based.

An important component of the adaptive immune system is the $CD8^+$ cytotoxic T lymphocyte (CTL). Directed principally at intracellular pathogens, including viruses, bacteria, and parasites, CTLs kill the infected cell, preventing the transmission of the pathogen to neighboring cells. CTLs are equipped with highly diverse T cell receptors (TCRs), which consists of a heterodimeric complex of proteins expressed on the surface of T lymphocytes responsible for recognizing and responding against a foreign antigen.

In the cytosol, proteasomes regularly degrade intracellularly expressed, self-derived proteins into small peptide fragments in order to regulate the concentration of certain proteins or eliminate misfolded proteins. Pathogen-derived proteins can also be degraded in the context of an infected cell (Figure 1.1). The short peptide fragments are shuttled into the endoplasmic reticulum (ER) via the TAP (transporter associated with antigen processing) complex. Inside the ER, the

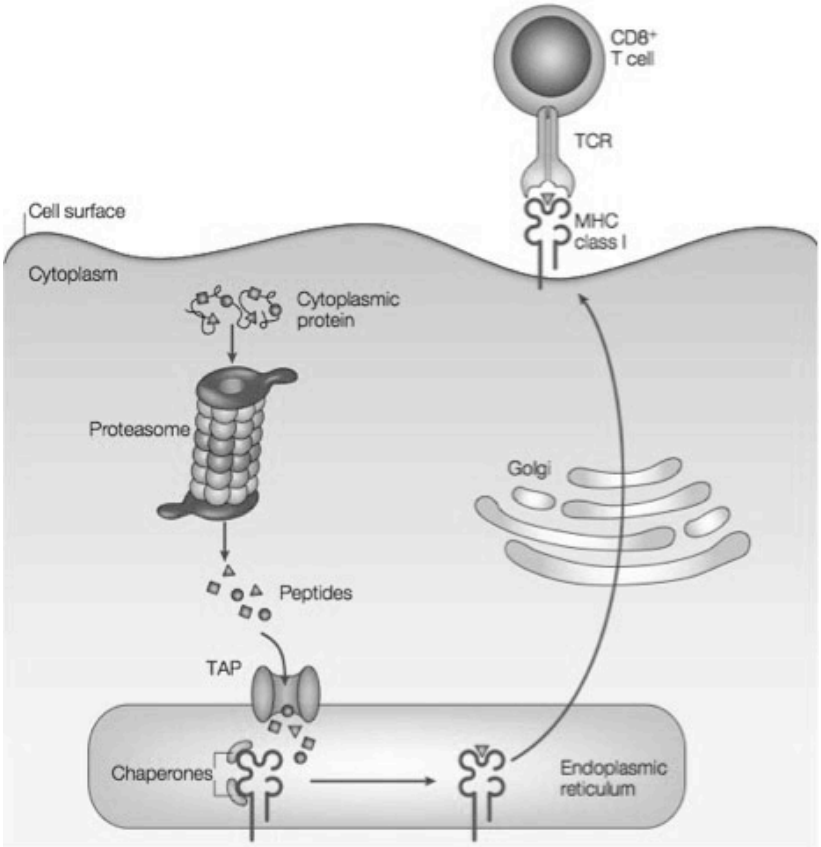

Figure 1.1 Schematic of antigen processing and presentation [adapted from Yewdell *et al* (83)]. Cytosolic proteins are degraded by the proteasome into short peptide fragments. The fragments are transported to the endoplasmic reticulum (ER) via TAP and loaded onto an MHC class I molecule present in the ER. Upon peptide binding, the peptide-MHC complex is transported to the cell surface and presented to T cells.

peptides are loaded onto a major histocompatibility complex (MHC) class I molecule and transported to the cell surface where the peptide-MHC complex (pMHC) is presented to CTL specific for the pMHC. Upon TCR recognition of a pMHC bearing a foreign antigen, preformed lytic granules (containing granzyme and perforin) in the CTL are released and effectively destroy the infected cell, followed by differentiation and proliferation of effector CTL. When the infection is cleared, the majority of effector CTL undergo apoptosis while a subset of memory cells persist long term in the event of a subsequent exposure.

1.2 Introduction to HIV-1 and HIV-1-specific CTL responses

1.2.1 HIV-1 pathogenesis and immune response –

Since the identification of Human Immunodeficiency Virus type-1 (HIV-1) as the pathogenic cause of Acquired Immune Deficiency Syndrome (AIDS), HIV-1 has emerged as a worldwide pandemic with an estimated 33.4 million infected individuals and 2.7 million new infections globally at the end of 2009 (72). HIV-1 spreads primarily by contact with body fluids, either through sexual transmission, intravenous drug use, blood transfusions, or mother-to-child transmission during childbirth. Specifically targeting the $CD4^+$ T lymphocyte population, HIV-1 infects cells by binding to CD4 and a co-receptor, either CCR5 or CXCR4 (1, 14). Throughout the course of infection, HIV-1 causes a progressive deterioration of the immune system, leaving the infected individual susceptible to opportunistic pathogens and, ultimately, AIDS and death. Although improvements in combination antiretroviral therapy have drastically reduced morbidity and mortality in patients with advanced HIV infection (42, 52), the search for a successful vaccine continues.

HIV-1 is a retrovirus that contains two copies of an RNA genome packaged with essential enzymes necessary for initial infection within each viral particle (Figure 1.2). The HIV-1 genome consists of nine genes with long terminal repeat sequences (LTR) on each end. Like all retroviruses, HIV-1 has three major genes—*gag*, *pol*, and *env*—that encode structural, functional, and viral envelope proteins, respectively. Additionally, HIV-1 consists of six smaller accessory genes—*tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*—essential for productive viral replication and infectivity *in*

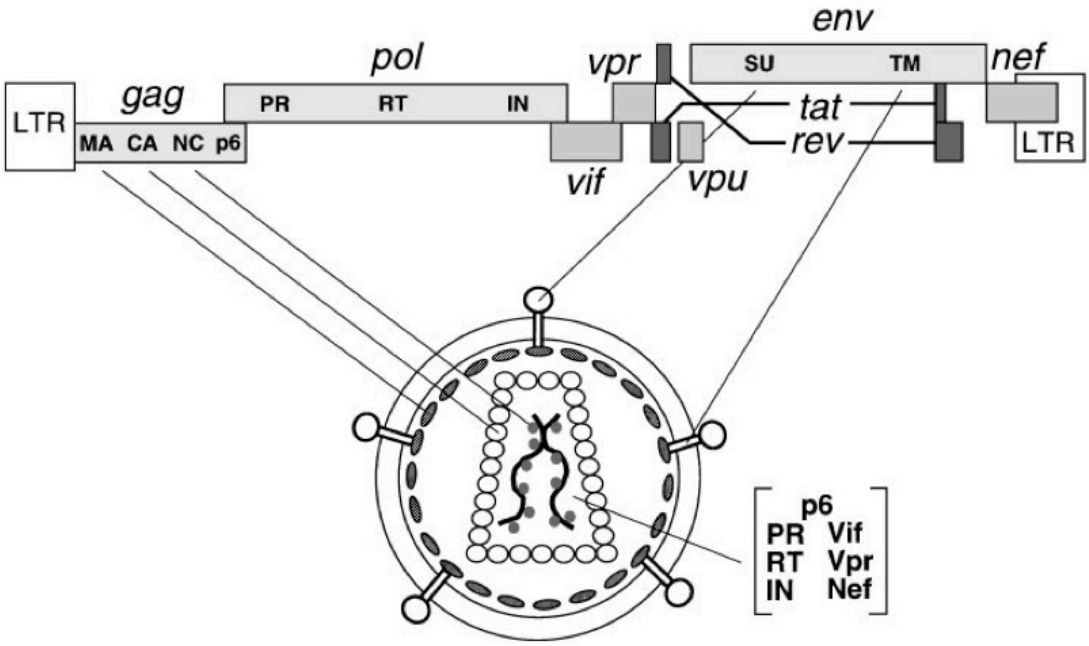

Figure 1.2 <u>Organization of the HIV-1 genome and viral particle [adapted from Frankel *et al* (21)]</u>. Two long terminal repeats (LTR) flank the HIV-1 genome, which encodes nine different proteins through overlapping reading frames. The surface of each virion is covered by heterodimer complexes composed of gp120 (SU) and gp41 (TM). A lipid bilayer surrounds the matrix (MA) proteins, which encapsulates the viral capsid. The capsid contains two copies of single-stranded RNA genomes and various viral-associated proteins.

vivo. HIV-1 exists as multiple viral subtypes and circulating recombinant forms on a global level and as a unique heterogeneous population, or quasispecies, on the individual level.

The major role of CTL over the course of HIV-1 infection is to control viral replication (24, 30). Studies have shown that CTLs can inhibit HIV-1 replication *in vitro* (9, 79), effective CTL responses can contain the virus *in vivo* (3, 37), and antibody-mediated $CD8^+$ cell depletion in Simian Immunodeficiency Virus (SIV)-infected macaques, the animal model for HIV-1 infection, leads to a spike in viral replication that diminishes once CTL repopulate (31, 45, 60). Moreover, there is a strong link between the emergence of CTL in the acute phase with the initial control of primary infection (37, 82) and the loss of HIV-1-specific CTL function with increased viral load and faster disease progression (55, 78). Although CTLs play an important function in controlling HIV-1 replication, the immune response does not eliminate HIV from the infected host. Viremia rises but then falls with the proliferation of HIV-1-specific CTL during acute infection. Subsequently, the CTL responses reach a stalemate with HIV (referred to as set point viremia) that continues over a long and relatively asymptomatic period, depending on the magnitude of viremia (47), to establish a chronic state of infection. Ultimately, the adaptive immune response becomes overwhelmed and fails to contain the infection as progression to disease ensues (Figure 1.3).

1.2.2 Failure of natural immunity under HIV infection –

The inability of CTL to successfully clear HIV can be attributed to a number of causes. HIV actively infects and destroys the $CD4^+$ T cell population, which is required for the development of functional $CD8^+$ memory T cells (5, 65, 68, 69). Furthermore, dysfunctional effects have been observed in HIV-1-specific CTL, including limited perforin and granzyme B expression (80),

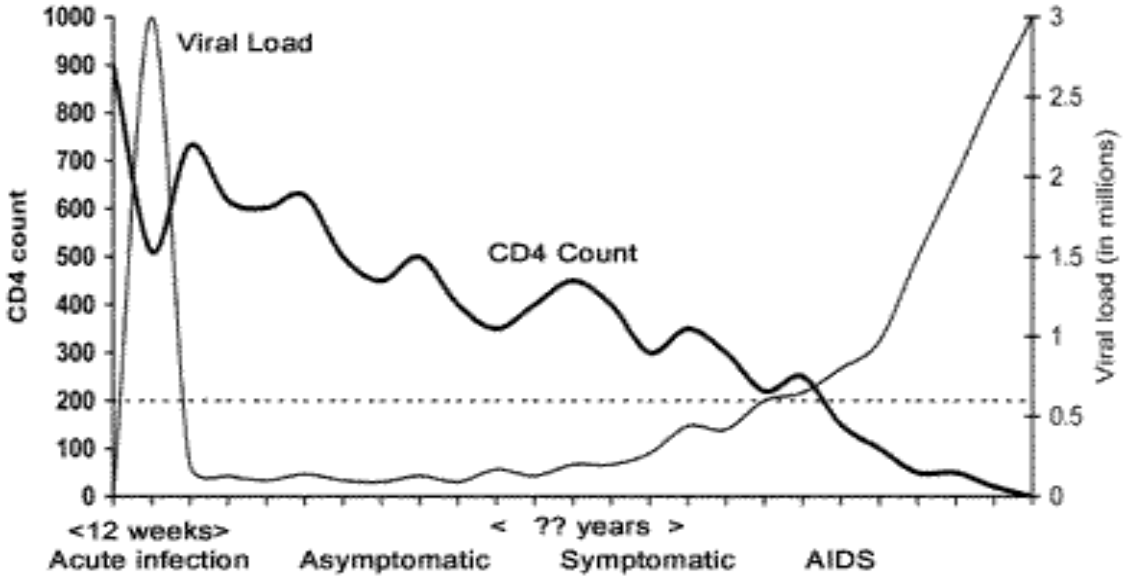

Figure 1.3 Course of HIV-1 infection. Following transmission, there is a burst of viremia and decrease in CD4 cells. The viral set point, which was shown to be temporally associated with the emergence of CTL, is established but gradually increases over the years. Time of progression to AIDS depends on the magnitude of set point viremia such that higher set point results in faster disease progression and vice versa. The CD4 count remains steady for a few years but gradually decreases, leaving the host vulnerable to opportunistic infections.

shortened telomere length (16), and increased PD-1 expression (17, 71). HIV-1 also avoids immune detection by remaining dormant in latently infected reservoirs (11-13, 63). Another reason for the diminished effectiveness of the CTL response is the ability of the HIV-1 protein Nef to downregulate cell surface expression of MHC class I, referred to as human leukocyte antigen (HLA) molecules in humans. By disrupting intracellular trafficking and sequestering HLA–A and –B molecules from reaching the plasma membrane (62, 77), Nef makes HIV-1-infected cells less likely be killed by CTL due to a reduced concentration of surface pMHC. Above all, the extraordinary mutational capacity of HIV-1 poses the biggest challenge to the immune response (36, 64) and to HIV vaccine design. The remarkable rate of mutation and adaptation allows the virus to evade adaptive immunity, forcing the immune system into a cyclical and exhaustive process of targeting and re-targeting of the virus.

1.3 Sequence plasticity of the HIV-1 genome

In a chronically infected individual, upwards of 10^{10} infectious virions are produced on a daily basis (27). Coupled with the error-prone nature of HIV-1 Reverse Transcriptase (43), this rapid turnover ensures that every possible combination of one to two mutations can be produced everyday in an infected individual (46, 53).

1.3.1 Mutations within Envelope (Env) gp41 tyrosine-based sorting signal –

The envelope glycoprotein (Env) of HIV-1 is a trimeric complex of heterodimers composed of gp120 and gp41, surface and transmembrane subunits, respectively. In the face of an evolving immune response, Env is capable of adapting and avoiding recognition by neutralizing antibodies (nAbs) while preserving the ability to mediate viral entry (58, 75).

The cytoplasmic tail of gp41 contains sequences that affect Env trafficking and targeting to the plasma membrane and the concentration of Env on the surface of infected cells (2, 19, 38). In T lymphocytes, a membrane-proximal tyrosine-based sorting signal (Y712xxϕ) is required for the release of both HIV and SIV from the plasma membrane (19). Mutations within the Y712xxϕ motif affect Env incorporation into virions (38) and overall infectivity (18). Interestingly, mutations in the sorting signal in SIV appeared to have minimal impact on viral replication, reflected by normal peak viremia *in vivo* and growth kinetic *in vitro*, and provided protection from challenge with wild type SIV (22) in macaques. Furthermore, *in vivo* CD8 depletion experiments indicated that $CD8^+$ T lymphocytes may contribute to the protective immunity (28).

The effect of analogous mutations in HIV-1 on viral replication and susceptibility to CTLs is described in **CHAPTER 2**.

1.3.2 Reduced synonymous mutation and co-variation in Pol –

The *pol* gene encodes the viral enzymes—Protease, Reverse Transcriptase and Integrase—that provide the essential machinery by which the virus can reproduce. The majority of antiretroviral drugs target protease and reverse transcriptase (67) and, thus, the structure and function of these proteins may influence the development of drug resistance.

A recent study (73) investigated this notion and uncovered what appeared to be an uncharacterized RNA secondary structure at the junction of protease and reverse transcriptase. Utilizing thermodynamic prediction programs, Wang *et al* provided compelling evidence of a novel RNA stem loop structure within HIV-1 *pol*. They reported markedly reduced synonymous variation, a hallmark of nucleotide secondary structures (66), and co-variation between synonymous mutations corresponding to predicted base-pairing sites. Lastly, they used a chemical-based method to interrogate between paired and unpaired nucleotides (76) to experimentally detect the RNA structure, which was independently validated through another study (74). **CHAPTER 3** is devoted to the work performed dissecting the functional importance of this structure and examining the impact of pairing-disruptive synonymous mutations on HIV-1 reproductive success.

1.3.3 Immune-mediated escape mutations –

A landmark study in 2002 (50) analyzed HIV Reverse Transcriptase sequences in a cohort of chronically infected individuals and noted that certain sequence polymorphisms were strongly associated with the expression of particular class I HLA alleles, which can have a significant impact on the outcome of patterns of escape (8, 10, 59). Moore *et al* were the first to describe HLA-associated selection of viral sequences in driving HIV-1 evolution at the population level. Thus, mutational escape from immune detection may be the strongest evidence supporting that HLA-restricted antigen-specific CTLs are a major determinant in controlling the virus.

A multitude of mutations can cause immune evasion. Mutations that may be classified as escape are those that interfere with intracellular antigen processing and presentation, including both intra- and extra-epitopic mutations that alter sequence-specific epitope processes (20, 70), cytosolic stability of the peptides (39), disruption of peptide-MHC binding (15, 56, 61), and reduced interaction of surface pMHC with TCRs (6, 44, 49). Remarkably, it has been shown that just a single amino acid substitution can impair CTL recognition and lead to escape (81). Although the magnitude of HIV variability may appear insurmountable for CTL to control, the end result must still be an infectious virus.

1.4 Fitness costs of HIV-1 mutations

Although HIV-1 frequently mutates to escape and poses a significant challenge for CTL response, escape mutations tend to exact a fitness cost of reduced replicative capacity (44, 54). We define the theoretical intrinsic ability of HIV to replicate in the absence of environmental factors as *replicative capacity* and the ability of the virus to grow within the context of a host environment as *viral fitness*. Depending on the timing and location of the mutation, the fitness costs may differ in severity.

1.4.1 Acute versus chronic infection: timing and occurrence of escape –

HIV-1 escape (23) and CTL evolution (7, 25) differ considerably in early versus chronic infection. Studies have shown that early CTL responses target variable sequences while CTL responses developed during chronic infection target more conserved sequences (35). As a consequence, early CTL responses often succumb to "original antigenic sin," or failure to increase CTL breadth (33). Contrary to acute infection, where viral escape and CTL re-targeting is commonly observed (4, 41, 51, 57), chronic infection has relatively stable CTL responses and minimal viral evolution (26, 29, 35). The differential fitness costs of escape in early versus late infection are addressed in **CHAPTER 4**.

1.4.2 HLA-associated fitness impairments: implications for control –

Several studies have identified CTL escape mutations in CTL-targeted epitopes that are associated with increased viral load and faster progression to AIDS (26, 32, 34). However, until relatively recently, escape mutations associated with substantial fitness impairment have

generally been limited to smaller observational studies and are reproducibly selected in the context of specific HLA alleles—B*13, B*27, B*57/5801—overrepresented among individuals able to maintain high CD4 counts and low viral load over a long period of time (6, 15, 44, 49, 56, 61). Further evidence of immune-mediated selection of such mutations have been demonstrated by the reversion of these mutations following transmission to non-HLA-matched individuals (40) and compensatory sequence changes that stabilize escape mutations selected elsewhere (15, 44). It has been observed in cohorts of long term non-progressors (LTNPs) that the frequency of HLA B*57 is disproportionately high (48) and that the aforementioned HLA-B alleles have strong genetic associations to slower HIV disease progression. Effectively targeting areas that are structurally constrained and selecting for escape mutations that severely affect viral fitness may partially explain why certain class I HLA molecules confer a protective advantage to their carrier. Although these reports are indicative of the overall benefit of having these particular alleles, these studies are also largely biased toward them. Thus it is imperative to understand the breadth of this phenomenon and if it extends to other HLA types not already implicated with superior control of replication. These issues are evaluated in **CHAPTER 4**.

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CHAPTER 2:

Disruption of an env tyrosine-dependent sorting signal does not affect the susceptibility of HIV-1 to cytotoxic T lymphocytes

2.1 ABSTRACT

Disruption of the gp41 tyrosine-dependent sorting signal has been shown to attenuate SIV infection to generate protective immunity in nonhuman primates. Given the key protective role of $CD8^+$ cytotoxic T lymphocytes (CTLs), we investigated whether disruption of this motif in HIV-1 might render the virus more susceptible to the antiviral activity of CTLs. Three previously studied viable mutations disrupting the conserved gp41 tyrosine-based sorting motif in SIV—EnvY712I, EnvY712S, and EnvΔGY—were introduced in the context of NL4-3, an HIV-1 molecular clone. These mutant strains were compared to the parental HIV-1 wild type strain for susceptibility to suppression by five HIV-1-specific CTL clones recognizing epitopes in Gag, Pol, Vpr, and Nef. EnvY712I and EnvY712S, but not EnvΔGY, demonstrated normal viability. Suppression of replication by the CTL clones was similar between mutants and wild type. Lastly, we found that disruption of the motif does not directly increase viral susceptibility to CTLs. This suggests that the attenuation of SIV infection *in vivo* occurs via another mechanism, such as reducing immunosuppressive effects of Env.

2.2 INTRODUCTION

Infections with live-attenuated viruses within SIV models of HIV-1 pathogenesis have offered the best evidence that a vaccine could generate protective immunity. Desrosiers and colleagues (7) first demonstrated this principle using *nef*-defective SIV, which established a chronic low-grade asymptomatic infection in macaques associated with protection against subsequent challenge with wild type SIV. At least in part, this phenomenon appeared to be due to attenuation of viral replication due to *nef* deletion.

