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Defining The Fitness Landscape of HIV-1 Escape from CD8+ Cytotoxic T-Lymphocytes

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Defining The Fitness Landscape of HIV-1 Escape from CD8+ Cytotoxic T-Lymphocytes

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Microbiology, Immunology, and Molecular Genetics

by

Aleksandr Gorin

2017

ABSTRACT OF THE DISSERTATION

Defining The Fitness Landscape of HIV-1 Escape

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Doctor of Philosophy in Microbiology, Immunology, and Molecular Genetics

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Professor Otto Orlean Yang, Chair

CD8+ Cytotoxic T-Lymphocytes (CTLs) are critical for control of viremia during Human Immunodeficiency Virus-1 (HIV-1) infection. While CTLs ultimately fail to fully suppress viral replication in most individuals, a small subset of infected persons can effectively control viremia for several decades. Understanding the factors that allow CTLs to mediate this control of viremia can provide a mechanistic basis for the failure of the CTL response in most individuals and help in the development of future HIV-1 vaccines.

Certain Major Histocompatibility-I (MHC-I) types are associated with better containment of viremia, but the mechanisms mediating control have been difficult to elucidate. In this dissertation, I construct HIV-1 libraries with saturation mutagenesis at several commonly targeted epitopes and utilize these libraries to demonstrate that epitopes presented by protective MHC-I types are highly constrained in their fitness landscapes and abilities to escape CTL clones

targeting the epitope. I then demonstrate that an epitope presented by a non-protective HLA type has multiple fit variants, allowing for mutational escape from CTL recognition with little or no loss in replicative capacity.

Next, I examine the CTL response induced by the Mrk/Ad5 HIV vaccine to understand how mutational escape may have contributed to the vaccine's failure. I demonstrate that the vaccine may have generated CTLs that were less efficient at cross recognizing fit epitope variants, and thus allowed for rapid mutational escape from CTL recognition, likely contributing to the failure of this vaccine trial.

In the final chapter of this dissertation, I focus on the HIV-1 Nef protein. Nef downregulates the expression of MHC-I on the surface of infected cells and represents another mechanism through which the virus can escape CTL recognition. I conduct a high throughput screen of an HIV-1 library containing thousands of point mutations throughout nef to identify amino acid residues essential for Nef's ability to downregulate MHC-I and demonstrate that these residues are highly conserved in circulating virus isolates.

These the results support a mechanism wherein protective MHC-I types elicit superior CTL responses through immunodominance of highly fitness-constrained epitopes and underscore the importance of escape pathways for successful vaccines and immunotherapies based on CTLs.

The dissertation of Aleksandr Gorin is approved.

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Gorin A, Du Y, Sun, R., and Yang, OO. HIV-1 Escape from Cytotoxic T-Lymphocytes Defined at Clonal Resolution. 2017. Presented at the Conference on Retroviruses and Opportunistic Infections. Seattle, WA, USA.

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Oral Presentations

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Chapter 1: Introduction

HIV and the natural history of AIDS

The Human Immunodeficiency Virus-1 (HIV-1) was first identified as the cause of Acquired Immunodeficiency Syndrome (AIDS) in 1983 (1). According to the World Health Organization, over 70 million individuals have become infected with HIV since the start of the epidemic in the 20th century, with approximately half of these dying as a result of the disease. HIV spreads through transfer of bodily fluids, in particular, blood and semen. The most common routes of infection are sexual transmission, along with mother-to-child transmission during birth, needle sharing by intravenous drug users, and blood transfusions.

HIV-1 is a lentivirus that encodes nine genes flanked by a long terminal repeat (LTR) on either side. The *gag*, *pol*, and *env* genes encode the structural, enzymatic, and envelope viral polyproteins, respectively, while *tat, rev, vif, vpr, vpu,* and *nef* encode accessory proteins which are necessary for replication and immune evasion of HIV-1 *in vivo*.

The life cycle of HIV-1 begins when the virion, containing two copies of its RNA genome, binds to the CD4 receptor (2, 3), and one of two coreceptors: CXCR4 (4) or CCR5 (5). HIV binding to the receptor triggers a conformational change in the envelope proteins that allows the virus to fuse to the cell. The virus then proceeds to reverse transcribe and integrate its genome into the host cell chromosomal DNA. Once integrated, the virus relies on host cell machinery to begin transcription and translation of the genome and packaging of new virions.