More recently, Shacklett *et al* (16) and Hoxie *et al* (8) have examined attenuated SIV infection via mutations in the transmembrane domain of gp41, which also subsequently protects from challenge by wild type SIV. Shacklett *et al* found that SIV containing multiple (stop and point) mutations disrupting this domain reduced viral replicative capacity *in vitro*, likely due to effects on the role of this domain for gp41 membrane trafficking in infected cells (5, 6). Hoxie and colleagues more specifically created mutations in the highly conserved Y712xxϕ tyrosine-based sorting motif (where Y is a Tyr, x is any amino acid, and φ is an amino acid with a bulky hydrophobic side chain) in the membrane-proximal cytoplasmic domain of gp41 (8) (Figure S2.1). Although this motif has a key role in gp41 trafficking (5, 6), the mutations appeared to have minimal impact on viral fitness as reflected by normal levels of peak viremia during acute infection *in vivo* and growth kinetics *in vitro*. Thus, the mechanism of attenuation and subsequent protection from wild type viral infection is unclear, but probably not due to markedly reduced capacity for SIV replication. Interestingly, Hoxie *et al* have preliminarily shown that $CD8^+$ T lymphocytes (CTLs) may have a role by *in vivo* CD8 depletion experiments (9).

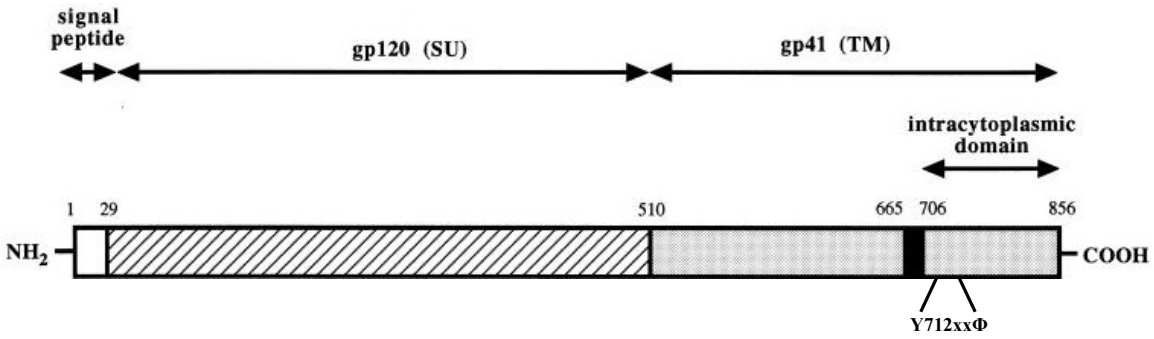

Figure S2.1 **Schematic view of HIV-1 envelope glycoprotein**. The membrane-proximal tyrosine-based sorting motif (Y712xxϕ) is located within the cytoplasmic domain of gp41.

2.3 MATERIALS AND METHODS

Target cell lines:

The HIV-1 permissive T1 cell line (14) was maintained in RPMI 1640 supplemented with 10% calf serum, L-glutamine, HEPES, and penicillin-streptomycin (R10). These were utilized as MHC-matched target cells for A*02-restricted CTL clones.

Creation of mutations within the gp41 tyrosine-based sorting signal (Y712xxϕ):

Mutations were generated by site-directed point mutagenesis with the QuikChangeIIXL kit (Agilent Technologies; Westlake Village, CA) in the context of HIV-1 NL4-3. Mutagenesis primers were designed using the QuikChange Primer Design Program (Agilent Technologies; Westlake Village, CA). All modifications were confirmed by the ABI Big Dye 3.1 Sequencing kit (Applied Biosystems; Carlsbad, CA) according to the manufacturer's protocol. The resulting mutations were named EnvY712I, EnvY712S and EnvΔGY (Table 2.I).

Production of infectious HIV-1 harboring Y712xxϕ mutations:

Infectious virus was produced by transfecting whole genome plasmids containing the mutations of interest. 293T cells were transfected with 10 μg of each plasmid using a standard calcium phosphate technique (3). Supernatant containing virus was collected on day 2 and 3 post-transfection and filtered through a 0.45 μM filter. Virus production was quantified using a p24 antigen-based ELISA (Perkin Elmer; Santa Clara, CA). Transfections typically produced greater than 200,000 pg/mL of p24.

Viral inhibition assays:

HIV-1 growth inhibition by CTL clones was assessed by co-culturing HIV-1-specific CTLs and target T1 cells infected with Y712xxϕ mutant viruses at a multiplicity of infection (MOI) of 10^{-2}. Using an effector-to-target ratio of 0.25:1, approximately 1.25×10^4 CTLs were co-cultured with 5×10^4 target cells per well in a 96-well flat-bottom plate in 200 μl R10-50 medium (R10 medium supplemented with IL-2), or with no CTLs (control), in triplicate wells. On days 1, 4, and 7 post-infection, 100 μl supernatant was removed for quantitative p24 ELISA (Perkin Elmer; Santa Clara, CA) and replaced with fresh R10-50 medium. The CTL clones were derived from HIV-1-infected persons under an IRB-approved protocol at the University of California, Los Angeles.

Env Wild Type	8355-	GGATATTCACCATTA -8369
	711-	**G Y S P L** -715
Env Y712I		---AT----------
		- I - - -
Env Y712S		----C----------
		- S - - -
Env ΔGY		XXXXXX---------
		X X - - -

Table 2.I **Nucleotide (NL4-3 residues 8355-8369) and amino acid sequences (NL4-3 Env 711-715) of Y712xxϕ**. Wild type and mutant forms of the conserved membrane-proximal tyrosine-based sorting motif (Y712xxϕ). Dash (-) indicates the same nucleotide or amino acid as wild type and cross (x) indicates a deletion of a nucleotide or amino acid compared to wild type.

2.4 RESULTS

To assess whether analogous mutations in HIV-1 might affect viral susceptibility to CTLs, mutations in the Y712xxϕ motif were constructed in HIV-1 NL4-3 (2) by point mutagenesis. These included EnvY712I, EnvY712S and EnvΔGY mutations in the cytoplasmic domain of gp41, inserted into the whole genome context of NL4-3, which had previously been engineered to contain clade B consensus sequence at amino acid positions 77-85 (HXB2 numbering system) of Gag (21). HIV-1 stocks were harvested from the supernatant fluid of 293T cells transfected with these plasmids two days post-transfection. When these viruses were examined for their ability to replicate in T1 cells (14), the EnvY712I and EnvY712S mutants had growth kinetics similar to wild-type NL4-3 (EnvY712), in agreement with findings using SIVmac239 from Hoxie *et al* (8) (Figure 2.1). In contrast, the EnvΔGY mutant was replication incompetent, suggesting that this mutation more severely interfered with viral replication in HIV-1 than SIV.

To assess the susceptibility of these mutants to CTLs, these viruses were tested in virus suppression assays using HIV-1-specific CTL clones as previously reported (4, 20) (Figure 2.2). The clones included: S1-SL9-3.23T recognizing the HLA A*02-restricted epitope SLYNTVATL in Gag p17 (a.a. 77-85) (1), S31-IV9-10.11T recognizing the HLA A*02-restricted epitope ILKEPVHGV in RT (a.a. 309-317), and S58-PL10-10.8 recognizing the HLA A*02-restricted epitope PLTFGWCYKL in Nef (a.a. 136-145). The HIV-1-permissive target cells utilized were A*02-expressing T1 cells (20). Comparisons of these viruses showed similar degrees of suppression by the three clones, with the wild type virus being suppressed equally or slightly

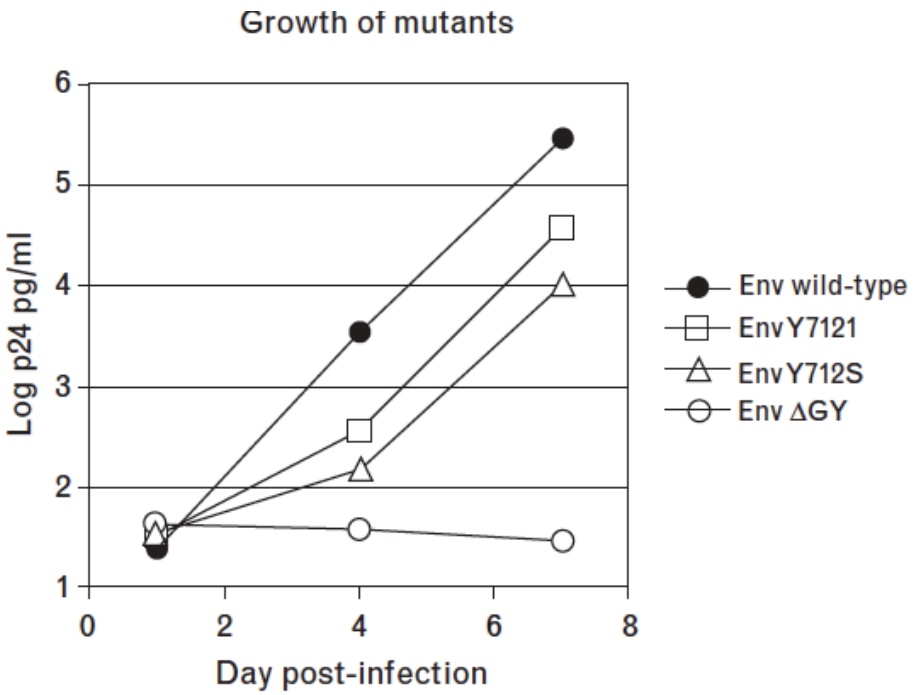

Figure 2.1 **Growth kinetics of wild type and mutant viruses**. 1x10^6 HIV-1 permissive T1 cells were infected with the indicated mutant or wild type virus at an input of 1000 pg p24 antigen and cultured in a 24-well tissue culture plate. Viral replication was measured by quantitative p24 ELISA (Perkin Elmer; Santa Clara, CA). These results are representative of four independent experiments.

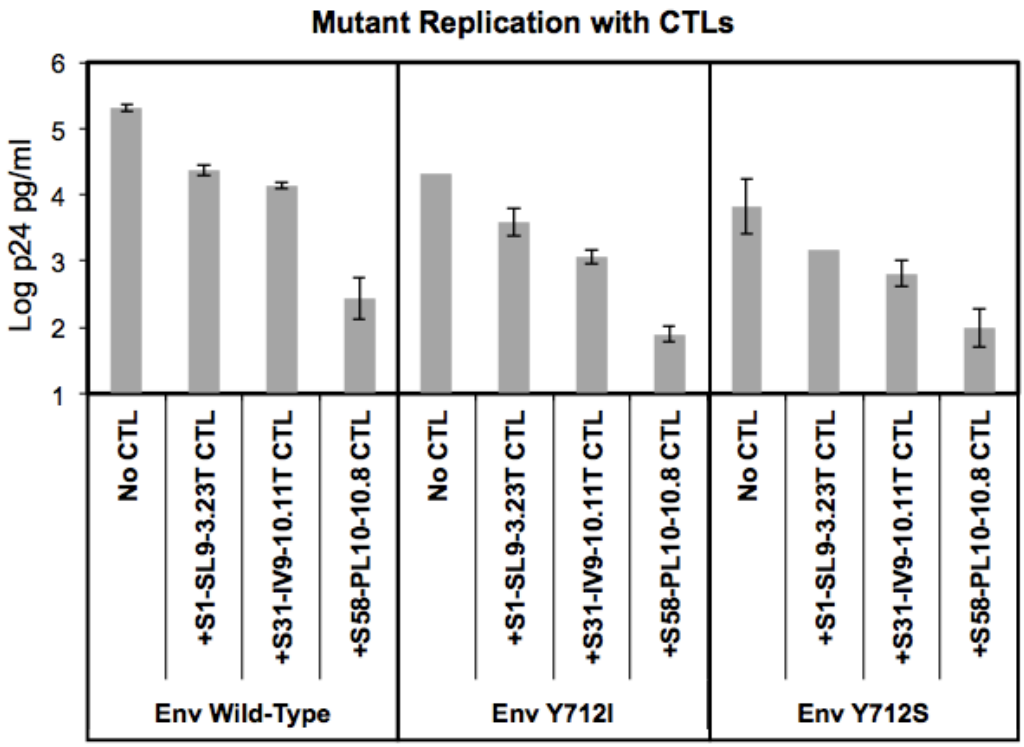

Figure 2.2 **Viral replication in the absence or presence of CTL**. T1 cells were infected with 500 pg p24/10^6 cells and cultured in triplicate in 96-well tissue culture plates with the indicated $CD8^+$ T lymphocyte (CTL) clones ($1.25x10^4$ CTLs with $5x10^4$ target cells). Viral replication was measured by quantitative p24 ELISA (Perkin Elmer; Santa Clara, CA) on day 4 after infection.

more than the two mutants (Figure 2.3). Similar results were noted in an independent experiment using two other CTL clones recognizing other epitopes (data not shown). Overall, these results suggested that directly increased susceptibility to CTL inhibition is not the mechanism of *in vivo* attenuation of infection with viruses containing these mutations.

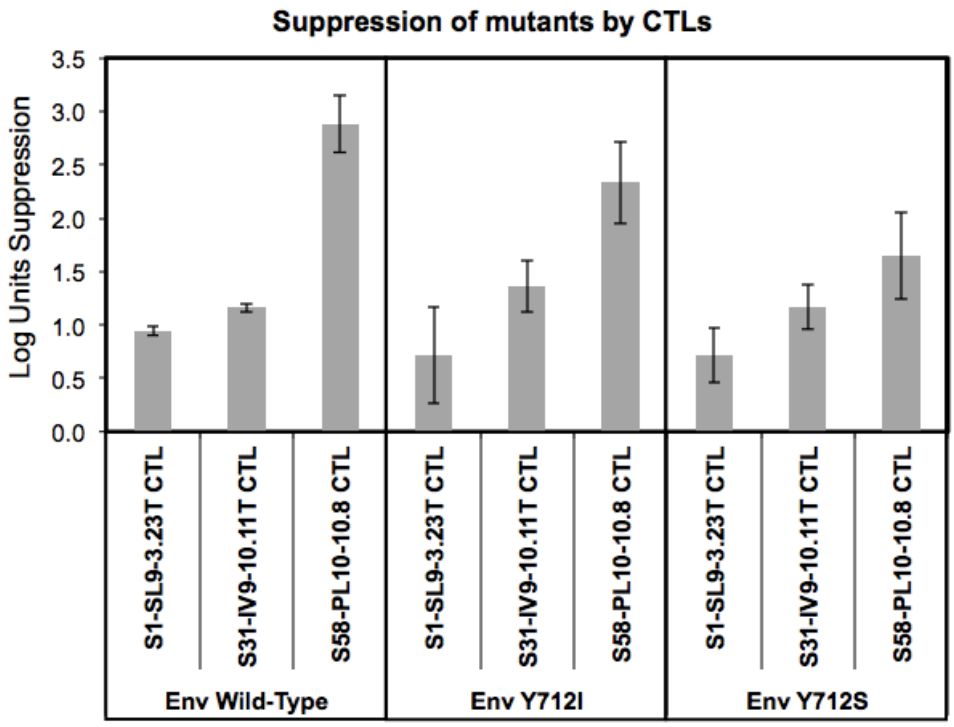

Figure 2.3 **Suppression of viruses by CTL**. Inhibition was calculated by comparing replication in cells with or without CTLs. The plotted data indicate means of triplicates (error bars indicate 1 SD) for one experiment, and the results are representative of two independent experiments with these clones. Similar results were seen using a B*57-restricted CTL clone recognizing the epitope TSTLQEQIGW in Gag p24 and an A*02-restricted CTL clone recognizing the epitope AIIRILQQL in Vpr in other experiments (data not shown).

2.5 DISCUSSION

Because attenuated SIV infection has been the most robust example of a protective vaccine in the SIV-macaque model of HIV-1 infection (7, 9, 11, 12, 19), understanding potential avenues of viral attenuation and the mechanisms of their impacts on antiviral immunity is clearly an important goal. The observation that SIV with mutations in the Y712xxϕ motif yields infections with high peak viremia but asymptomatic low viremia set-points in macaques with subsequent protection from wild type virus challenge (8, 9, 16) suggests that viral replication is not markedly affected by the mutations *in vivo,* and that infection is attenuated during the chronic phase of infection. A major determinant of set-point viremia during this phase is the CTL response, as demonstrated by CD8 depletion experiments in SIV-infected rhesus macaques (10, 13, 15). Furthermore, it has been suggested through analogous CD8 depletion experiments that the low set-point viremia of macaques infected with SIV containing disruption of the Y712xxϕ motif may be related to the CTL response (9). Thus, a simple explanation could be that disruption of the motif somehow renders SIV directly more susceptible to CTL.

However, our results suggest that this is not the case for HIV-1; disruption of the Y712xxϕ motif did not appear to increase susceptibility of HIV-1 to CTLs in direct tests of viral growth suppression. Although it is possible that our *in vitro* assay does not predict the interaction of virus and CTLs *in vivo*, it seems likely that the mechanism of attenuation is either not mediated by CTLs, or that if it is, an increase in the antiviral activity of CTLs is indirect. Interestingly, it has been suggested that the HIV-1 Env may facilitate viral escape from CTLs in lymph nodes (17, 18). Although our data do not address this issue directly, they are compatible with an

indirect mechanism whereby reduced levels of Env could reduce viral escape from CTLs. Further work would be required to explore this possibility.

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CHAPTER 3:

Conservative mutations within a highly conserved RNA secondary structure in HIV *pol* do not interfere with HIV-1 viral replication

3.1 ABSTRACT

Important RNA secondary structures in Human Immunodeficiency Virus type-1 (HIV-1) include the *trans*-activating responsive (TAR) element, Rev response element (RRE) and the *gag-pol* frameshift hairpin. Wang *et al* have provided compelling evidence of a novel RNA stem loop structure within HIV-1 *pol* (nt 382-494), noting reduced synonymous variation and co-variation between synonymous mutations for the predicted base-pairing nucleotides, suggesting strong sequence conservation for pairing to maintain the predicted RNA structure. To evaluate the biological importance of this structure, we assessed the effect of pairing-disruptive synonymous mutations on HIV-1 reproductive success. Synonymous mutations were generated to disrupt selected base pairs implicated in the co-variation analysis. Viral replication of mutant viruses—Stem A, Stem ABC, Stem ABC+(L)—was monitored over several days and, in parallel, selective 2'-hydroxyl acylation by primer extension (SHAPE) analysis was performed to confirm the disruption of the novel stem loop structure. According to SHAPE analysis, the stem loop structure of the mutant virus appeared to have been modified by the synonymous mutations. Interestingly, the mutant virus showed similar growth kinetics compared to the wild type virus. Thus, the synonymous mutations created within the highly conserved RNA structure in HIV-1 *pol*, though predicted to alter the stem loop structure, did not interfere with viral replication.

3.2 INTRODUCTION

The genomes of all single-stranded RNA viruses contain internal secondary structures that play fundamental roles in viral replication. Some of these viral RNA structures include internal ribosomal entry sites, packaging signals, pseudoknots, and ribosomal frameshift motifs. In Human Immunodeficiency Virus type-1 (HIV-1), a number of RNA secondary structures have been well documented for their roles in HIV-1 transcription: the *trans*-activating responsive (TAR) element at the 5'-end of the genome (3); the Rev response element (RRE) present in the *env* gene (8, 13); and the *gag-pol* frameshift stem-loop (16). Several more RNA regulatory motifs have been identified within the 5' and 3' untranslated regions (4), suggesting the possibility that most regulatory structures within the viral RNA genomes remain uncharacterized (6, 14, 15, 17, 18).

A more recent study (20) utilized a high throughput RNA analysis technique called selective 2'-hydroxyl acylation and primer extension (SHAPE) (22) to interrogate the RNA architecture of the entire HIV-1 genome. SHAPE exploits the fact that unpaired, or conformationally flexible, nucleotides are more accessible to chemical modification. An anhydride was used to acylate unpaired nucleotides at the 2'-OH position, resulting in adducts that are detected as stops to primer extension using fluorescently-labeled primers and capillary electrophoresis. In this study, they confirmed at least 10 previously uncharacterized structured regions that exhibited low SHAPE reactivity and high pairing probability.

A highly conserved RNA stem-loop proposed by Wang *et al* (19) was among those validated by Watts *et al*. Using a series of thermodynamic prediction methods, synonymous variability analysis, and co-variation analysis on approximately 20,000 HIV-1 *pol* sequences, a novel RNA secondary structure at the junction of the protease and reverse transcriptase genes was uncovered. Within the 107 nucleotide region, there are 3 stem loop structures—stems A, B, and C—that show markedly reduced synonymous variation and co-variation between synonymous mutations corresponding to predicted base-pairing sites. However, the exact biological function of this region remains unclear.

To dissect the functional importance of this structure, we introduced a series of pairing-disruptive synonymous mutations throughout each stem loop structure within the context of NL4-3. We monitored the effect of these mutations on the replicative capacity of HIV-1 *in vitro* and performed SHAPE analysis on our panel of mutated RNA secondary structures to confirm the base-pairing disruption.

3.3 MATERIALS AND METHODS

Creation of mutations in the stem regions of HIV-1 pol 418-494 RNA structure:

Mutations were generated at the wobble position by site-directed point mutagenesis with the QuikChangeIIXL kit (Agilent Technologies; Westlake Village, CA) in the context of HIV-1 NL4-3. Mutagenesis primers were designed using the QuikChange Primer Design Program (Agilent Technologies; Westlake Village, CA). All modifications were confirmed by the ABI Big Dye 3.1 Sequencing kit (Applied Biosystems; Carlsbad, CA) according to the manufacturer's protocol. The resulting mutations are summarized in Table 3.I; the unmodified stem structure was designated as Stem WT.

Thermodynamic prediction, free energy calculation and base-pair probabilities:

RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi), an online tool that predicts the free energy of the most stable RNA structure for a given sequence, predicted the stem structures containing our mutations of interest with the established default parameters. Furthermore, RNAfold was utilized to measure the minimal free energy (MFE) for each sequence with default parameters. Lastly, RNAfold determined the base-pair probabilities for each mutated stem structure.

Generation of HIV-1 containing mutated stem structures:

Infectious virus was produced by co-electroporation of NL4-3 half-genome plasmids into T1 cells (1). Briefly, modified p83-2.1 plasmid containing stem mutations and p83-10 plasmid were

Virus	Nt Position	WT nt	Mutant Nt
Stem A1	387	U	A
Stem A2	390	A	U
Stem A3	399	A	U
Stem A4	402	A	G
Stem A1A2	387	U	A
	390	A	U
Stem A3A4	399	A	U
	402	A	G
Stem A	387	U	A
	390	A	U
	399	A	U
	402	A	G
Stem ABC	387	U	A
	390	A	U
	399	A	U
	402	A	G
	414	C	U
	417	C	U
	420	A	U
	444	G	A
	447	U	A
Stem WT(L)	***445***	***A***	***C***
Stem ABC+(L)	387	U	A
	390	A	U
	394	U	C
	396	A	C
	399	A	U
	402	A	G
	414	C	G
	417	C	U
	420	A	U
	435	G	C
	436	T	C
	444	G	A
	445	***A***	***C***
	447	U	A
	470	T	A
	471	A	T
	472	G	C
	473	T	G
Stem ABC+(I)	387	U	A
	390	A	U
	394	U	C
	396	A	C
	399	A	U
	402	A	G
	414	C	G
	417	C	U
	420	A	U
	435	G	C
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	471	A	T
	472	G	C
	473	T	G

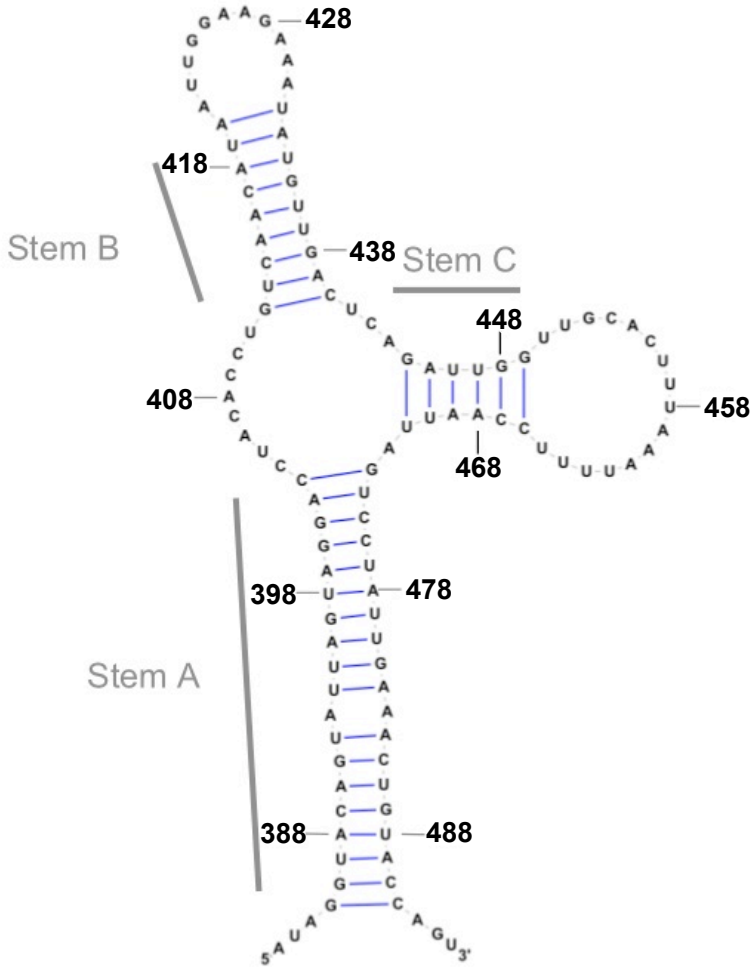


Table 3.I <u>Panel of viruses representing several variants of the novel stem loop structure (bottom, adapted from (19))</u>. Nucleotide (HIV-1 *pol* 382-494) positions of the novel RNA stem loop structure and the new nucleotides* that were engineered via site-directed mutagenesis.

* Substitutions marked with (*) result in non-synonymous amino acid change.

linearized with *Eco*RI (New England Biolabs; Ipswich, MA) and electroporated into T1 using GenePulser Xcell (Biorad; Hercules, CA). Mutant viruses were tagged with the HSA reporter and wild type virus was tagged with the HSA-HA reporter (2). The p24 in the supernatants of the electroporated cells was monitored by quantitative p24 ELISA (Perkin Elmer; Santa Clara, CA) for 7-14 days and virus stocks were harvested at the peak.

Assessment of the growth kinetics for the mutant stem loop viruses:

To determine the reproductive success of each mutant virus, T1 cells or primary $CD4^+$ were separately infected with each variant using 500 pg of p24 antigen/10^6 cells (approximately an MOI of 0.01) in triplicate in a 24-well plate. On days 4, 7, and 10 post-infection, 1 mL supernatant was removed for quantitative p24 ELISA (Perkin Elmer; Santa Clara, CA) and replaced with fresh R10 medium (for T1) or R10-50 (for primary $CD4^+$).

Real-time PCR quantitation of mixed HIV-1 infected T1 cells:

The T1 cell line was co-infected with an index reporter virus containing the consensus clade B sequence for the stem loop sequence and tagged with HSA-HA in the *nef* locus and mutant stem loop virus was tagged with HSA in the *nef* locus at an MOI of 0.005 each. At days 2 and 5 post-infection, genomic DNA was isolated using Puregene Blood Core Kit B (Qiagen; Valencia, CA) for analysis by real time PCR (2x universal premix, Applied Biosystems; Carlsbad, CA) in a 25 μl reaction volume, followed by analysis using StepOne Plus (Applied Biosystems; Carlsbad, CA). HSA-HA and HSA was detected separately using the following primers: HSA93F (HSA-specific) 5'-GCACCGTTTCCCGGTAACCAGAATAT; HA95F (HSA-HA-specific) 5'-CCCGTATGATGTACCGGATT; HSA210R (reverse primer for both forward primers) 5'-

AGAGAGAGAGAGAGCCAGGAGA. Simultaneously, β-actin was detected using the following primers: Actin171752F 5'-TCACCCACACTGTGCCCATCTACGA; Actin172246R 5'-CAGCGGAACCGCTCATTGCCAATGG. We used a probe specific for both HSA and HSA-HA, HSA76FAM 5'-FAM-TCCCTGCAGTCCACAGCTGG-Tamra, and a separate probe for actin, ACTIN 5'-VIC-ATGCCCTCCCCCATGCCATCCTGCGT-Tamra. Plasmid standards were used to generate curves of Ct versus copy number, after analysis using StepOne Software v2.0 (Applied Biosystems; Carlsbad, CA).

Long term passaging of mutant stem loop viruses to detect subtle fitness costs:

For the first round passage, T1 cells were co-infected with wild type and mutant stem loop viruses at a total MOI of 0.01 (approximately 500 pg of p24 antigen/10^6 cells) in triplicate in a 24-well plate. On day 4 post-infection, half of the infected cells were pelleted and genomic DNA was extracted from each for sequencing of the region with the mutation. On day 6 post-infection, the infected cells were pelleted, washed twice with R10, resuspended in 2 mL fresh R10, and transferred to a new 24-well plate. On day 7 post-infection, the infected cells were pelleted and genomic DNA was extracted for sequencing; additionally, supernatant was removed for quantitative p24 ELISA (Perkin Elmer; Santa Clara, CA) and virus stocks were harvested for second round infection.

For the subsequent round of passage, virus stock collected at day 7 was used to infect fresh T1 cells at an MOI of 0.01. On day 4, 1 mL supernatant was removed and replaced with fresh R10. On day 7 post-infection, the infected cells were pelleted and genomic DNA was extracted for sequencing; supernatant was removed for quantitative p24 ELISA and virus stocks were

harvested for another round infection. This process was repeated for a total of five rounds of passaging.

As per a modification of the method used Martinez-Picado *et al* (9), relative replicative fitness in each virus co-culture was performed by PCR amplification of the mutant region and subsequent sequencing. The relative proportions of the two competing viruses were determined at each passage based on the ratios of the specific mutations. Ratios were estimated based on the relative peak heights in electropherograms obtained by automated sequencing. Genotyping was performed by using the ABI Big Dye 3.1 Sequencing kit (Applied Biosystems; Carlsbad, CA) followed by capillary electrophoresis in the AB3130 Automated Sequencer (Applied Biosystems; Carlsbad, CA).

Selective 2'-hydroxyl acylation and primer extension (SHAPE) *analysis*:

Cloning and transcription –

The SHAPE method (10, 12, 21-23) was used to determine unpaired regions in the predicted secondary structure. The sequences of the wild type and mutant stem structures were cloned into the pCR2.1_TOPO-TA vector (Invitrogen; Carlsbad, CA). In addition to the HIV sequence, a 5' linker, and a 3' linker containing the RT primer binding site (22). Each plasmid was linearized with *Xba*I and used in a run-off transcription by T7 RNA polymerase (11). Transcriptions were precipitated in ethanol and purified on a 6% denaturing polyacrylamide gel. The RNA was resuspended in 0.5x TE buffer (5 mM Tris pH 7.5, 0.5 mM EDTA).

N-methylisatoic anhydride (NMIA) modification –

Approximately 12 pmol of each RNA was heated to 95°C for 3 minutes, then chilled at 4°C for 1 minute. 3.33x annealing buffer (333 mM HEPES, pH 8.0, 20 mM $MgCl_2$, 333 mM NaCl) was added to the RNAs, then incubated at 37°C for 20 minutes. The annealed RNA was treated with 6 mM NMIA in DMSO or DMSO alone for 45 minutes at 37°C, then ethanol precipitated for reverse transcription.

Primer extension –

RNA pellets were mixed with 0.4 μM SHAPE primer. The (+) and (-) NMIA reactions were labeled with FAM and TET, respectively. Primers were annealed to the RNA at 65°C for 5 minutes, 35°C for 5 minutes, and 4°C for 1 minute. Next, 6 μl of SHAPE enzyme mix (SuperScript III buffer, 0.1 M DTT, 10 mM dNTP mix; Invitrogen; Carlsbad, CA) was added and incubated at 52°C for 1 minute. 1 μl SuperScript III RT (Invitrogen; Carlsbad, CA) was added and immediately incubated at 52°C for 10 minutes. 1 μl 4 M NaOH was added to degrade the RNA and heated to 95°C for 5 minutes and cooled to 4°C. 3 M sodium acetate was added to each tube, (+) and (-) tubes were combined, and absolute ethanol was added to precipitate the cDNA products. The reactions were pelleted, washed with 70% ethanol, and dissolved in H_20.

Sequencing and data processing –

cDNA fragments were resolved by capillary electrophoresis (AB3130 instrument, Applied Biosystems; Carlsbad, CA). Raw electropherograms, containing fluorescence intensity versus elution time information, were aligned using Sequencing Analysis Software (Applied Biosystems; Carlsbad, CA) to compare the (+) and (-) NMIA reactions. Modified unpaired

regions were selected based on their peak heights relative to those of the 5'/3' linker regions, which have a known RNA stem loop structure.

3.4 RESULTS

To determine the effects of synonymous mutations on the reproductive success of HIV-1, a series of mutations were created that would disrupt the base pairing needed to sustain the novel stem loop structure. According to the RNAfold program, which predicts the most thermodynamically stable RNA structure for a given sequence, disruption of stem loops drastically altered the base pairing ability throughout the entire structure (Figure 3.1A-H). Surprisingly, electroporation of these mutant constructs produced viable virus, as demonstrated by a second round of infection of both an immortalized T cell line and primary $CD4^+$ cells (Figure 3.2).

To determine the fitness costs for viral replication of each mutant, target T1 cells were co-infected with wild type and each mutant virus and the reproduction of both viruses were compared using real time PCR. Relative to wild type, there were no significant differences in replication found in our panel of stem A mutant viruses or the Stem ABC virus, the variant in which, in theory, each stem loop structure should be disrupted (Figure 3.3A-B).

To examine the likelihood that these mutations elicited a subtle effect on fitness, a modified fitness assay surveying the co-infection over several rounds of passaging was performed. For comparison, SL9_82I/85I, a virus with previously determined loss of fitness (2), was included in the analyses. It was observed that the Stem A virus had a modest fitness impairment compared to wild type virus over a period of several weeks (Figure 3.4A). Additionally, a long-term assessment of Stem ABC+(L), the stem loop variant with the most disrupted pairings including

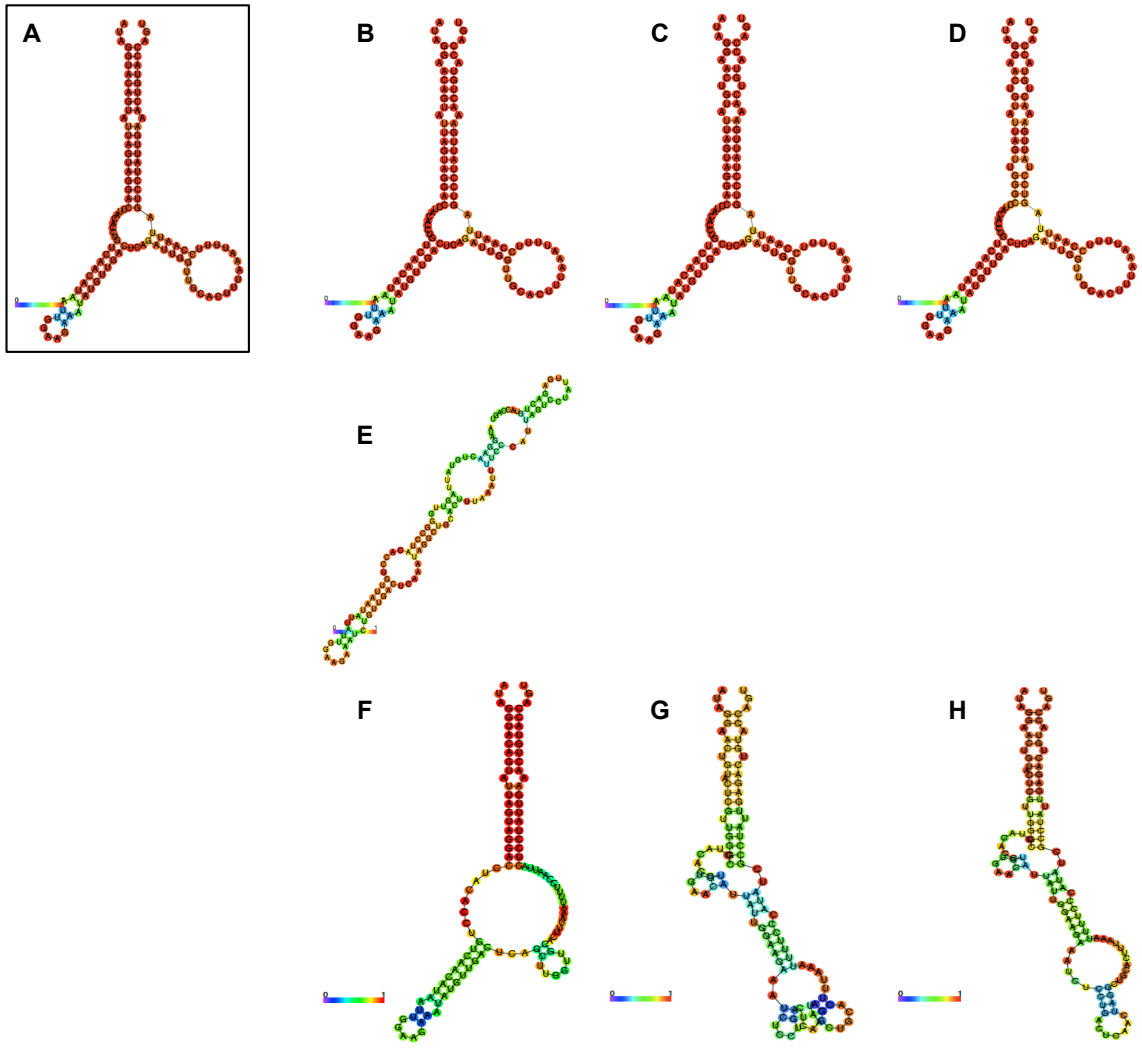


Figure 3.1 The predicted RNA secondary structures of HIV-1 *pol* 382-494. A) Stem WT, B) Stem A1, C) Stem A1A2, D) Stem A, E) Stem ABC, F) Stem WT(L), G) Stem ABC+(I), and H) Stem ABC+(L), using RNAfold with default parameters. The structure is colored by base-pairing probabilities, with red indicating a high probability and blue indicating a low probability. For predicted unpaired regions, the color denotes the probability of being unpaired (RNAfold, http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

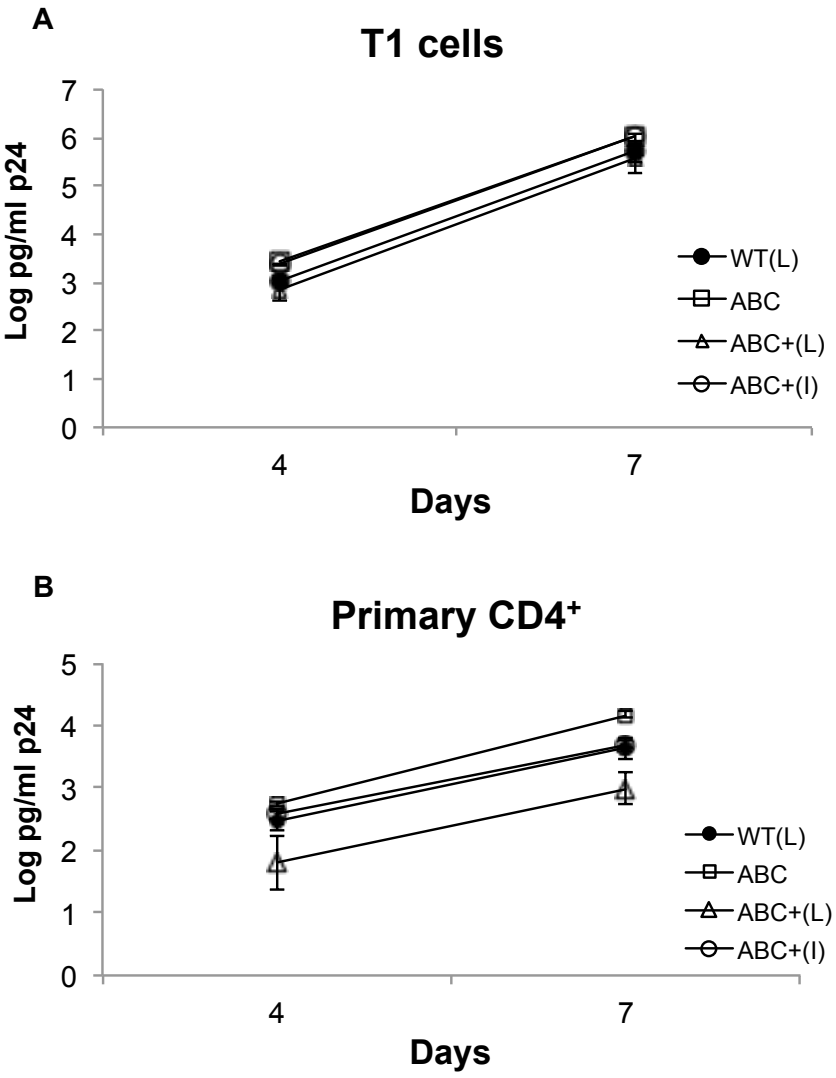


Figure 3.2 **Growth kinetics of mutant viruses**. $1x10^6$ HIV-1 permissive A) T1 cells or B) primary $CD4^+$ were infected with the indicated mutant at an input of 1000 pg p24 antigen and cultured in a 24-well tissue culture plate. Viral replication was measured by quantitative p24 ELISA (Perkin Elmer; Santa Clara, CA).