The progression of HIV infection is characterized by the depletion of CD4+ T-cells. Clinically, HIV infection begins with an acute phase characterized by fever, chills, and malaise during which the virus rapidly replicates and depletes CD4+ cells. The acute phase generally resolves within several weeks when the adaptive immune response suppresses viremia to much lower levels. This period of clinical latency generally lasts several years. During this period the

infected individuals show no symptoms. However, the virus persists throughout this period, and in most individuals slowly depletes CD4+ cells until the individual develops an immune dysfunction (AIDS), which predisposes them to opportunistic infections and cancers. Without treatment the overwhelming majority of HIV-infected individuals eventually die as a result of AIDS-related pathologies. Despite the modern antiretroviral therapy successfully controlling viremia in most treated individuals, a cure or vaccine for HIV-1 still remains elusive.

CD8+ cytotoxic T-lymphocytes and the T-cell receptor

The ability of the mammalian immune system to kill cells that are actively infected by viruses is critical for control of infection. As early as the 1960s and 70s immunologists had recognized that in addition to antibodies conferring protection from infection, there was a class of immune cells capable of killing virus-infected target cells (6). This class of cells, which would eventually be recognized as CD8+ cytotoxic T-lymphocytes (CTL) play a critical role in the control of viral infections.

In nearly all human cells, both self and foreign (viral) cytoplasmic proteins are degraded by the proteasome into smaller peptides. These peptides are then transported into the endoplasmic reticulum where they can be loaded onto the human leukocyte antigen class I (HLA I). This complex of the peptide-HLA complex is then transported to the cell surface and presented to CTLs that have been selected to recognize non-self-antigens.

CTLs recognize virus-infected cells through binding of their heterodimeric T-cell receptor (TCR) to recognized viral peptides bound to the HLA I molecule (7-10). Upon activation of the TCR, the CTL can kill the target cell by expressing Fas, and secreting perforin and granzyme (11) as well as several pro-inflammatory cytokines including IFN-γ and TNF-α.

The ability of CTLs to recognize foreign antigens via their TCR is established by specifically weeding out TCRs that can recognize self-antigens. CTL development begins with the semi-random rearrangement of the germ-line encoded V, D, and J segments of the TCR locus (12). These CTLs, bearing random TCRs with unknown specificity, then migrate the thymus to undergo further maturation. In the thymus, the TCRs are first tested for their ability recognize self MHC. In this process, termed positive selection, CTLs possessing TCR incapable of binding to self MHC will undergo apoptosis. Next, CTLs possessing TCR that recognize self-antigens are deleted in a process termed negative selection: CTLs possessing TCR that bind strongly to self-antigens presented in the thymus undergo apoptosis. This process of thymic maturation produces a repertoire of CTLs that have been selected to recognize non-self-antigens (13).

Clinical relevance of CTL in HIV infection

In 1986, only three years after the identification of HIV as the virus causing AIDS, several groups independently discovered that CTLs isolated from infected individuals could suppress HIV replication in seropositive individuals (14-16). In the thirty years since this discovery, the CTL response to HIV has been studied in great detail and has been recognized as one of the single most important factors controlling HIV replication *in vivo*.

The first evidence for CTL control of HIV infection came from studies investigating the immune response during the acute phase of HIV infection. Acute HIV infection is characterized by a period of rapid viral replication and high levels of viremia, followed by a precipitous drop in viremia several weeks later. In 1994 Richard Koup *et al*. first demonstrated the temporal association of CTL recognizing HIV-antigens and the drop in viremia that occurs during the acute phase of infection (17). These results showed that the detection of a CTL response to HIV

corresponds with the drop in viremia, whereas antibodies against HIV are not detected until many weeks after this drop. These results were supported by the work of Persephone Borrow who also demonstrated the correlation between a CTL response to HIV and drop in viremia, and who additionally found that two individuals who did not develop an HIV-specific CTL response showed prolonged levels of viremia (18).