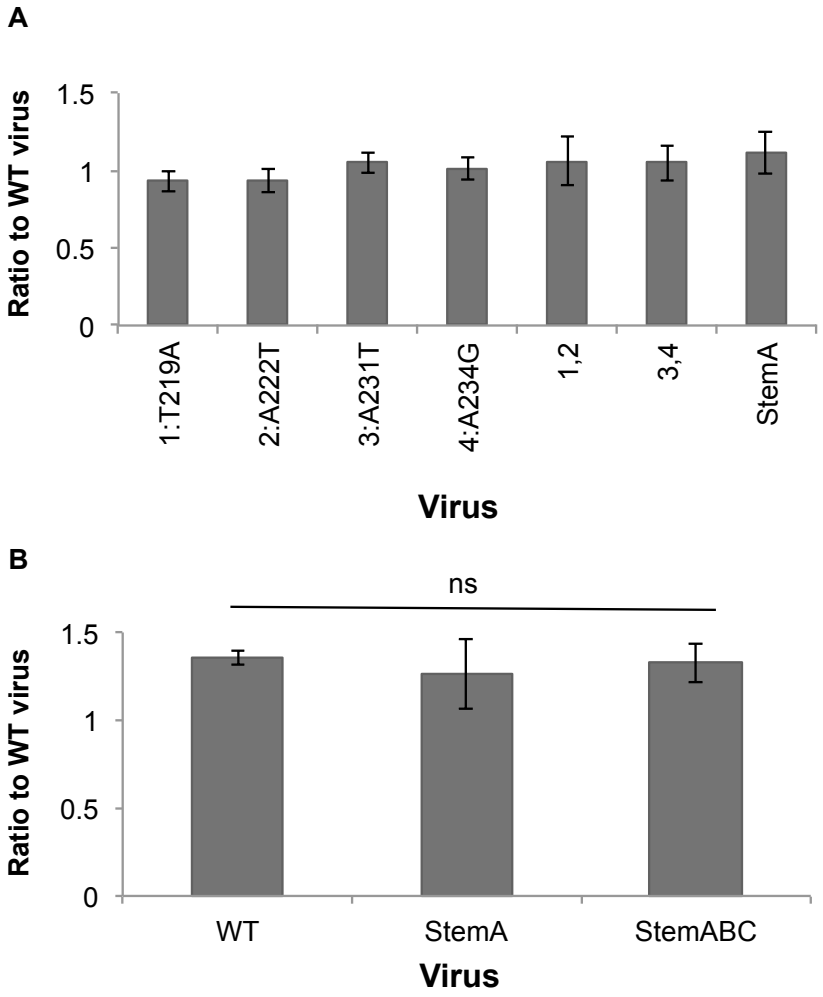


Figure 3.3 **Real time PCR analysis of short term fitness**. T1 cells were co-infected with wild type virus and A) panel of stem A mutant viruses or B.) Stem ABC virus at an MOI of 0.005 each. At days 2 and 5 post-infection, genomic DNA was isolated for analysis by real time PCR. These results are representative of two independent experiments.

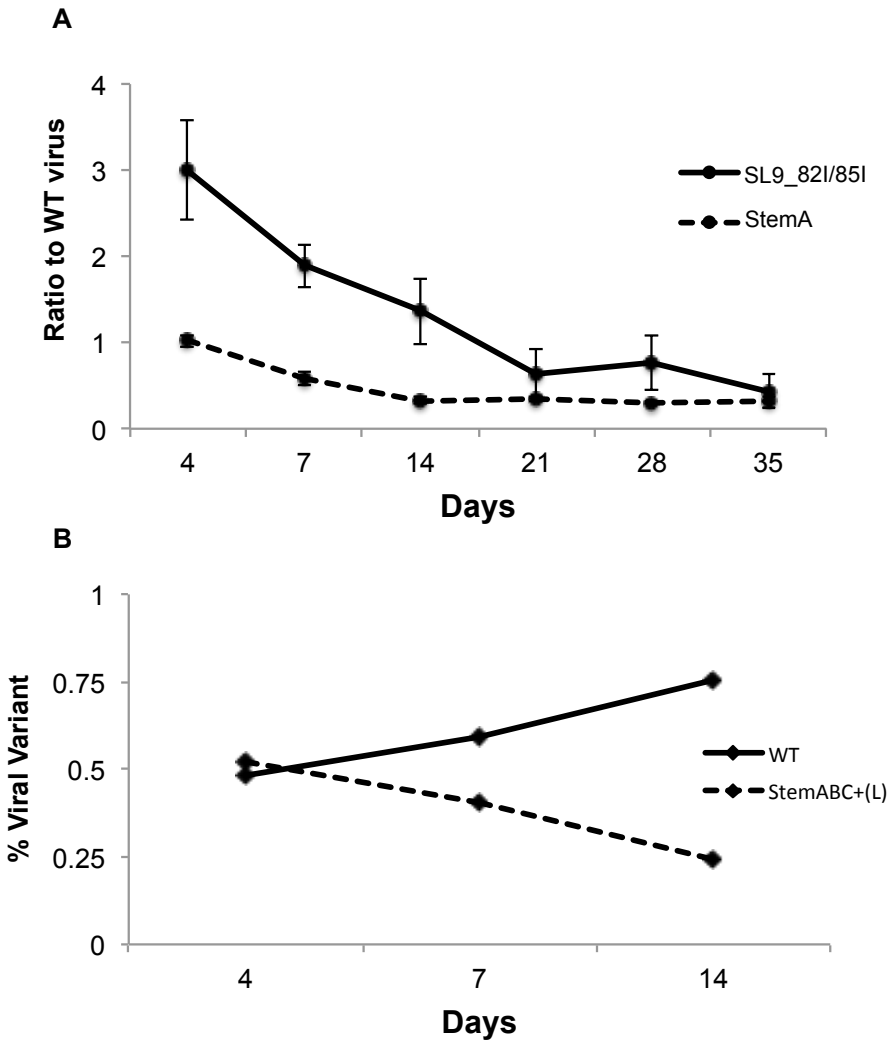


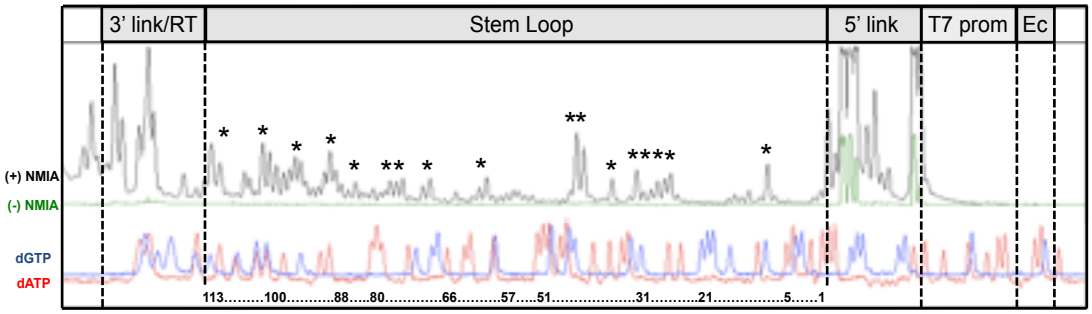
Figure 3.4 Long term fitness assessment of viruses. A) Stem A virus and SL9_82I/85I, a virus containing mutations within the p17-SL9 epitope that carry a known loss of fitness (2). The ratio to wild type virus is shown for each mutant. B) Long term fitness assessment of Stem ABC+(L) versus wild type virus, monitoring the percentage of each viral variant over the indicated days.

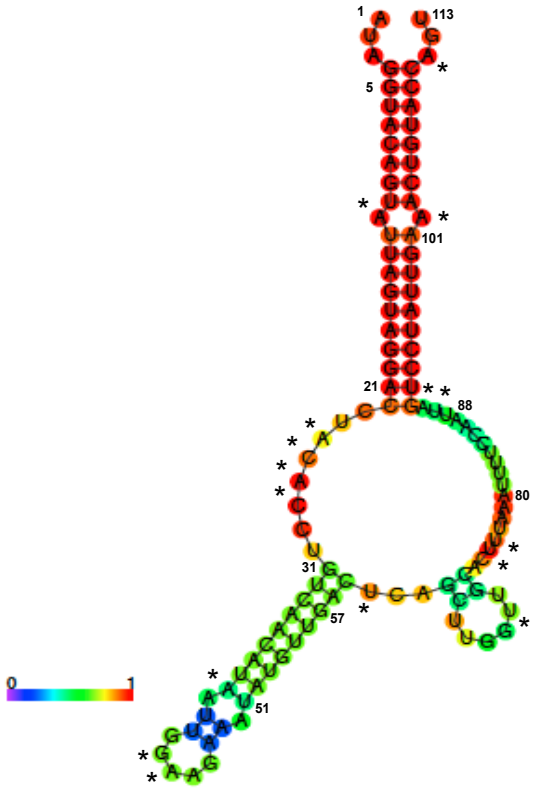
one non-synonymous mutation, demonstrated an altered replication pattern compared to wild type virus (Figure 3.4B). Interestingly, these mutant viruses were not completely overtaken by the wild type, suggesting that these viruses can productively replicate over an extended period of time despite the mutations to the conserved stem loop structure.

In parallel to the fitness assessment of the mutant viruses, we determined whether the engineered mutations actually disrupted the stem loop structure. With SHAPE, it is possible to distinguish between paired and unpaired nucleotides through a chemical modification specific for unpaired nucleotides. Following SHAPE analysis, the predicted structures for Stem WT(L), Stem ABC, and Stem ABC+(L) were validated (Figure 3.5A-C), suggesting that the point mutations that were generated were sufficient to disrupt the wild type stem loop structure.

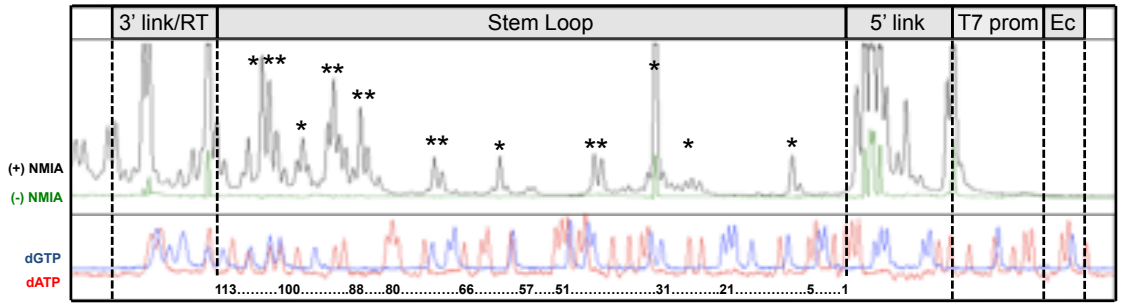
Overall, these results indicated that either: 1) this novel structure was not fundamental to the reproduction with HIV-1, 2) this structure serves an important function *in vivo* that was not reflected in our *in vitro* system, and/or 3) the secondary structure was maintained in infected cells despite our mutations and SHAPE predictions.

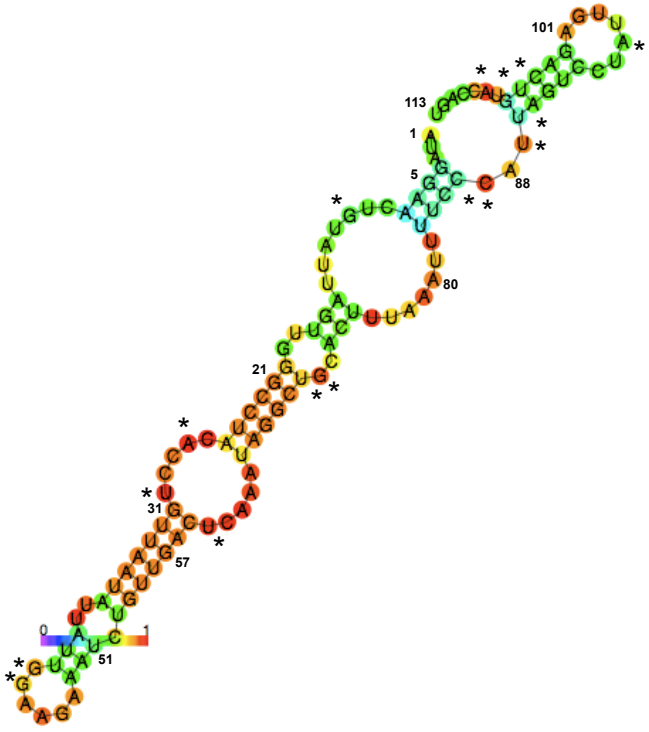
A


3' link/RT
Stem Loop
5' link
T7 prom
Ec
(+) NMIA
(-) NMIA
dGTP
dATP
113.....100.....88.....80.....66.....57.....51.....31.....21.....5.....1


0
1

B


3' link/RT
Stem Loop
5' link
T7 prom
Ec
(+) NMIA
(-) NMIA
dGTP
dATP
113.....100.....88.....80.....66.....57.....51.....31.....21.....5.....1


101
113
1
5
88
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21
31
57
51
0
1

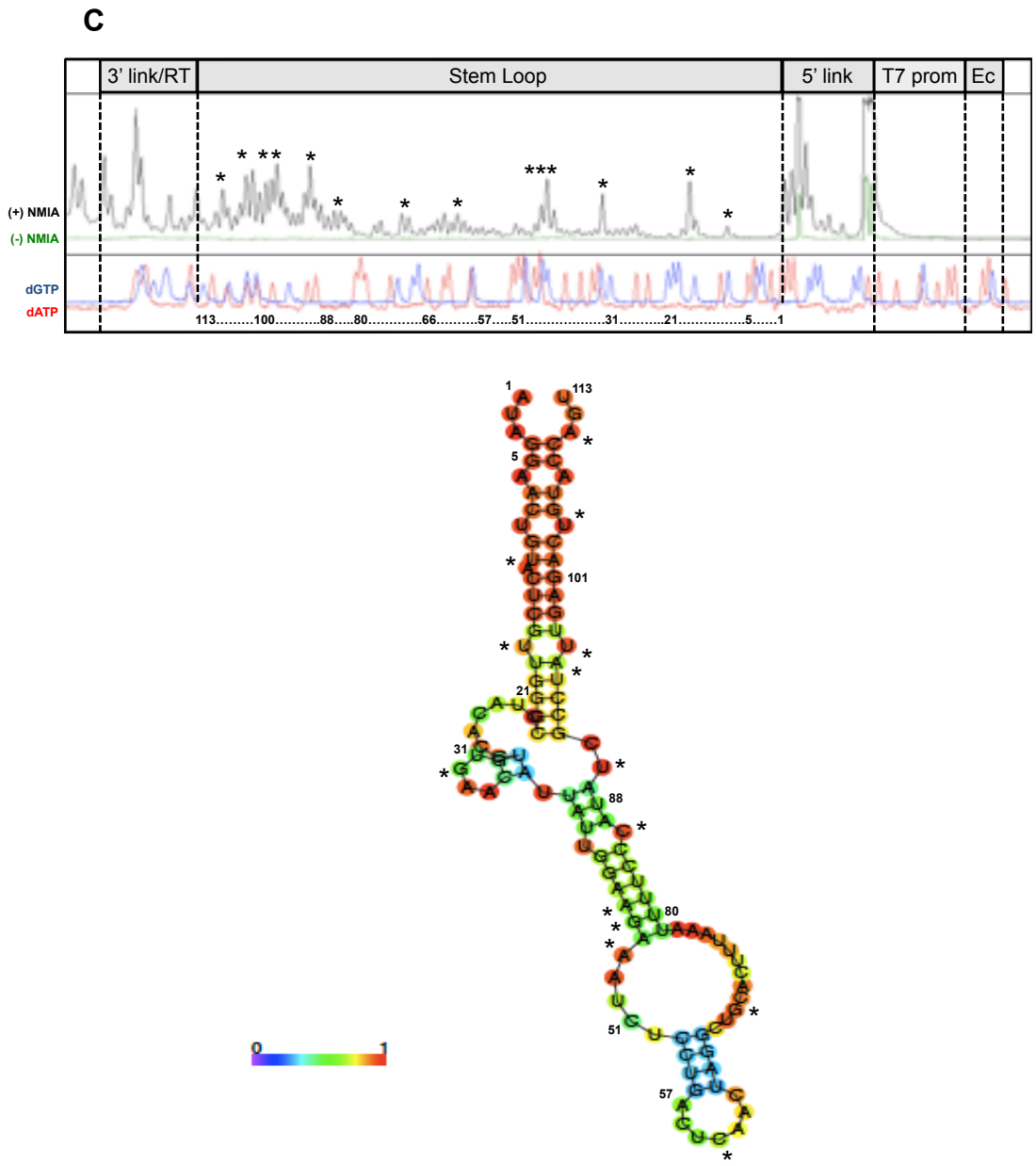

Figure 3.5 **SHAPE analysis of the predicted HIV secondary structures**. A) Stem WT(L), B) Stem ABC+(I), and C) Stem ABC+(L). The raw electropherogram showing only dGTP and dATP of the sequenced region of the SHAPE cassette is numbered according to the RNA secondary structure. The (*) indicates the site of an NMIA-modified, unpaired nucleotide and are labeled in the corresponding predicted secondary structure.

[*Ec = EcoRI site, T7 prom = T7 promoter sequence, 5′ link = 5′ linker region, Stem Loop = RNA stem loop region, 3′ link/RT = 3′ linker and reverse transcription site*.]

3.5 DISCUSSION

A number of well-characterized RNA secondary structures exist throughout the HIV-1 genome, including common regulatory motifs and regions specific for productive transcription of HIV. Interestingly, the majority of these structures have been identified within the 5' and 3' untranslated regions, suggesting that the structures within the genome have yet to be described. Studies performed by Watts *et al* (20) offered a glimpse of the RNA secondary structures throughout the HIV-1 genome. Their observations validated a study by Wang *et al* (19) who previously reported the structure of a novel RNA stem loop located within HIV-1 *pol*. However, the exact biological function of this proposed structure remains to be unknown.

From the data presented in the Wang *et al* paper, we postulated that the disruption of this structure, which appears to be conserved under an evolutionary selective pressure, would exact an immediate deleterious effect. However, our results indicated that this was not the case. The comprehensive panel of viruses representing variants of the wild type RNA stem loop structure produced viable virus. Although we have evidence that these mutations may potentially elicit a subtle fitness cost, the mutant viruses successfully reproduced and were not outcompeted by wild type virus over an extended duration. Studies have show that just a single mutation within HIV-1 RNA secondary structures, such as the psi packaging signal (7) or the TAR element (5), severely impacts the replicative capacity of the virus. However, despite introducing 12 pairing-disruptive mutations in Stem ABC+(L), this variant remained capable of replicating throughout several lifecycles with identical growth kinetics in both primary $CD4^+$ and immortalized T cells.

Although the novel secondary structure may play a functional role *in vivo*, these results suggested that it was not fundamental to the reproduction of HIV-1 in our *in vitro* system.

In addition to the *in vitro* assays, we performed SHAPE analysis on selected mutants with particular focus on the Stem ABC and Stem ABC+(L) variants due to their high number of theoretically disrupted pairings. Although we did not reach single nucleotide resolution using SHAPE followed by capillary electrophoresis, a pattern consistent with the thermodynamically predicted structures was observed. These data suggested that, although the stem loops were disrupted, the structure may be maintained within infected cells. One caveat of using SHAPE, however, is that it experimentally validates an RNA structure but only within an *in vitro* setting, suggesting that our observations may not exactly represent what occurs in an *in vivo* context. Further experimentation would be required to fully characterize the function of the secondary structure *in vivo*.

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CHAPTER 4:

Assessing the fitness costs of HIV-1 escape from $CD8^+$ cytotoxic T lymphocytes in acutely and chronically infected individuals

4.1 ABSTRACT

Growing evidence indicates that beneficial effects of Human Leukocyte Antigen class I (HLA-I) types associated with superior immune control of HIV-1 infection are mediated by immunodominant $CD8^+$ T lymphocyte (CTL) targeting of epitopes that force mutational escape causing loss of viral replicative capacity (RC). In contrast, it is unclear whether there is any role for escape and RC loss for other CTL responses. To evaluate this issue, *gag-pol* sequences were analyzed after *ex vivo* HIV-1 passaging of bulk virus from nine chronically infected persons. Over ~10 weeks of passaging in allogeneic PBMC (without HIV-1-specific CTLs), there was evolution of CTL epitope sequences, loss of quasispecies diversity, and altered Gag-Pol contribution to RC that correlated to the number of changed codons seen to be under selective pressure. This demonstrates that HIV-1 during chronic stable infection has impaired fitness under pressure by the CTL response. For comparison, HIV-1 adaption to mounting immune responses *in vivo* was followed using longitudinal samples collected from four individuals identified in acute infection, noting a modest loss of fitness with a concomitant reduction in diversity over time. Overall, these findings indicate that CTL selective pressure generally reduces HIV-1 RC over the course of infection, which represents a potential beneficial impact of CTL during the chronic phase.

4.2 INTRODUCTION

$CD8^+$ cytotoxic T lymphocytes (CTLs) play a pivotal role in the immune response against infection with Human Immunodeficiency Virus type-1 (HIV-1). Evidence has shown that CTLs can inhibit viral replication *in vitro* (8, 59), effective CTL responses *in vivo* result in viral containment (3, 5, 30), and experimental monoclonal antibody-mediated depletion of $CD8^+$ cells in Simian Immunodeficiency Virus (SIV)-infected macaques leads to a spike in viremia that resolves as the CTL repopulate (26, 38, 50). However, immune control is incomplete in most persons and disease progression eventually occurs.

HIV-1 sequence variation is a key challenge to immune containment. The high replication and mutation rates *in vivo* are expected to generate all single and double nucleotide mutations daily within quasispecies (44), allowing viral escape from CTL recognition (60). This is most evident in acute infection, when rapid outgrowth of viral epitope escape mutations and CTL retargeting are commonly observed (6, 35), due to decay of the CTL responses against the original epitope sequence (6, 25). Indeed, CTLs are the key driver of viral sequence evolution after infection (32, 35). In chronic infection, however, CTLs and HIV-1 appear to settle in a quasi-steady state, with CTL targeting and HIV-1 epitope sequences that remain stable (29) or develop escape slowly (19, 25).

Certain Human Leukocyte Antigen class I (HLA-I) types are associated with better immune containment of HIV-1 (10, 13, 15, 19, 32, 46), and it has become apparent that the protective effect is related to CTL targeting and loss of HIV-1 replicative capacity associated with epitope

mutations to escape the CTL response. Immunodominant epitopes for B*13 (46), B*27 (51), and B*57 (7, 12, 37, 40) have been shown to develop CTL escape mutations that cause impaired RC (loss of fitness), likely through structural (37) and/or functional constraints (4, 53) on protein sequences. Moreover, viral escape mutations in some constrained epitopes appear to be associated with disease progression (15, 19, 27, 28). It therefore appears that the fitness costs incurred to allow evasion of these CTL responses is a key benefit that contributes to immune control.

Although the CD8 depletion data in SIV-infected macaques indicates that the overall CTL response reduces viral replication *in vivo*, it is unclear whether this protective mechanism of reduced RC from CTL escape mutations also plays a role for other HLA types, versus direct antiviral activity without escape. To address this issue, we examined HIV-1 from chronically infected individuals representing a broad panel of HLA types. These viruses were assessed for sequence changes in Gag-Pol and the RC of HIV-1 containing the observed Gag-Pol variants that arose when cultured *ex vivo* in the absence of CTL pressure. In parallel, we assessed the effect of viral evolution on RC during early infection. Our findings demonstrated marked differences in fitness costs of mutational escape between early and chronic infection.

4.3 MATERIALS AND METHODS

Study participants:

Nine chronically HIV-1-infected subjects with detectable viremia off antiretroviral treatment (Table 4.IA) from the Los Angeles area enrolled and provided informed consent under a protocol approved by the UCLA Institutional Review Board. Additionally, four acutely infected subjects identified early in infection (Table 4.IB) were recruited by Dr. Eric Daar through the Primary Infection Network. These individuals provided information on their most recent absolute CD4 count and viral load at the time of study visit. Class I HLA typing was performed through the UCLA Medical Center clinical laboratories.

Mapping of HIV-1-specific CTL responses:

Peripheral blood mononuclear cells (PBMCs) were collected by standard Ficoll separation. Bulk $CD8^+$ were expanded from 5 x 10^6 PBMC by the addition of a CD3:CD4 bispecific antibody that allows for polyclonal expansion of the $CD8^+$ T lymphocyte subset, as previously described (56, 57). HIV-1-specific targeting was defined by standard IFNγ-ELISpot assays with overlapping 15mer peptides spanning the entire HIV-1 consensus B subtype proteome (NIH AIDS Reagent Repository; Germantown, MD) and analyzed using an automated ELISpot reader (AID; Strassberg, Germany) as previously described (58). Recognized epitope regions were defined as either 1) singly targeted peptides or 2) region of overlap between consecutive, overlapping peptides, in which a minimal epitope could be inferred from the participant's HLA type and known epitopes reported in the Los Alamos National Laboratory (LANL) HIV Immunology Database. Spot-forming cells (SFCs) were normalized to SFC/10^6 $CD8^+$ cells. Counts were

A.

Subject	Duration (years)	Viral Load[a]	CD4[b]	HLA type		
				A	B	C
9	>10	15600	449	3, 26	15, 38	3, 12
59	>5	6408	394	2, 26	44, 52	3, 4
66	>10	21000	400	2, 23	7, 45	7, 16
68	>10	5810	904	1, 29	44, 57	5, 6
70	>10	130234	311	2, 74	44, 53	4, 5
77	>10	23510	895	1, 23	53, 57	6
80	8-9	9570	643	2, 3	52, 61	10, 12
82	>4	24300	368	2, 31	44, 60	2, 10
83	>10	2180	1346	2, 33	15, 64	14, 16

[a] Viral load (VL) is given in copies/ml; value obtained at the most recent study visit.
[b] CD4 count is absolute no./ μl; value obtained at the most recent study visit.

B.

Subject: HLA Haplotype	Visit	Date	Viral Load	CD4
A:	1	6/11/2001	403508	837
A03,31	2	6/18/2001	74137	706
B14,38	3	7/3/2001	14272	743
C08,12	4	7/16/2001	7687	787
	5	8/27/2001	5462	714
B:	1	1/5/2004	340000	319
A01,32	2	1/14/2004	355000	526
B08,64(14)	3	1/21/2004	1210000	504
C07,08	4	3/1/2004	18000	430
	5	3/31/2004	40700	517
C:	1	6/2/2004	749000	445
A01,02	2	6/14/2004	83900	534
B08,18	3	6/23/2004	1150000	538
C07,08	4	8/2/2004	1420000	419
	5	8/30/2004	1250000	441
D:	1	10/23/2001	477003	328
A01,68	2	10/30/2001	685299	241
B08,40	3	11/6/2001	10008400	345
C02,07	4	11/13/2001	356929	360
	5	12/12/2001	1760000	280

Table 4.I <u>Study subject information</u> A) chronic and B) acute infection cohorts.

considered positive if they were >3 SDs above the mean of the quadruplicate negative control wells, and >50 SFC/10^6 $CD8^+$ T lymphocytes.

Ex vivo passaging of virus:

Virus was recovered from PBMCs by expansion of $CD4^+$ T lymphocytes using a CD3:CD8 bispecific antibody (56, 57). Recovered viruses were passaged weekly in freshly expanded $CD4^+$ T lymphocytes from multiple, healthy HIV-1 uninfected donors (without regard for HLA-I) for 10 to 12 weeks. All cells were maintained in RPMI 1640 (Lonza; Walkersville, MD) supplemented with supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 HEPES and 50 U/ml recombinant human interleukin-2 (NIH AIDS Reagent Repository; Germantown, MD). At the conclusion of each 7 to 10 day round of passaging, infected cells were pelleted and genomic DNA was isolated for PCR amplification and HIV-1 sequencing.

Creation of an NL4-3 Δgag-pol cloning vector:

First, site-directed mutagenesis (QuikChangeIIXL, Agilent; Westlake Village, CA) was performed to change nucleotides 781-786 into an *Stu*I site (AGG^CCT). Second, nucleotides 5044-5049 were mutagenized to contain an *Hpa*I site (GTT^AAC), which also created a premature stop codon to eliminated virus production from plasmid without the desired *gag-pol* replacement. The resultant vector was named p83-2Δ*gag-pol* (Figure 4.1A). Additionally, a whole genome version was constructed by engineering an *Nru*I site at the junction of the HIV-1 insert and the plasmid backbone of p83-2Δ*gag-pol*. After cutting with *Nru*I and *EcoR*I (New England Biolabs; Ipswich, MA), the resulting HIV-1 fragment was ligated into either p83-

10/HSA or p83-10/HSA-HA plasmids containing the 3' end of the HIV-1 genome with reporter genes in the *nef* locus as previously described (2), producing pNL4-3/HSAΔ*gag-pol* and pNL4-3/HSA-HAΔ*gag-pol* whole genome plasmids (Figure 4.1B). All modifications were confirmed by sequencing.

Primary HIV-1 gag-pol amplification and cloning into NL4-3:

Genomic DNA (gDNA) was isolated from the cells from the initial (t_1) and concluding (t_{10}) rounds of *ex vivo* passaging using the DNeasy Blood and Tissue Kit (Qiagen; Valencia, CA) according to the manufacturer's protocol. With the longitudinal samples of acute infection, viral RNA was isolated using the QIAamp Ultrasens kit (Qiagen; Valencia, CA) and reverse transcribed using Superscript III First Strand Synthesis (Invitrogen; Carlsbad, CA) with AGPolR gene-specific primer according to the manufacturer's protocol. Using gDNA or cDNA as template, hemi-nested PCR was performed using KOD hi-fidelity DNA polymerase (EMD; Billerica, MA). The first PCR used primers 737F and AGPolRnst, and the second PCR used primers F2nst and AGPolRnst (Table 4.II). Both PCRs were conducted under the following conditions: 20 seconds at 95°C, 40 cycles of 95°C for 20 seconds plus 62°C for 30 seconds plus 70°C for 2.5 minutes, followed by a final extension at 70°C for 10 minutes. The second PCR primers included overhang sequences that corresponded to the HIV-1 sequences flanking the *Stu*I and *Hpa*I sites in the Δ*gag-pol* vectors. Each primary *gag-pol* PCR product (in bulk, uncloned) and an appropriate Δ*gag-pol* vector after excision of the *Stu*I – *Hpa*I restriction fragment (New England Biolabs; Ipswich, MA) were combined using the In-Fusion kit (Clontech; Mountain View, CA) to create NL4-3 genomic quasispecies containing subject-derived *gag-pol*.

A. B.

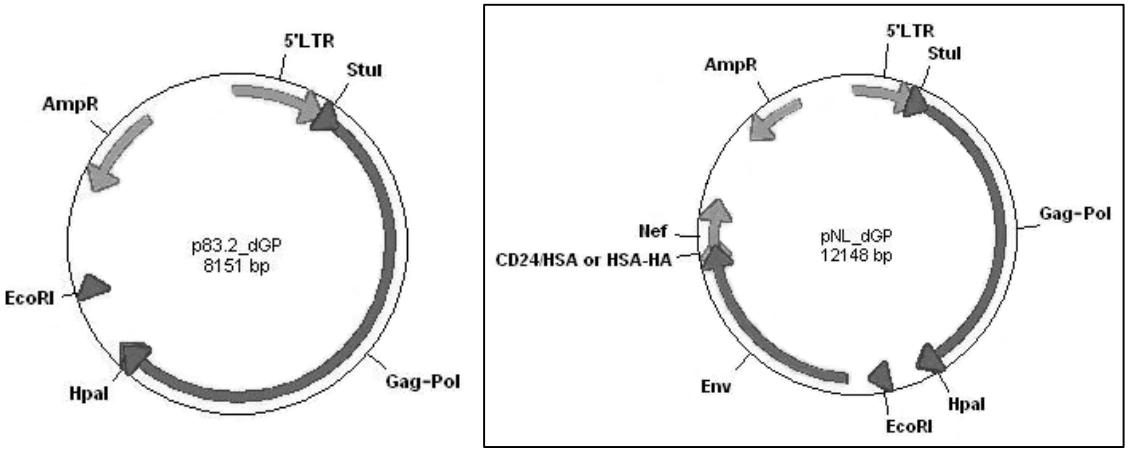


Figure 4.1 <u>Vector map p83-2Δ*gag-pol* and pNL4-3/Δ*gag-pol*</u>. A) The 5' half-genome NL4-3-based plasmid, p83-2Δ*gag-pol*. NL4-3 Gag-Pol is digested away and replaced with subject-derived Gag-Pol via Infusion cloning method (Clontech; Mountain View, CA). B) The reconstructed whole genome version of the combined parent p83-2Δ*gag-pol* and p83-10 with HSA or HSA-HA reporters.

Full-length gag-pol *sequencing and analysis for fixed changes over time*:

Nine or more full-length *gag-pol* clones per time point were isolated per subject by picking bacterial colonies after transformation of the final plasmid constructs. Full-length *gag-pol* DNA sequencing was completed with 10 primers (Table 4.II) using the Big Dye 3.1 sequence terminator kit (Applied Biosystems; Carlsbad, CA) on an AB3130 genetic analyzer, and edited with ChromasLite. Edited sequences from each clone were assembled into the full-length *gag-pol* sequence, translated, and aligned to the Los Alamos HIV Sequence Database 2010 clade B consensus sequence, manually adjusted using BioEdit, and analyzed for fixed amino acid changes following passaging. Codon changes overlapping with reported HLA-matched epitopes were determined with the Epitope Location Finder (ELF) program via the LANL database. Epitope prediction analyses per subject HLA were conducted using two algorithms: 1) Bioinformatics and Molecular Analysis Section (BIMAS) (43) and 2) SYFPEITHI database (48, 52). The threshold for predicted HLA-I binding was set as previously described (20, 21).

Phylogenetic analyses:

Neighbor-joining trees containing all *gag-pol* sequences were constructed using the F84 model of nucleotide substitution using PHYLIP 3.69 software (16). The trees were rooted to consensus B and 100 bootstrap replications were performed. Average pairwise nucleotide diversity and divergence was calculated between time points using SENDBS (41) and standard errors were estimated using 100 bootstrap replicates. Differential selective pressure between the initial and concluding passages were examined using the random effects likelihood (REL) program of HyPhy (45) to determine positive selection *ex vivo* by ratio of the non-synonymous to synonymous mutation ratio (dN/dS>1).

	Primer	Sequence (5'→3')
Gene-specific RT	AGPolR	TGCCACACAATCATCACCTGCCATC
Hemi-nested PCR	737F	GCGACTGGTGAGTACGCC
	F2nst	GCGGAGGCTAGAAGGAGAGAGATGG
	AGPolRnst	CATCACCTGCCATCTGTTTTCCATA
Gene sequencing	MSF12	AAATCTCTAGCAGTGGCGCCCGAACAG
	G60	CAGCCAAAATTACCCTATAGTGCAG
	MW1500R	CGCTGCCAAAGAGTGATTTGAGGG
	Pol1	AGGCTAATTTTTTAGGGA
	Pol2	GGACCTACACCTGTCAACATAATTGG
	Pol3	GCATTCACCATACCTAGTATAAACAATG
	Pol4	AGCTGGACTGTCAATGATATACAG
	Pol5	TACCCATCCAAAAAGAAACATGGG
	Pol6	CTACCTGTCATGGGTACCAGCAC
	GP2R	GTGATGTCTATAAAACCAGTCCTTAGC
Real-time PCR	HSA93F	GCACCGTTTCCCGGTAACCAGAATAT
	HA95F	CCCGTATGATGTACCGGATT
	HSA210R	AGAGAGAGAGAGAGCCAGGAGA
	Actin171752F	TCACCCACACTGTGCCCATCTACGA
	Actin172246R	CAGCGGAACCGCTCATTGCCAATGG
	HSA76FAM	FAM-TCCCTGCAGTCCACAGCTGG-Tamra
	ACTIN	VIC-ATGCCCTCCCCCATGCCATCCTGCGT-Tamra

Table 4.II <u>Primer and probe sequences</u>. Utilized for gene-specific reverse transcription nested PCR amplification of *gag-pol*, whole *gag-pol* sequencing, and real-time PCR detection of reporter HIV-1 constructs.

Production of Gag-Pol recombinant reporter virus:

Recombinant reporter virus was constructed using half genome plasmids, as previously described (1). The p83-10 plasmid with HSA reporter (inserted within the *nef* reading frame) was used to tag t_{10} viruses and p83-10 plasmid with HSA-HA reporter was used to tag t_1 viruses. The p83-2Δ*gag-pol* plasmid was used as the vector for insertion of the *gag-pol* quasispecies. Viruses were produced with these constructs (in parallel with a p83-2 control containing NL4-3 *gag-pol*) by co-electroporation with either p83-10 plasmid (2). Briefly, 10 μg of each half plasmid genome construct was digested with *Eco*RI Both digested, gel-purified plasmids were mixed and electroporated into 10 x 10^6 T1 (49) cells using GenePulser Xcell (Biorad). After 15 minute recovery on ice, cells were cultured in RPMI 1640 medium (Lonza; Walkersville, MD) supplemented with 20% fetal calf serum. Supernatant containing virus was collected between days 7 to 9 post-electroporation and quantified for p24 by ELISA (Perkin Elmer; Santa Clara, CA). gDNA from infected cells was isolated and confirmed that no cross-culture contamination had occurred and that they contained expected polymorphisms and substitutions of the previously sequenced quasispecies at the respective time points.

With respect to the longitudinal samples of acute infection, bulk *gag-pol* from the first time point was inserted into the pNL4-3/HSAΔ*gag-pol* vector while bulk *gag-pol* from the remaining subsequent time points were inserted into pNL4-3/HSA-HAΔ*gag-pol*. 293T cells were transfected with 10 μg of each plasmid with Bio-T transfection reagent (Bioland; Paramount, CA) according to the manufacturer's protocol. Supernatant containing virus was collected on day 2 post-transfection and filtered through a 0.45 μM filter. A second round of infection of T1 cells

using transfection supernatant was performed and virus was collected on d5 post-infection. Virus production was quantified using a p24 antigen-based ELISA (Perkin Elmer; Santa, Clara, CA).

Quantitation of viral replication capacity:

Paired t_1 and t_{10} viruses were used to co-infect 2 x 10^6 T1 cells in triplicate using 500 pg total of p24 antigen/10^6 cells (approximate multiplicity of infection of 0.005 for each). At days 2 and 5 post-infection, gDNA was isolated using the Puregene Blood Core Kit B (Qiagen; Valencia, CA) for analysis by real-time PCR (2x universal premix, Applied Biosystems; Carlsbad, CA) in a 25 μl reaction volume, followed by analysis using StepOne Plus (Applied Biosystems; Carlsbad, CA), as previously described (2). HSA-HA and HSA were detected separately using the following primers: HSA93F (HSA-specific), HA95F (HSA-HA-specific), HSA210R (reverse primer for both forward primers). Simultaneously, β-actin was detected using the following primers: Actin171752F, Actin172246R. We used a probe specific for both HSA and HSA-HA, HSA76FAM, and a separate probe for actin, ACTIN (refer to Table 4.II for primer and probe sequences). Plasmid standards were used to generate curves of Ct versus copy number, after analysis using StepOne Software v2.0 (Applied Biosystems; Carlsbad, CA). Relative fitness was expressed as the ratio of the $\log_{10}$ growth slope of the t_{10} viruses to that of the t_1 viruses.

Similarly, the recombinant viruses generated for each longitudinal sample per acute subject were paired with NL4-3 reporter virus and followed the same infection protocol. Absolute fitness was expressed as the ratio of the $\log_{10}$ growth slope of the acute viruses to that of NL4-3.

Statistical analyses:

Gag-Pol amino acid changes during *ex vivo* passaging were evaluated for statistical significance by Fisher's exact test and the significance of proviral diversity changes was evaluated using the unpaired Student's *t* test. Linear regression analyses were performed to compare the number of sequence substitutions versus the relative fitness of t_{10} to that of t_1 viruses. P values are shown only where significant relationships were found ($p<0.05$). Lastly, confidence intervals (95%) were calculated for all correlation analyses.

4.4 RESULTS

4.4.1 Chronic HIV-1 Infection

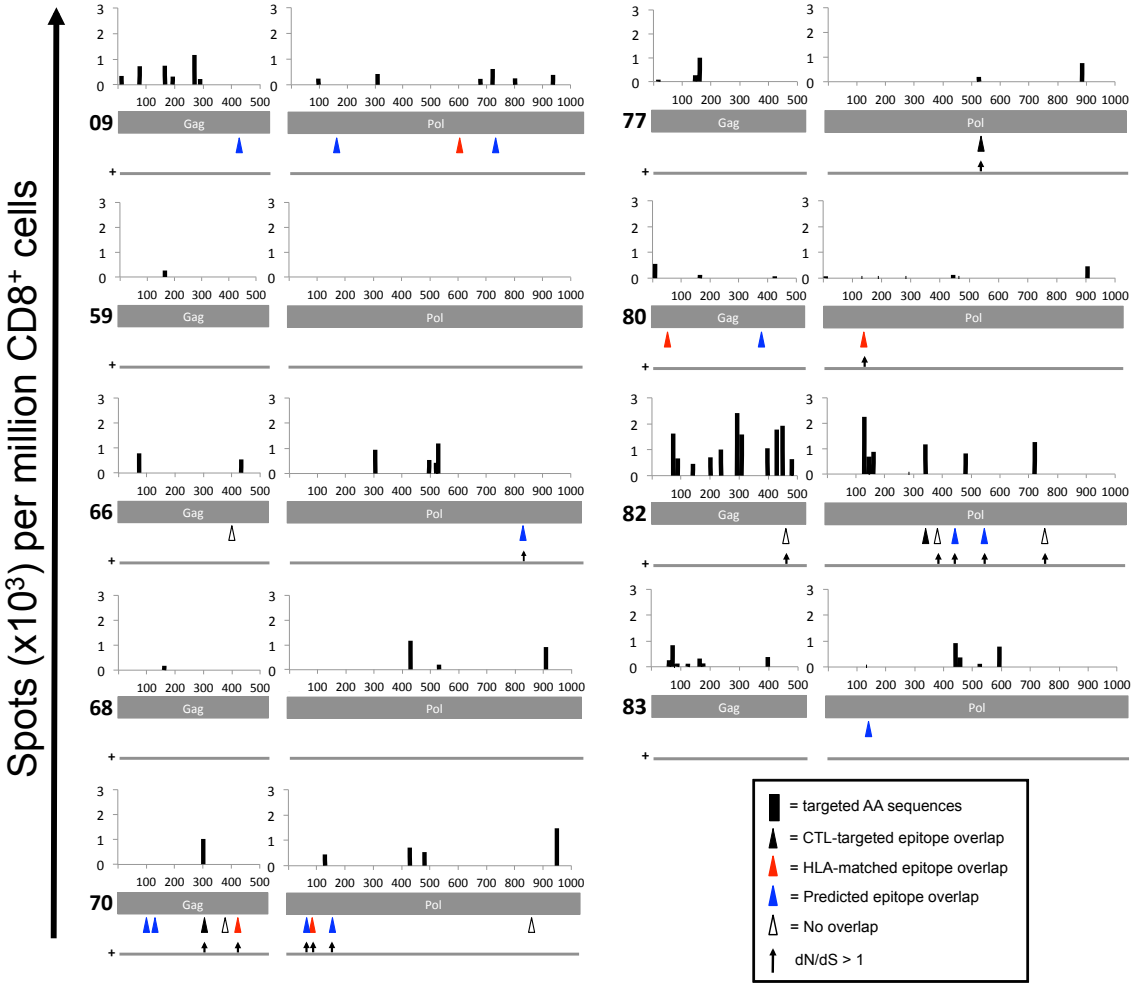
All HIV-1 subjects had CTL responses against Gag-Pol.