Studies with nonhuman primates further reinforced the importance of CTLs to control of HIV infection. Rhesus macaques infected with SIV (a closely related Simian lentivirus used as a model for HIV) were found to have an exponential rise in viremia and increased death rate when depleted of CD8+ cells (19, 20), directly demonstrating that CTLs are largely responsible for the suppression of viremia during chronic SIV (and likely HIV) infection.

CTL-mediated suppression of HIV replication

CTL control of HIV is thought to be largely mediated by the ability of HIV-specific CTLs to directly kill infected cells. The ability of PBMC (16, 21, 22) and CTL clones (23) isolated from HIV seropositive individuals to lyse target cells presenting exogenous HIV antigens was reported in some of the first publications on HIV-specific CTLs. Shortly thereafter, Otto Yang *et al*. demonstrated that productively HIV-infected CD4+ cells could in fact be lysed by CTL clones specific for HIV antigens (24).

Despite general consensus that the main mechanism of HIV control by CD8+ T-cells is through cytotoxic mechanisms, there is also substantial evidence that CD8+ T-cells can suppress HIV infection through non-lytic mechanisms. In fact, the first report of suppression of HIV replication by CD8+ T-cells argued that control of viral replication was due to a mechanism other than lysis of target cells (14). This noncytolytic mechanism of viral suppression has been

attributed to a still unidentified antiviral secreted factor (25). This factor can be found in the supernatant of CD8+ T-cells activated with HIV antigens and can slow virus replication to a certain degree. However, at least *in vitro*, the cytotoxic response provides the overwhelming majority of the antiviral suppression exhibited by CTLs (26)

HIV escape from the CTL response

CTLs are ultimately unable to fully suppress viral replication in most individuals. A multitude of reasons for CTL failure have been recognized including Nef-mediated downregulation of HLA class I molecules on infected cells (27), preferential infection and killing of HIV-1 specific CD4+ cells (28) resulting in loss of help to CTLs, and exhaustion of virus specific CTLs (29). However, the major factor is likely viral escape mutations in regions targeted by CTLs.

CTL recognition of HIV antigens depends on an HIV protein sequence being cleaved by the proteasome, transported into the ER, trimmed by aminopeptidases, loaded onto the MHC I complex, and then recognized by a TCR specific for that antigen. If HIV can mutate a targeted epitope in such a way that it is able to preserve viability, but alter any of the above processes in such a way that the TCR can no longer detect that antigen on an infected cell, then the virus can escape CTL-mediated immune pressure. Such a mechanism of immune escape is particularly relevant in HIV-1 owing to the intrinsically high mutation rate (10^{-3} to 10^{-5} mutations/base pair/cycle) of its reverse transcriptase (30, 31), high levels of viral replication in infected individuals, and the capacity of the virus to tolerate mutation. In fact, HIV mutates so rapidly that it is predicted that every single and double mutation of the virus is made every day within an infected individual (32).

HIV escape from CTLs was first observed *in vivo* in 1991 (33) and since then the reported mechanisms of escape have encompassed altered antigen processing (34-37), abrogation of HLA binding (38, 39), and lack of TCR recognition (40, 41). Furthermore, HIV escape from CTLs is a major driving force of viral evolution both within a host and in the general population. The majority of HIV genomic evolution during acute infection outside of env is driven by the CTL response (42, 43). At the population level, several groups have demonstrated HLA-associated "footprints" – mutations likely driven by the CTL response – that can be identified in multiple cohorts of infected individuals (44-46).

Effects of HIV-1 escape from CTL recognition

The effect of immune escape on the CTL response has been clearly established: several studies in humans (47, 48) and primates (49) have demonstrated that the CTL response against the viral epitope fades away after a majority of circulating viral sequences have mutated the targeted epitope. An early clinical trial demonstrated that once the virus mutates and escapes, CTLs recognizing the virus have no clinical effect. In the trial, an infected individual received an infusion of *ex-vivo* expanded HIV-specific CTL clones. This infusion lead to rapid mutation of the targeted epitope, and no decrease in viremia or CD4+ T-cell counts (50).