HIV-1 Gag-Pol-specific targeting by bulk $CD8^+$ from the nine chronically infected persons was defined to the level of 15-mer peptides by standard IFNγ-ELISpot assays (Figure 4.2A), as previously described (58). There was a median of 6 (range 1 to 17) targeted epitope regions (peptides targeted in isolation or overlap of two consecutive targeted peptides). Numbers of targeted epitope regions were similar for Gag (median 3, range 1 to 11) and Pol (median 4, range 0 to 6) despite the larger size of Pol, similar to previously reported distributions of CTL targeting (17, 24).

HIV-1 sequences demonstrated significant codon changes after *ex vivo* viral passaging.

Bulk HIV-1 from the nine subjects was passaged serially in allogeneic PBMC from HIV-1-uninfected donors for approximately 10 weeks. The *gag-pol* region was assessed from the initial (t_1) and concluding (t_{10}) passages, with an average of 9 clonal sequences obtained per time point per individual. Across the nine subjects, 26 statistically significant codon changes were observed after passaging (Table 4.IIIA). Of the 26 changes, 7 showed significant positive selective pressure (non-synonymous to synonymous mutation rate, dN/dS >1). There were 14 changes from a non-consensus to the clade B consensus amino acid (54%), suggesting optimization of HIV-1 sequence toward the presumed fittest sequence overall. Additionally, neighbor-joining phylogenetic trees were constructed to determine the relationship between the initial and passaged virus *gag-pol* sequences. For 5 of the 9 subjects, the passaged virus sequences clustered

A.


Spots (x10³) per million CD8⁺ cells
09
59
66
68
70
77
80
82
83
Gag
Pol
= targeted AA sequences
= CTL-targeted epitope overlap
= HLA-matched epitope overlap
= Predicted epitope overlap
= No overlap
dN/dS > 1

B.

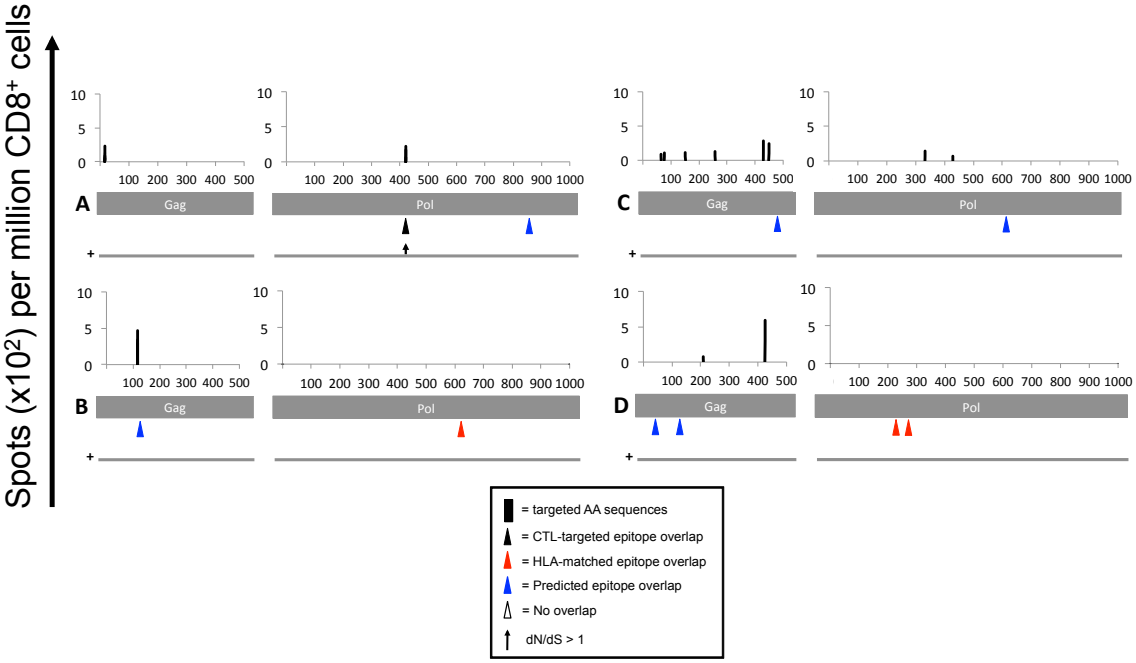


Figure 4.2 <u>Summary of CTL targeting and associated amino acid substitutions</u>. A) chronic and B) acute infection cohorts. The black columns mark the location and magnitude of CTL responses within the HIV-1 Gag-Pol region as determined by IFNγ-ELISpot mapping of $CD8^+$ T lymphocytes using a consensus B peptide library. Amino acid sites that changed with *ex vivo* passaging are marked with arrowheads and defined as follows: overlap with CTL-targeted epitope (black), overlap with HLA-matched epitope (red), overlap with predicted epitope (blue), no overlap (open). The sites that displayed evidence of positive selection with *ex vivo* passaging are labeled with a black arrow. For acute infection cohort, data represent cumulative responses observed throughout longitudinal sampling.

A.

Subject	Protein	Amino acid change[a]	Fisher's result[b]	CTL target overlap[c]	Evolve to consensus[d]	dN/dS >1[e]
9	Gag	S440P	0.0007	No	No	ns
	Pol	R175K	0.0007	No	Yes	ns
	Pol	T605I	0.0001	No	No	ns
	Pol	I714V	0.0007	No	Yes	ns
59	n/a	n/a	n/a	n/a	n/a	n/a
66	Gag	R403K	0.0128	No	Yes	ns
	Pol	T827A	0.0128	No	No	Yes
68	n/a	n/a	n/a	n/a	n/a	n/a
70	Gag	I104V	0.0034	No	No	ns
	Gag	H126N	0.009	No	No	ns
	Gag	S310T	0.0023	Gag 301-315	No	Yes
	Gag	N382K	0.0004	No	No	ns
	Gag	S427T	0.0034	No	Yes	Yes
	Pol	V71I	0.0152	No	Yes	Yes
	Pol	K99R	0.0034	No	No	Yes
	Pol	V163I	0.0152	No	No	Yes
	Pol	V837I	0.0004	No	No	ns
77	Pol	S534G	0.0033	Pol 529-538	No	Yes
80	Gag	L51I	0.0001	No	No	ns
	Gag	R387G	0.0023	No	No	ns
	Pol	I141V	0.0001	No	No	Yes
82	Gag	S464R	0.0001	No	Yes	Yes
	Pol	V344I	0.0001	Pol 341-355	No	ns
	Pol	R378K	0.0498	No	Yes	Yes
	Pol	I448V	0.0011	No	No	Yes
	Pol	K545R	0.0001	No	No	Yes
	Pol	R757K	0.0031	No	Yes	Yes
83	Pol	M149I	0.0215	No	Yes	ns

[a] Amino acid numbering is according to HXB2 reference sequence.

[b] P values are given for Fisher's exact test, $p<0.05$.

[c] Changes occurring within a targeted 15-mer peptides.

[d] Evolution toward consensus clade B sequence.

[e] dN/dS – ratio of the rate of non-synonymous (N) to synonymous (S) replacements, as determined by HyPhy random effects likelihood (REL) test for positive selection; ns – not significantly >1.

B.

Subject	Protein	Amino acid change	Fisher's result	CTL target overlap	Timing of emergence (weeks post-onset)	dN/dS >1
A	Pol	V431I	0.0001	Pol 421-435	7; visit 4	Yes
	Pol	I856V	0.0001	No	14; visit 5	ns
B	Gag	L147I	0.009	No	16; visit 5	ns
	Pol	V621I	0.009	No	16; visit 5	ns
C	Gag	K481Q	0.0108	No	4; visit 3	ns
	Pol	S623P	0.0001	No	9; visit 4	ns
D	Gag	V54G	0.0003	No	3; visit 2	ns
	Gag	I138M	0.0003	No	3; visit 2	ns
	Pol	K238R	0.0031	No	4; visit 3*	ns
	Pol	K277E	0.0031	No	4; visit 3*	ns

Table 4.III **Statistical analysis of amino acid changes**. A) *ex vivo* passaging of HIV-1 from individuals with chronic infection and B) *in vivo* evolution within individuals with acute infection.

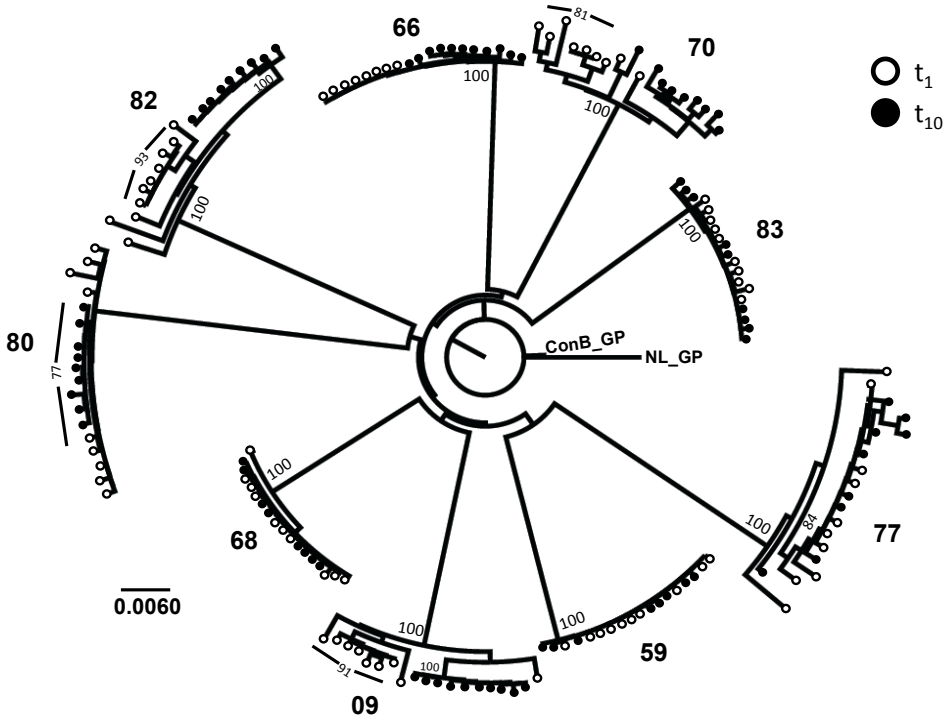
* Reverted to original residue by subsequent visit.

distinctly from the baseline virus sequences with strong bootstrap support (Figure 4.3A), suggesting directed evolution of the viruses during passaging.

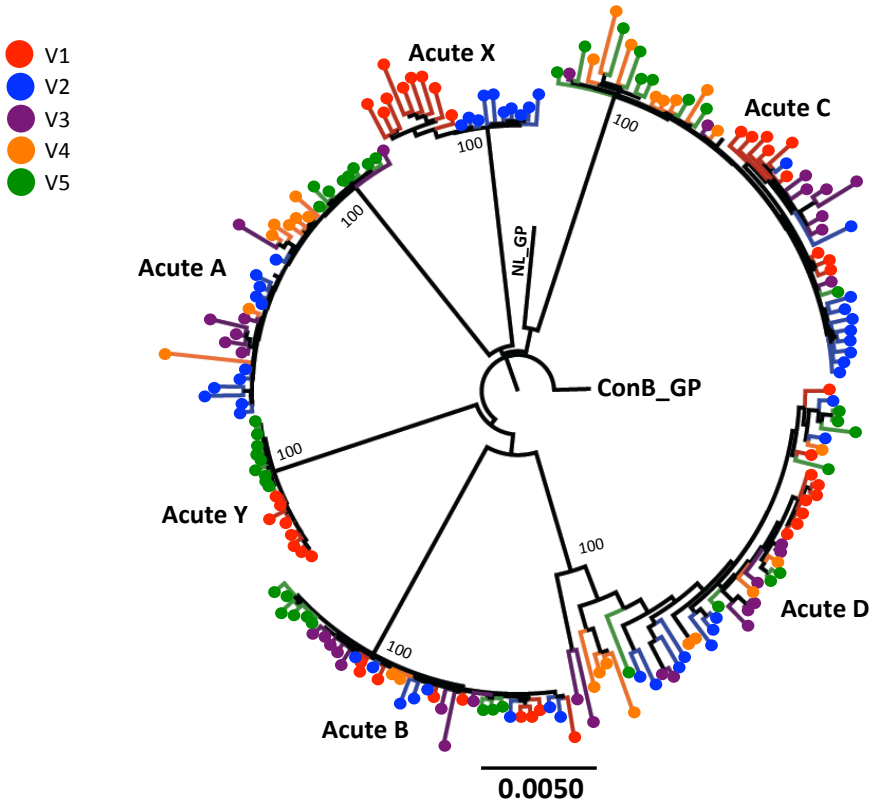
The diversity of HIV-1 *gag-pol* quasispecies *in vivo* is driven by CTL immune pressure.

The t_1 and t_{10} quasispecies diversity of *gag-pol* was examined for each subject. The t_1 quasispecies (assessed shortly after culture of *in vivo* virus) varied across subjects (range 0 to 13.0 x 10^{-4}), with a mean of 7.0 x 10^{-4} ± 4.9 (mean ± S.D.). When these baseline diversity values were compared to Gag-Pol-specific CTL breadth (number of targeted epitope regions) and magnitude as assessed by IFNγ-ELISpot, there were trends for correlation between higher breadth/magnitude to diversity (Figure 4.4A-B). After passaging *ex vivo* in the absence of HIV-1-specific CTLs, *gag-pol* diversity dropped in the quasispecies from 8 of the 9 subjects (Figure 4.4C), and was significantly reduced across all nine subjects, dropping to a mean of 2.3 x 10^{-4} ± 2.6 (range 0 to 8.9 x 10^{-4}) (Figure 4.4D). Furthermore, we observed differential changes in diversity when we evaluated the Gag and Pol proteins separately (Figure 4.5). Notably the diversity for all but one subject appeared to converge on a low baseline, which was reflected by tight correlation between t_1 diversity after passaging *ex vivo* (Figure 4.4E), and a trend of correlation between Gag-Pol CTL breadth and magnitude compared to the observed change in diversity after passaging (Figure 4.4F and 4.4G). As a whole, these data suggest that Gag-Pol-specific CTLs drive diversity of *gag-pol* quasispecies *in vivo*, and that this diversity is shed when the virus is removed from the influence of CTLs.

A.


66
70
82
83
80
68
77
59
09
ConB_GP
NL_GP
t1
t10
0.0060

B.


V1
V2
V3
V4
V5
Acute X
Acute C
Acute A
Acute Y
Acute D
Acute B
NL_GP
ConB_GP
0.0050

C.

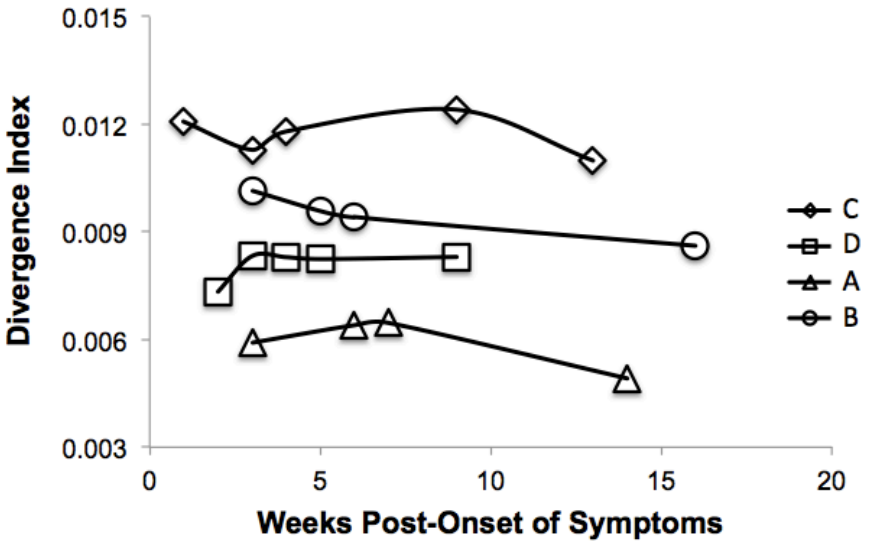
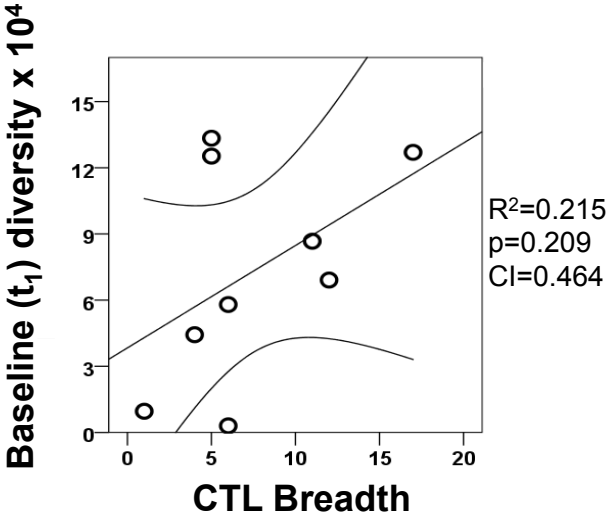
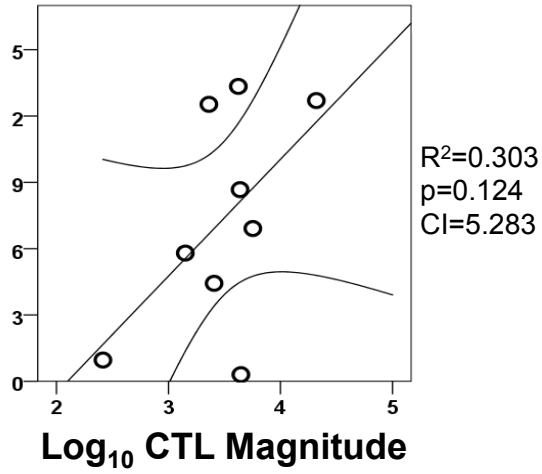


Figure 4.3: **Phylogenetic analysis**. A) *ex vivo* passaged viruses from individuals with chronic infection and B) *in vivo* evolved viruses from longitudinal acute infection samples. The neighbor-joining phylogenetic trees of *gag-pol* sequences are rooted on clade B consensus and evaluated with 100 bootstrap replicates. Only significant bootstrap values (>70) are shown. C) Average genetic divergence of acute HIV-1 from consensus clade B. For part A, isolates from the initial (t_1) passage are labeled with open circles and isolates from the concluding (t_{10}) passage are labeled with closed circles. For 3B, the color labels are as follows: visit 1 (red), visit 2 (blue), visit 3 (purple), visit 4 (orange), and visit 5 (green). Acute X and Y to be completed.

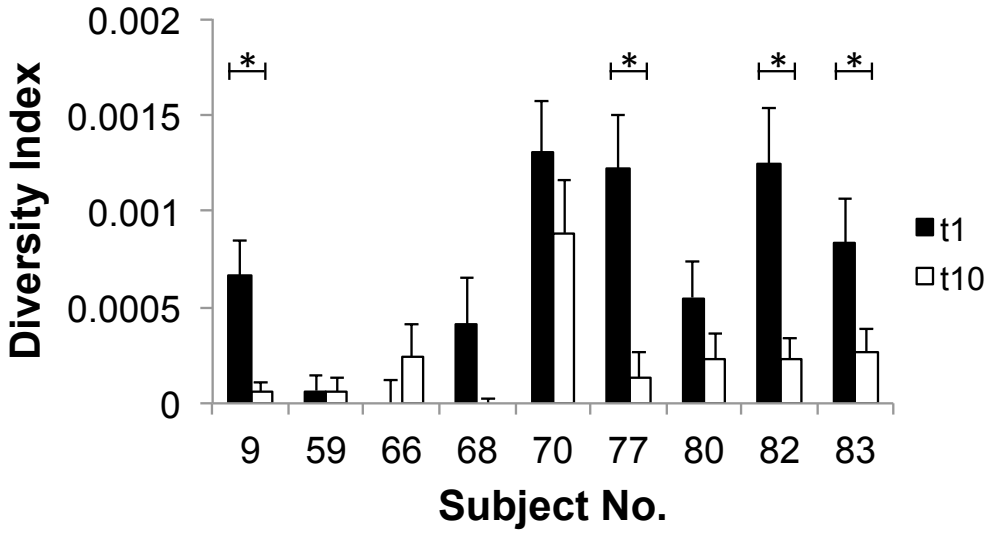
A.