While the ability of the virus to grow uninhibited after mutational escape occurs has been clearly demonstrated, it's effect on clinical disease progression has been far more controversial. Thus far, only case reports of escape at a highly conserved epitope (KK10, Gag 263-272) have been associated with loss of viremic control and rapid progression to AIDS (51). Markers of CD8+ T-cell polyfunctionality (52) and immune exhaustion are generally far better predictors of disease progression than CTL escape (53, 54). However, since exhaustion is highly dependent on

viral persistence, it is likely that continued escape from CTL recognition allows for viral persistence and may in fact be driving immune exhaustion (55).

Fitness costs of mutations escape from CTL recognition

Mutating targeted viral epitopes can result in a cost to viral fitness, and several CTL epitope escape mutations have been documented to incur significant costs to viral replicative capacity (56-61). Additionally reversion of such escape mutants has been observed upon transmission to HLA mismatched individuals, indicating these escape mutations decreased overall virus fitness in the absence of a CTL response directed against the epitope (58, 62-64).

Overall, viral escape mutations likely represent a balance between viral replicative capacity and pressure exerted by CTLs, wherein the virus will escape to a mutant with lower replicative capacity if the escape variant confers higher overall fitness to the virus in the presence of the CTL response. Sunshine *et al.* demonstrated that over the course of the infection HIV will mutate a targeted epitope until the most fit escape variant arises and predominates in the population (65). This dynamic interplay between fitness cost and immune escape has also been well demonstrated by work from Arne Schneidewind *et al*, wherein mutations within a Gag epitope were shown to significantly decreased viral replicative capacity, but greatly increased overall fitness of the virus when grown in the presence of CTL clones targeting the epitope (59, 60).

Different TCRs targeting the same epitope vary in their ability to cross-recognize variants

Each T-cell has a distinct TCR that only recognizes a certain subset of peptides presented by a specific HLA molecule. While highly conserved epitopes such as KK10 (Gag 163-172),

KF11 (Gag 162-172), and TW10 (Gag 240-249) have a generally predictable pattern of escape (likely due to few viable options for escape), many epitopes exhibit a large degree of variability in escape in different individuals, and even from different CTL clones isolated from the same individual (26, 40, 41, 66-68). *In vitro* passaging of HIV in the presence of different CTL clones targeting the same epitope leads to the development of different escape patterns within the epitope (26). *In vivo,* twin studies have demonstrated the clinical significance of such "private escape" routes as twins infected at birth develop different patterns of viral escape (69).

The exact variants that a TCR can cross recognize may be somewhat random, however it appears that TCR cross recognition of epitope variants is at least partially dependent on the virus variants present in an individual. One study showed that CTL clones isolated from an individual will not cross-recognize a common variant of a targeted epitope until that variant appears in the virus population of the infected individual, indicating that the immune system needs to be exposed to a variant before CTLs able of cross-recognizing it can arise (40, 41). Furthermore, infected individuals have multiple CTL clones targeting the same epitope (70, 71), and unpublished work from our group has demonstrated that the HIV-specific clonal population is constantly shifting over the course of the infection. We have found that clones targeting conserved epitopes are more stable than those targeting variable epitopes, suggesting that the CTLs response against variable epitopes is constantly shifting to adapt to newly arising variants.

"Elite" controllers and protective HLA types

Although the majority of untreated infected individuals inevitably progress to AIDS, a small minority are able to suppress viremia to very low or undetectable levels for a prolonged periods of time. These "elite" or "viremic" controllers have been a focus of intense research in

the hopes that understanding the factors that allow this group to effectively suppress HIV-1 may provide insight on an effective immune response to HIV-1 and perhaps to an effective vaccine.

Early work into these viremic controllers found a correlation between certain HLA types and the odds of suppressing viremia (72). Further evidence to support the importance of CTLs and HLA types in elite control came from the association of HLA heterozygosity (73) and expression of rarer HLA types (74) with superior viral control. The impact of the HLA type on disease progression has been confirmed by genome-wide association studies of viremic controllers which found that the only region of the human genome in which polymorphisms are significantly associated with elite control is the HLA locus (75-77). The SNPs most significantly associated with viremic control in these studies were those associated with increased expression levels of HLA C, and expression of the HLA B*57 allele (a well-recognized protective HLA type). The importance of the HLA type to progression of HIV disease strongly implicates the CTL response in successful control of HIV and raises the question of what about these protective HLA types allows for successful CTL-mediated control of HIV.