Baseline (t_1) diversity x 10^4
15
12
9
6
3
0
0
5
10
15
20
CTL Breadth
R^2=0.215
p=0.209
CI=0.464

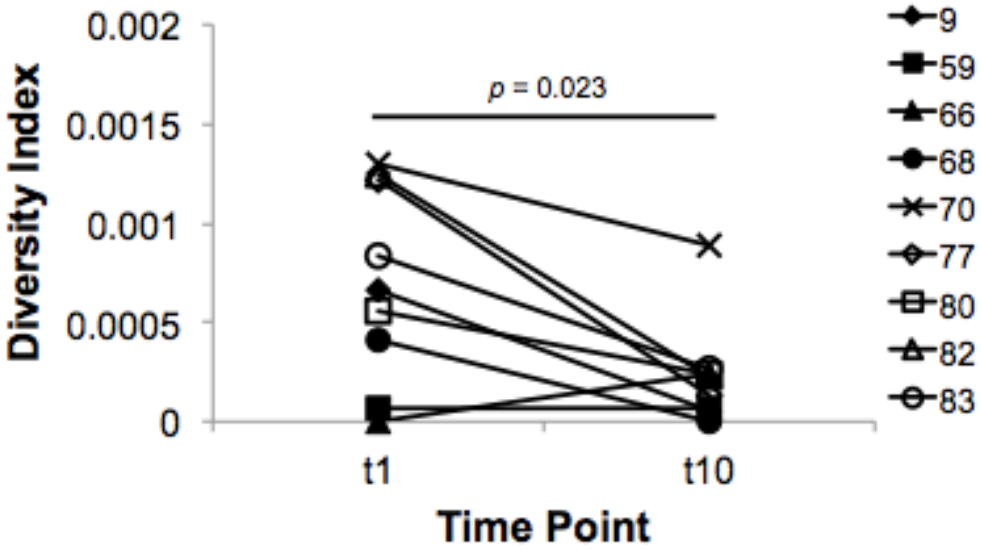
B.


5
2
9
6
3
0
2
3
4
5
Log_{10} CTL Magnitude
R^2=0.303
p=0.124
CI=5.283

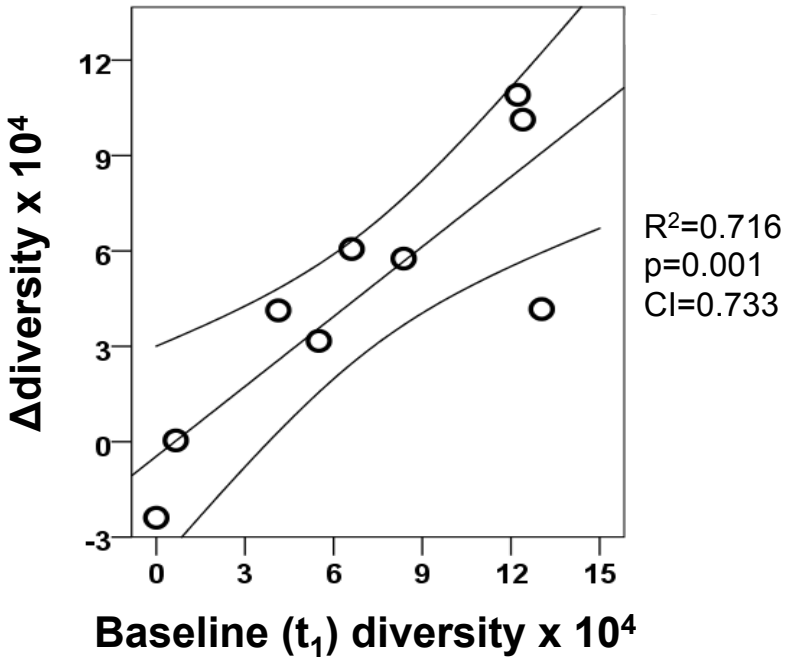
C.


0.002
0.0015
0.001
0.0005
0
Diversity Index
*
*
*
*
9
59
66
68
70
77
80
82
83
Subject No.
t1
t10

D.


0.002
0.0015
0.001
0.0005
0
Diversity Index
p = 0.023
t1
t10
Time Point
9
59
66
68
70
77
80
82
83

E.


12
9
6
3
0
-3
Δdiversity x 10⁴
0
3
6
9
12
15
Baseline (t1) diversity x 10⁴
R²=0.716
p=0.001
CI=0.733

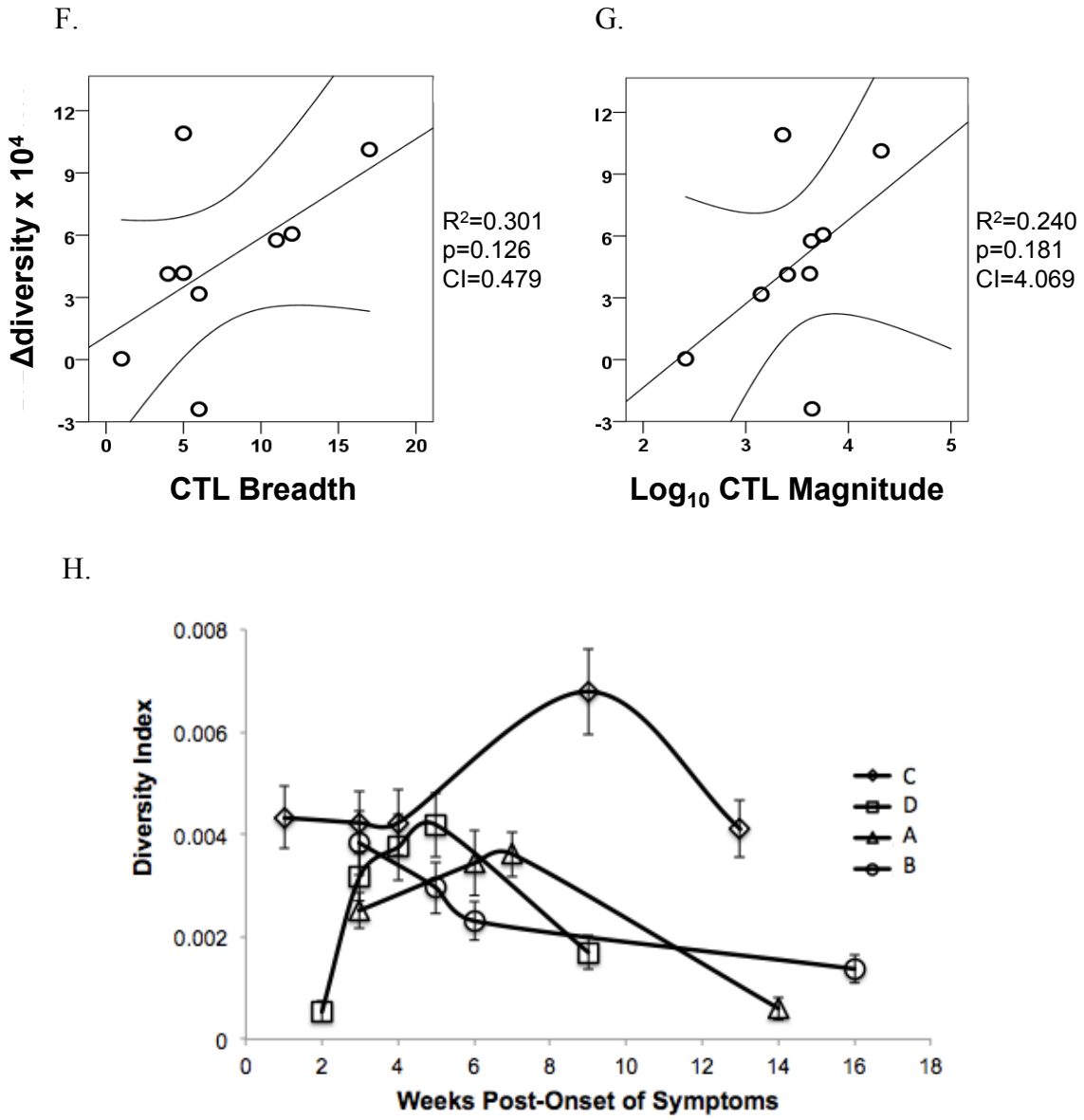


Figure 4.4 **Diversity of *gag-pol* quasispecies**. The diversity of the HIV-1 quasispecies was assessed at baseline (t_1) with respect to A) Gag-Pol CTL breadth and B) magnitude. The reduction of diversity during *ex vivo* culture was examined C) per subject (*, $p<0.05$) and D) across all subjects, converging toward E) a low diversity index. F) The Gag-Pol CTL breadth and G) magnitude correlated with change in diversity after passaging. H) Change in diversity post-onset of symptoms for acute subjects.

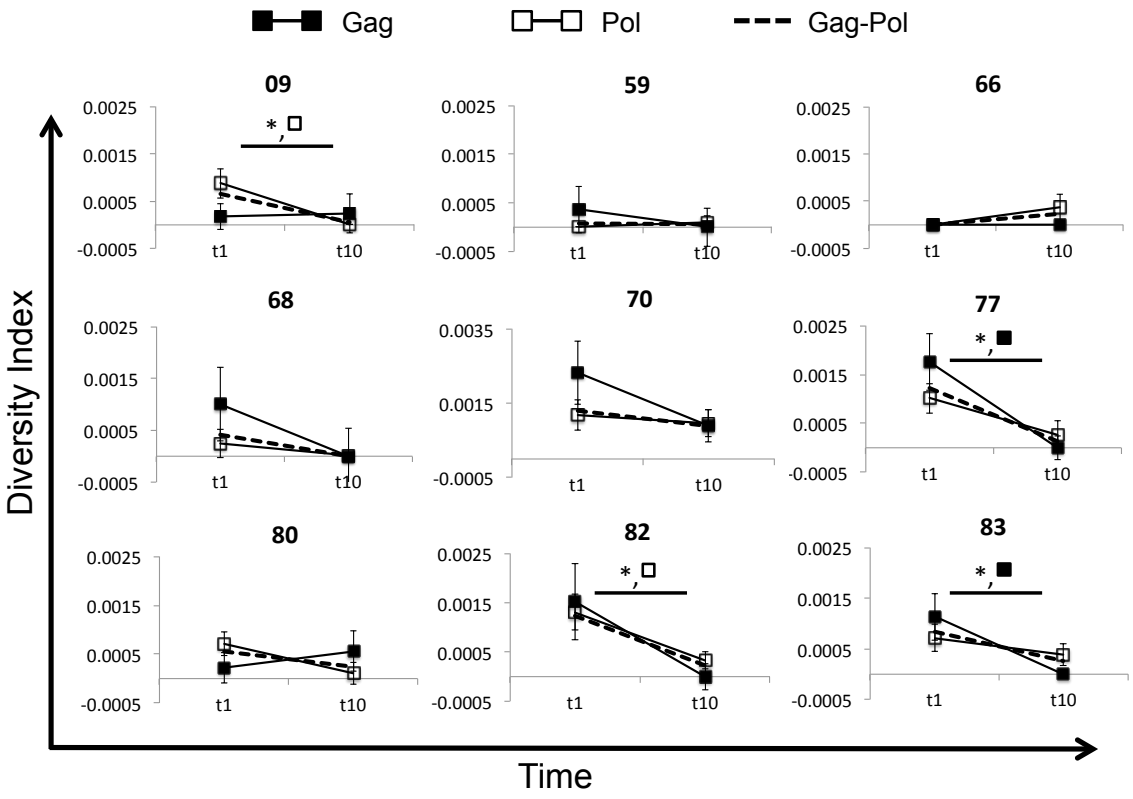
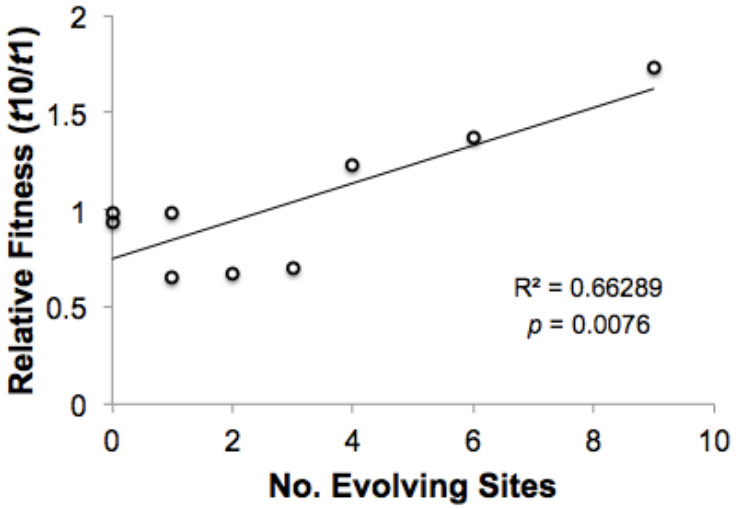


Figure 4.5 **Assessment of diversity change within Gag and Pol subunits**. Diversity for Gag (■), Pol ( ), and whole Gag-Pol (---) are plotted per time point. P values were determined by unpaired Student's *t* test. Significant differences are marked with an asterisk and symbol according to the associated protein (*, $p<0.05$).

The replicative capacity of HIV-1 is reduced by the presence of CTLs.

Development of fixed codon changes and reduced diversity in *gag-pol* after passaging in the absence of CTLs *ex vivo* suggests convergence on a fitter sequence. The replicative capacities (RCs) of HIV-1 with the *gag-pol* quasispecies of all subjects at t_1 and t_{10} were evaluated to assess the fitness cost imposed by CTLs *in vivo*. The sequences from t_{10} were compared to those of t_1 in the controlled context of the HIV-1 molecular clone NL4-3 in competition assays and relative fitness was calculated as a ratio of the $\log_{10}$ growth rates of each virus. Although this assay suggested loss of fitness for some subjects (likely due to the change in context of the *gag-pol* sequences), the change in fitness after *ex vivo* passaging in the absence of HIV-1-specific CTLs was correlated to the amount of observed evolution (Figure 4.6A). To demonstrate the *in vivo* relevance of the expressed viral mutations, we compared the absolute fitness of each virus to that of an M184V antiretroviral resistant variant, which has reportedly diminished replicative capacity *in vitro* and viral load *in vivo* (Figure 4.7A). Relative to M184V, the noted mutations imposed varying degrees of impact on absolute fitness, the values of which were consistent with the calculated relative fitness of the aforementioned virus pairs. Overall, these data indicated that the sequence changes associated with CTL pressure have a fitness cost that was alleviated as the virus was allowed to evolve without that selection.

A.


Relative Fitness (f10/f1)
No. Evolving Sites
R² = 0.66289
p = 0.0076

B.

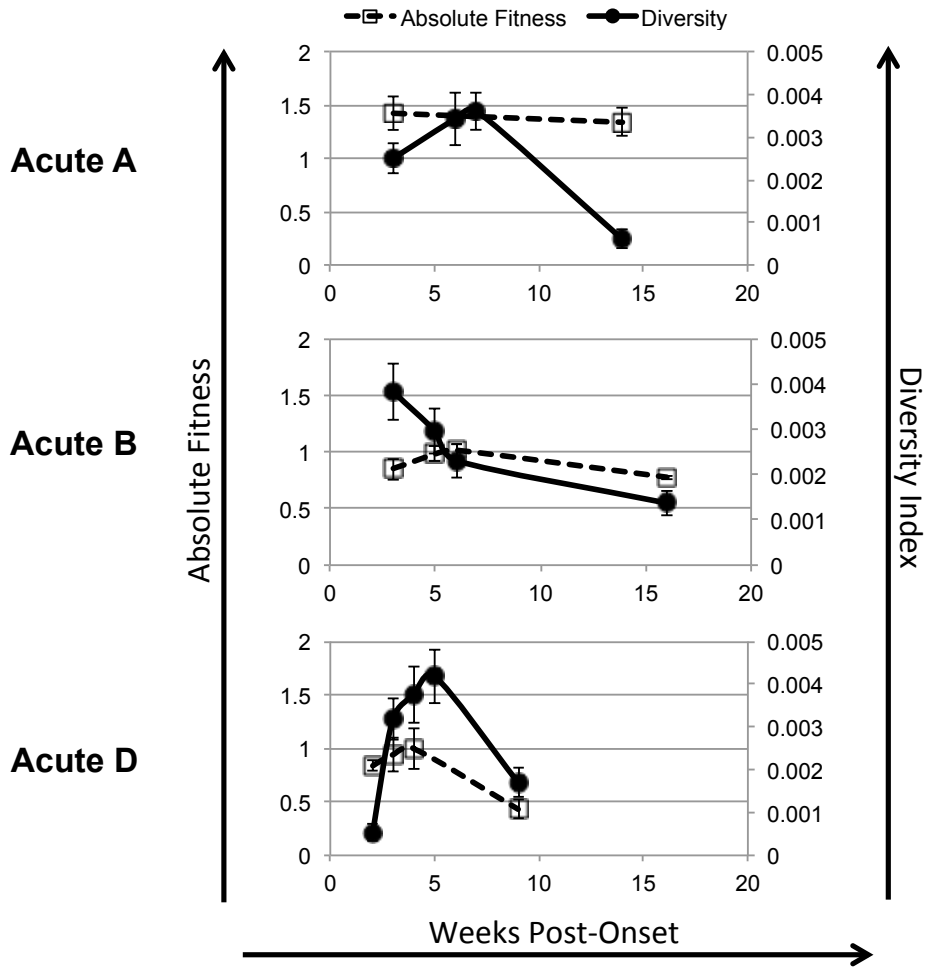
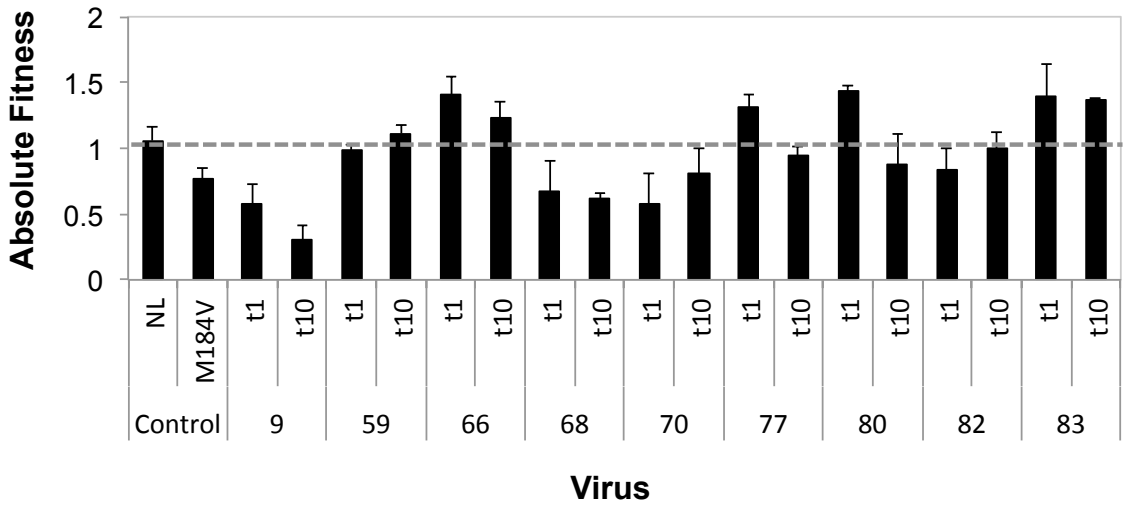

Absolute Fitness
Diversity
Acute A
Acute B
Acute D
Absolute Fitness
Diversity Index
Weeks Post-Onset

Figure 4.6 Fitness assessment of recombinant reporter viruses. A) t_1 and t_{10} viruses representing *ex vivo* passaged HIV-1. Correlation of the number of amino acid substitutions with the relative fitness of t_{10} viruses, defined by the ratio of the rate of replication of t_{10} to t_1, per subject. Statistical significance was evaluated using linear regression analyses (p=0.0076). B) Recombinant viruses for acute HIV-1 per indicated longitudinal time points, showing change in viral diversity and viral fitness over time.

A.



B.