Mechanisms of CTL-mediated control of viremia: targeting of conserved epitopes

The observation that protective HLA types are associated with better outcomes in HIV infection has led to the conclusion that either these protective HLAs can present viral epitopes in a manner that the virus has a more difficult time escaping or that the CTLs raised in individuals expressing protective HLAs are somehow superior at recognizing and controlling HIV-1.

In support of the former hypothesis is evidence that frequently targeted immunodominant epitopes in individuals with the protective HLA B*27 and B*57 are highly conserved (78), and that common escape mutations within these epitopes greatly diminish viral replicative capacity

(57, 59) and require several compensatory mutations to allow viral replication (64, 79-81). Additionally, CTL targeting of the more conserved Gag protein in is loosely associated with a lower viremia (82-84). Finally, several studies that correlated viremia with targeting of individual epitopes found that viral load correlated inversely with CTL targeting of conserved epitopes (85, 86). These studies found that several of the epitopes whose targeting was most significantly associated with lower viral loads were highly conserved epitopes presented by protective HLA types.

Mechanisms of CTL-mediated control of viremia: promiscuity of the TCR

While the importance of fitness constraints to viral escape has been clearly demonstrated for several epitopes presented by protective HLA types, several groups have proposed that an additional mechanism of protection from these HLAs comes from highly cross-reactive TCR raised in individuals with these HLA types (87). This model is particularly interesting in that it could explain the observation that one protective HLA type, B*27, is associated with several autoimmune diseases.

In one computation model of HLA binding, it was suggested the HLA B*57, a protective HLA type, was able to bind fewer self-peptides than other HLA types. The group predicted that this would lead to deletion of fewer highly promiscuous TCR during thymic negative selection (88) and the establishment of a CTL repertories better able to cross-recognize HIV escape mutants and control viremia. Further support for this hypothesis comes from a study of elite controllers and progressors expressing protective HLA alleles HLA B*27 and HLA B*57. In this work, Chen *et al* found that CTLs targeting the immunodominant epitope in elite controllers were better at cross recognizing common escape variants in these epitopes (67).

However, some doubt was cast on these results in a recent publication showing that the controller and progressor TCRs tested in *Chen et al*. were equal in their ability to recognize and control HIV when introduced into healthy CD8+ cells, and that the difference observed previously may have been due to the functional state of the CTL in the progressors and not inherent to the TCR (89). The notion of TCR promiscuity in elite controllers also contradicts recent results that showed that CTL from elite controllers are actually less promiscuous that those in progressors. Sunshine *et al.* demonstrated that CTL from controllers can effectively cross-recognize the most common escape variants, thus likely blocking off the most common routes of escape, while CTL from progressors cross recognize more variants, but do not specifically target the most frequency variants. (90)

The HIV-1 Nef protein and its relevance to evasion from CTL recognition

In addition to escaping the CTL response by mutating targeted epitopes, HIV also encodes the Nef protein, which decreases CTL recognition of HIV-infected cells and contributes to immune evasion. Nef is a 27-kDa myristoylated protein that is not essential for HIV replication but is required for virulence and pathogenesis *in vivo*. Nef contributes to HIV pathogenesis through effects on multiple proteins and pathways, most notably the protein downregulates CD4 to prevent superinfection and facilitate release budding virus (91), modulates cell activation status (92-95), and downregulates expression of the peptide-MHC I complex on the surface of infected cells (96).

Nef's effect on the CTL response in likely central to its role in pathogenesis. Nef specifically downregulates HLAs A and B (97, 98), and results in a significantly decreased ability of CTLs targeting HIV-infected cells to kill HIV-infected cells (99, 100). The importance

of HLA downregulation to HIV pathogenesis is demonstrated by the following. First, mutations that decrease the ability of nef to downregulate the HLA are selected against when the virus is grown in the presence of HIV-specific CTL (101, 102). Second, infection with nef-deficient virus results in a robust CTL response that often control viremia (27, 103, 104). Third, there is strong evolutionary pressure during acute infection for nef to maintain its ability to downregulate the MHC (105). Finally, the ability of nef to downregulate MHC-I *in vivo* is correlated to the breadth of the CTL response in an individual, and loosely associated with viral load and CD4+ cell depletion (106).