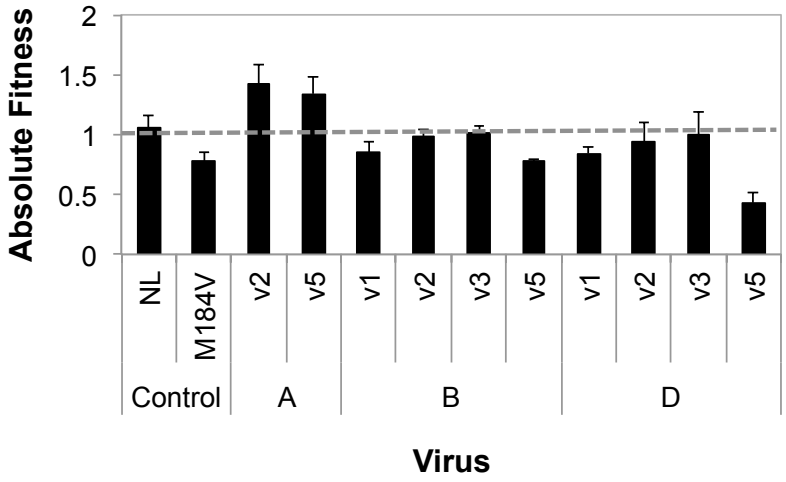


Figure 4.7 **Comparison of absolute viral fitness to M184V**. *In vivo* relevance of mutations were evaluated by comparing the recombinant Gag-Pol viruses generated per A) chronic and B) acute infection subject to the M184V mutant virus. The horizontal line denotes the wild type absolute viral fitness, which is 1 by definition.

4.4.2 Acute HIV-1 Infection

HIV-1-specific targeting was defined to the level of 15-mer peptides by utilizing standard IFNγ-ELISpot assays, as previously noted (58). The median number of epitope regions targeted within Gag-Pol was 2, with a range of 1 to 6 (Figure 4.2B). Numbers of targeted epitope regions were similar for Gag (median 1.5 epitope regions, range 1 to 4) and Pol (median 0.5 epitope regions, range 0 to 2).

Bulk HIV-1 from four acutely infected subjects was isolated from plasma and reverse transcribed for bulk amplification. An average of 10 individual clones were sequenced for whole autologous *gag-pol* per longitudinal visit per individual. Across the four subjects, 10 statistically significant amino acid changes occurred between the variants isolated from the first longitudinal sample and the indicated time point thereafter (Table 4.IIIB). Of the 10 changes, 1 showed evidence of significant positive selective pressure by dN/dS>1. Furthermore, a neighbor-joining phylogenetic tree evaluated with 100 bootstrap replicates for full-length Gag-Pol was constructed to determine the relationship among the variants isolated at each time point per subject (Figure 4.3B), noting convergence toward clade B for 3 of 4 subjects (Figure 4.3C).

The change in diversity in *gag-pol* quasispecies *in vivo* for each acute infection subject was assessed. As immune responses developed and stabilized, we found reduced diversity over time (Figure 4.4H), suggesting that CTL pressure contributed to the observed changes in diversity in early infection. Moreover, the overall effect of early viral evolution *in vivo* on viral RC was examined. Full-length *gag-pol* coding regions for subjects A, B, and D were cloned in bulk into respective pNL4Δ*gag-pol* whole genome cloning vectors. Viral growth competitions

demonstrated differential absolute fitness longitudinally (Figure 4.6B) and compared to the M184V mutant virus (Figure 4.7B). In 2 of 3 subjects, there was a modest loss of fitness in parallel with decreasing viral diversity, suggesting that early viral evolution occurred with limited consequences to replication capacity.

4.5 DISCUSSION

Mutational escape represents a significant challenge for CTL to control viral replication, as it has been shown to interfere with immune recognition via extra-epitopic (13, 31, 55) and intra-epitopic mutations (12, 37, 51). However, these CTL escape mutations can come at a substantial cost to the replicative capacity of the virus. It has been observed that certain epitopes escape at a high fitness cost to the virus and may contribute to the protective effect of particular HLA-I types (7, 12, 37, 40, 46, 51). It is unclear, however, whether escape in other epitopes plays a role in the overall impact on viral RC. Given the stability of the immune response and seldom, if at all, viral evolution during chronic infection, it is important to assess the fitness costs of such mutations to gain a better understanding of the dynamics of escape and the contribution of CTL to control of viremia during late infection.

In the absence of autologous CTL pressure, viral fitness was optimized. After several rounds of *ex vivo* culture, passaged virus clustered with strong phylogenetic support with concomitant reduction in viral diversity, suggesting directed evolution toward the fittest sequence. However, sequences from a few subjects did not follow these trends, likely due to lack of CTL pressure, potential replicative fitness constraints for reversion, minimal fitness cost for escape, and or the presence of compensatory mutations (i.e. H219Q compensatory mutation for T242N) (12, 37) that would delay reversion. 14 of 26 (~54%) fixed codon changes after passaging evolved toward consensus clade B, providing further support of sequence optimization toward the fittest variant. The changes that did not match the consensus sequence likely represent a strain-specific amino acid or a common escape mutation in the consensus (39). Finally, diversity of the *gag-pol* quasispecies *in vivo* positively correlated with mounting CTL pressure, defined by breadth and

magnitude of the CTL responses. Confidence intervals (CI) for these correlations were calculated and, for predictive purposes, these bands must be interpreted with caution due to the relatively small sampling of chronically infected persons. However, as a general trending tool, the bands present an overall positive CI slope.

Since Gag is immunodominant (18, 54), Gag-specific CTL play an effective role in controlling HIV-1 (14, 23), and interactions between Gag and Pol (11, 34) suggest the fitness constraints for these proteins are likely to be similar *in vivo* and *in vitro*, the impact of escape mutations within whole *gag-pol* on viral RC was examined. A caveat to this approach, however, is the efficiency of subject-derived Gag-Pol incorporation into the context of the HIV-1 molecular clone NL4-3, which may explain the unexpected loss of fitness for some subjects following *ex vivo* culture. Overall, dual inoculation of target cells with paired t_1 and t_{10} viruses demonstrated that the changes observed in Gag-Pol after *ex vivo* passaging result in an gain of fitness within the t_{10} group, supporting the notion that HIV-1 is less fit in chronic infection and persisting CTLs induce a quantifiable fitness cost to these viruses even in presence of non-protective HLA alleles. These results are in agreement with a recent study by Lewis *et al* (33) in which "partial escape," whereby an epitope mutation reduces CTL antiviral pressure yet still drives CTL persistence, imposes a fitness cost that contributes to the global antiviral effects of CTL during chronic infection.

An inherent issue with *in vitro* fitness assays is the ability to deduce viral fitness *in vivo* using the *in vitro*-derived values. Therefore, the absolute fitness of all viruses was compared to that of a virus harboring the M184V antiretroviral drug resistance mutation. The M184V mutation

signifies a clinically relevant point of reference against which the net fitness loss of our viruses can be compared. Our results showed a reduction in viral fitness of our M184V virus consistent with other studies (7, 36) and validated the *in vivo* relevance of the viruses containing various CTL escape mutations. Interestingly, some viruses appear to have greater viral RC than wild type (RC=1, by definition) and this may be due to differing capacities to replicate that is to certain Gag-Pol variants.

Viral diversity in individuals with acute infection followed a unimodal pattern with respect to the development and stabilization of the immune response. Following infection, there was low diversity due to lack of dominant CTL responses. In the intermediate time points, the mounting immune selection pressured HIV-1 to diversify and escape. As the immune response stabilized, fewer fit variants were selected and reduction in diversity was restored. As diversity ultimately decreased over time, we observed a modest loss of fitness for 2 of 3 acute subjects. This result corroborates previous studies that observe rapid early escape during primary infection (5, 9, 42, 47), likely due to low impact on viral RC. Furthermore, in agreement with previous findings that HIV recovers certain ancestral traits shortly after transmission to a new host (22), analysis of *gag-pol* over this period demonstrated convergence of viral sequences toward clade B consensus, which carries strong implications for vaccine design that favor inclusion of viral features present in early infection. Importantly, a larger sampling is required to evaluate the consistency of these trends.

In order to successfully design a vaccine therapy to induce potent CTL immune responses toward HIV-1, a sufficient understanding of the impact of CTL escape mutations to the

immunopathogenesis of disease is desperately needed. Our work examined accrued mutations within the region of whole Gag-Pol in persons with acute and chronic infection and quantified the net effect of these mutations on viral replicative capacity. The results indicated that CTL-associated mutations associated with a direct fitness impairment was a phenomenon observed within our panel of individuals representing a broad array of class I HLA types and was not exclusively limited to those linked to protection. Future studies investigating specific mutations individually or in combination will provide further insight into the relative impact of such mutations on viral fitness and the eventual development of an effective approach to manipulate or diminish the frequency of CTL escape.

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CHAPTER 5:

Conclusion

5.1 Overview of Dissertation

This dissertation sought to examine the effects of novel mutations on the ability of HIV-1 to reproduce and their overall impact on viral fitness over time. Chapter 2 described specific mutations in the HIV-1 Env glycoprotein gp41 membrane-proximal tyrosine-based sorting signal that did not affect viral replication nor make them more susceptible to CTL inhibition. Chapter 3 focused on pairing-disruptive mutations to a newly discovered conserved RNA structure in HIV-1 *pol* that interrupted the assembly of the stem loops but did not have an immediately detectable phenotype on infection and viral replication. Lastly, Chapter 4 investigated immune-mediated escape mutations developed within HIV-1 Gag-Pol that induced fitness constraints for viral replication, providing a minimal estimate of antiviral pressure exerted by CTL *in vivo*, regardless of HLA allelic background.

5.2 Summary of Results

5.2.1 *Disruption of an env tyrosine-dependent sorting signal does not affect the susceptibility of HIV-1 to cytotoxic T lymphocytes –*

First demonstrated by Desrosiers and colleagues (16), infections with live-attenuated *nef*-defective viruses within SIV models of HIV-1 pathogenesis could generate protective immunity. Thus, understanding potential avenues of viral attenuation and the mechanism of their impact is an important goal for therapy design. More recent studies examined attenuated SIV infection, highlighting mutations and truncations to the transmembrane domain of gp41 (51) specifically within the highly conserved membrane-proximal tyrosine-based sorting motif (Y712xxϕ) (21). Mutations within this domain had minimal impact on viral fitness as demonstrated by normal peak viremia *in vivo* and growth kinetics *in vitro*. Interestingly, infection with these attenuated viruses conferred protection against subsequent wild type SIV challenge *in vivo*, suggesting that disruption of the motif somehow renders SIV more susceptible to CTL inhibition. Via CD8 depletion experiments, Hoxie *et al* (27) demonstrated that $CD8^+$ T lymphocytes may play a role in the *in vivo* attenuation and protection from wild type viral infection.

To examine whether mutations within the gp41 tyrosine-based sorting signal affect viral susceptibility to CTLs, we first engineered analogous mutations into the context of HIV-1 and generated infectious virus (Chapter 2). In agreement with results with SIVmac239 from Hoxie *et al* (21), the EnvY712I and Y712S mutant viruses had growth kinetics similar to wild type. On the contrary, the non-parallel behavior of the specific corresponding truncation mutation of EnvΔGY suggested that this mutation had a more severe impact on viral replication in HIV-1

than SIV. Although analogous mutations in HIV-1 and SIV did not result in the same phenotype, this phenomenon is not uncommon. Next, we directly tested their susceptibility to CTLs through *in vitro* viral growth suppression assays and noted similar degrees of suppression using three different HIV-1-specific CTL clones (6, 64). Thus, it is likely that the mechanism of attenuation is either not due CTLs *in vivo* or that the increase in antiviral activity of CTLs is indirect. Consistent with this latter notion is that HIV-1 Env may facilitate viral escape from CTLs (26, 53, 54) and that reduced levels of Env could potentially reduce viral escape from CTLs. It would be of interest to address this possible indirect mechanism of attenuation and explore whether the level of Env expression on the surface of cells infected with either wild type or mutant viruses significantly differs, leading to differential susceptibility to CTL.

5.2.2 *Conservative mutations within a highly conserved RNA secondary structure in HIV do not interfere with HIV-1 viral replication –*

The study by Wang *et al* (57) reported a previously uncharacterized RNA secondary structure located in between the genes encoding protease and reverse transcriptase of HIV-1 *pol*, noting reduced synonymous variation and co-variation between synonymous mutations for predicted base pairing nucleotides. A subsequent study by Watts *et al* (58) independently validated the presence of the structure genome-wide selective 2'-hydroxyl acylation and primer extension (SHAPE) analysis (60, 61) of HIV-1. Given the evidence that there is strong selective pressure to maintain the integrity of this RNA structure across clade B, the biological importance of this region was investigated. Thus, we assessed the effect of pairing-disruptive synonymous mutations on HIV-1 reproductive success (Chapter 3).

It was anticipated that, given the selective maintenance of the RNA structure, disrupting the predicted base pairing would immediately present an observable phenotype and restoring the base pairing would rescue the phenotype. However, the initial set of mutations disrupting stem A (Stem A mutant) yielded infectious virus without an evident impact on viral replication. Additional disruptive mutations were engineered to interfere with stem B and stem C pairing (Stem ABC mutant), including one non-synonymous change (Stem ABC+(L)), yet viable virus was generated.

Viral growth assays examined the ability of each mutant virus to replicate short term, approximately a period of 2 to 3 replication cycles. However, it was not until certain mutants were passaged over several weeks that a potential subtle fitness cost was observed. Still, the mutant viruses were able to successfully reproduce over an extended duration and were never completely overtaken by the wild type virus. Furthermore, each virus sustained throughout several lifecycles with identical growth kinetics in both T1 cells and primary $CD4^+$ cells, disproving the notion that a possible artifact in the immortalized T1 cell line compensated for the potential adverse effects of the mutations. Interestingly, it has been shown that even a single mutation within certain HIV-1 RNA secondary structures, such as the psi packaging signal (25) and TAR element (24), severely impairs the ability of HIV-1 to replicate. Although the novel RNA secondary structure in *pol* may play a functional role *in vivo* that was not reflected in our analyses, these results suggested that it was dispensable for viral reproduction *in vitro*.

In parallel with the growth assays, SHAPE analysis was performed on the Stem ABC and Stem ABC+(L) mutants due to the number of theoretically disrupted base pairings. Although single

nucleotide resolution was not attained using SHAPE analysis, a pattern consistent with the thermodynamically predicted structures was detected. For further validation of these SHAPE results, simple thermal melting analyses can evaluate whether the RNA denaturation profile across a temperature gradient differs between the wild type and stem loop variants (18, 59). Although SHAPE experimentally validates RNA structure in an *in vitro* context, it may not accurately predict the disrupted RNA stem loop in the setting of infection. Thus, further experimentation would be required to fully determine the function and conformation of the secondary structure *in vivo*.

5.2.3 *Assessing the fitness costs of HIV-1 escape from CD8$^+$ cytotoxic T lymphocytes in acutely and chronically infected individuals –*

$CD8^+$ cytotoxic T lymphocytes (CTL) play an integral role in the immune response against infection with HIV-1. Due to high replication and mutation rates *in vivo*, HIV-1 readily generates numerous escape variants that can potentially evade immune recognition (8, 23, 28), though it has been observed that the process occurs more rapidly during early (8, 12, 36, 43, 45) versus late infection (23, 28, 32). Certain epitopes have been shown to develop mutations that impair viral replication capacity (RC), likely due to structural and functional constraints (5, 38, 52), which appear to associate with subsequent disease progression (23, 29, 31). In the past decade, the identification of escape mutations with an associated loss of fitness has generally been limited to studies largely biased toward protective HLA-I alleles (9, 15, 38, 39, 44, 50). The fitness costs incurred to allow immune evasion must therefore represent a key factor for control, although it remains unclear if this mechanism pertains to other HLA-1 types. To address this

issue, we investigated CTL escape in acutely and chronically infected persons without class I HLA alleles known to be protective.

Using HIV-1 from persons with chronic infection, we examined viral evolution by culturing the virus *ex vivo* in $CD4^+$ T lymphocytes from multiple, non-HLA matched, healthy HIV-1-uninfected donors over a period of 10 to 12 weeks in the absence of autologous CTL pressure (Chapter 4). Across all 9 individuals, we observed 26 amino acid changes that occurred within whole Gag-Pol following *ex vivo* culture, 14 of 26 (54%) evolving toward subtype B consensus, and 7 of 26 showing evidence of strong positive selection (dN/dS>1), altogether suggesting rapid optimization of HIV-1 toward the presumed fittest sequence. Consistent with these results, phylogenetic analysis suggested directed evolution of the passaged viruses with concomitant decrease in viral diversity. Viral sequences from some subjects evolved differently from the others, possibly due to lack of CTL pressure, potential replicative fitness constraints for reversion, minimal fitness cost for escape, or presence of compensatory mutations that would delay reversion. Lastly, a trend of correlation was observed between *gag-pol* diversity and immune pressure. As a whole, these data suggested that Gag-Pol-specific CTLs drive diversity *in vivo* and this diversity is shed when CTL pressure is removed.

In parallel, viral evolution *in vivo* was examined using longitudinal collections spanning a period of approximately 3 months from individuals identified very early in infection (some before peak viremia) (Chapter 4). Across 4 individuals, we observed 10 fixed codon substitutions within Gag-Pol with 1 site showing strong positive selection. Phylogenetic analysis demonstrated a

convergence toward consensus for subtype B and a unimodal pattern of viral diversification across these subjects over time.

To quantitate the effect of the observed Gag-Pol mutations, we utilized an *in vitro* system to measure viral fitness (2). Bulk, subject-derived *gag-pol* was inserted into the controlled context of HIV-1 molecular clone NL4-3 and differential growth kinetics between paired t_1 and t_{10} viruses (chronic) or visit 1 and visit 5 viruses (acute) were examined. A caveat to this approach is the efficiency of subject-derived *gag-pol* insertion into the NL4-3 backbone, which may explain the suggested loss of fitness for some subjects following *ex vivo* culture. Nonetheless, the data indicate that the codon changes associated with CTL pressure have fitness costs alleviated in the absence of selection, supporting the idea that HIV-1 is pressured to be less fit in chronic infection and persisting CTLs impose a measurable fitness cost to these viruses even in the presence of class I HLA alleles not associated with protection. This result is consistent with Lewis *et al* (35) in which "partial escape," defined as an epitope mutation that reduces CTL antiviral pressure but continues to drive CTL persistence, exacts a fitness cost contributing to the antiviral effects of CTL present during chronic infection. Additionally, a modest loss of fitness for 2 of 3 acute subjects was observed in our longitudinal analyses, which supports the notion that escape occurs rapidly during acute infection due to low cost to RC. Furthermore, the *in vivo* relevance of all viruses and their expressed escape mutations was determined through direct comparisons to the impact of the M184V drug resistance mutation, which has been shown to cause a clinically important reduction of viral RC *in vivo* (14, 19) and *in vitro* (9, 37).

Fitness assessment was performed on HIV-1 from persons with acute or chronic infection, although it is absolutely required to obtain larger samples sizes to better define the consistency of the observed trends. Our work thus far has produced a robust cloning system and optimized a method of analysis to easily integrate additional subjects. Furthermore, it would be of great interest to examine the effects of the identified mutations in isolation (or in combination), wherever possible, to distinguish individual contributions to the net impact on viral fitness. Lastly, these studies warrant further exploration of the pathways of escape and identification of factors determining fitness loss to uncover potential avenues for vaccine development that would manipulate CTL escape to the benefit of the host.

5.3 Perspective: HIV-1 mutational escape and keys to vaccine design

Mutational escape appears to play a significant role in the immunopathogenesis of HIV-1. However, the factors that influence both the kinetics and frequency of viral escape remain unclear. Indeed, it has been reported that escape can lead to disease progression (23, 29, 31) but studies have shown that CTLs can cross-recognize epitope variants to retain partial recognition (35, 55) and that the immune system can generate *de novo* CTL responses against escape mutants (3). Furthermore, it is known that escape mutations occur in the context of a variety of other changes, both compensatory or reversions of transmitted mutations (13). Thus, the likelihood for any given mutation amongst a network of evolving sites to be linked to a detectable impact on HIV-1 disease progression is fraught with difficulty.

Given the dynamics of HIV-1 adaptation, is the hope for an effective HIV vaccine unrealistic? A variety of vaccine strategies have been proposed to cope with the staggering mutational capacity of HIV-1 (30, 42, 47, 56, 65), including an emphasis on CTL-based approaches (4, 10, 20) [refer to review and commentary (34, 62, 63)]. Despite the recent failure of a major CTL-based HIV-1 vaccine trial (1), the CTL response remains the most defined feature of the immune system associated to the control of HIV, as demonstrated by the evidence that CTL drive viral evolution (36, 40) and that highly potent CTL responses correlate with slower disease progression (7). Rather than including whole proteins in a vaccine, which has the potential of duplicating the failure of our natural immune response against HIV-1, excluding variable sequences and focusing the response on conserved epitopes, for which escape exacts a loss of fitness to the virus, would be most desirable. Recent advancements in the development of mosaic vaccines (20,

33) that elicit broad cross-clade immune responses *in vivo* (48, 49) and *in vitro* (41) demonstrate promising movement toward the prospects of a new vaccine. The studies outlined in this dissertation may warrant further discussion regarding alternative regions in HIV-1 to utilize with which to further limit epitope variation and maximize CTL targeting. To date, only the RV144 Vaccine Trial has yielded progress toward a vaccine (17, 46), though flawed and modestly effective, at best (11, 22). Therefore, an improved understanding of the relationship between CTL mutational escape and disease progression may provide insight toward designing future treatment that simultaneously induces potent and persistent CTL immune responses and exploits the inherent weaknesses of HIV-1.

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