The most convincing evidence for Nef's importance to HIV pathogenesis comes from studies of individuals infected with nef-deficient virus. In one famous example, a cohort receiving blood transfusions in Sydney, Australia all became infected with the same HIV strain containing large deletions in nef. The infected individuals all seroconverted, but did not progress to AIDS and maintained low or undetectable viral loads (107) as well as a strong CTL response against HIV (108). This long term non-progression and control of viremia was seen again in another individual infected with a nef-deficient virus (109). Similarly SIV with a truncated version of nef was highly attenuated in nonhuman primates (110), and resulted in an immune response capable of preventing subsequent infection with highly pathogenic strains of SIV (111).

Overall, these studies of nef have demonstrated that while the absence of nef does not greatly affect the ability of the virus to replicate *in vitro*, it is necessary to blunt the CTL response and allow the virus to maintain full virulence *in vivo*.

HIV-1 vaccines: failure of CTL based vaccines

The importance of inducing of an antiviral CTL response was recognized early on as an important factor in the design of an HIV vaccine. In fact, the very first trial with an HIV vaccine attempted to induce a CTL response (112). Unfortunately, this early trial started and ended in controversy – the lead scientist injected himself with the vaccine as the first subject, and later the trial was shut down because of adverse effects in children immunized with a vaccine intended for nonhuman primates. However, the push for an HIV vaccine dependent on a CTL response continued and culminated with the STEP/MRKAd5 vaccine trial. The vaccine consisted of a mixture of three replication-incompetent Ad5 vectors that expressed HIV gag, pol, and nef. Despite successfully inducing a CTL response in most vaccine recipients, the vaccine showed no efficacy in either preventing HIV infection, or lower viral load after infection(113, 114). Another vaccine trial, HVTN 505, which included a DNA prime, followed by an Ad5 boost (this time including env in the vaccine), also induced a CTL response in the majority of vaccinated individuals, but ultimately also failed at both preventing infection and lowering viral load in infected individuals(114, 115).

HIV-1 vaccines: previous pitfalls and promising new approaches

The failure of these vaccines (and the limited success of the RV144 trial (116), which did not elicit a strong protective CTL response) has shifted vaccine development efforts away from inducing antiviral CTL responses and towards an approach focused on humoral immunity. However, while work on inducing broadly neutralizing antibodies through vaccination is highly promising, there is no reason to believe that CTL based vaccines are all doomed to fail. There are

several clues as to why the STEP vaccine may have failed, and promising new vaccine strategies that show promised in eliciting a protective CTL response.

The first issue that any HIV vaccine will need to overcome is the high rate of mutation and extreme sequence diversity of HIV-1 (32). Since HIV can rapidly mutate targeted epitopes, a vaccine that induces CTL incapable of cross-recognizing common escape mutants will likely lead to rapid viral escape and have little overall effect. In fact, an analysis of the STEP vaccine trial found that in vaccinated individuals who later became infected, the vaccine did just this and acted as a "sieve" – causing the virus to mutate commonly targeted epitopes and escape immune recognition (117).

Another barrier that is a result of HIV's high rate of mutation is diversity of HIV-1. The sequence diversity of HIV-1 in a single chronically infected individual is approximately equal that of all influenza viruses worldwide in a single year (118). For a vaccine to be successful across a population, it will need to effectively recognize most variants of this highly diverse virus, and past vaccine trials have largely ignored this issue. Both the STEP and HVTN505 vaccines included several full length HIV genes as immunogens. While rationale for utilizing full length consensus genes was that it would induce a broader response against the virus, including the entire gene could actually have been detrimental. Inclusion of highly variable protein regions increased the chance that epitopes in these regions would be targeted leading to CTL responses that would likely be of little help if the individual was infected with an unrecognized variant.

Building on the failure of these two vaccine trials, several groups have made promising steps towards a successful CTL based vaccine including mosaic and conserved region HIV vaccines to attempt to overcome the failure of previous trials.

Mosaic vaccines contain multiple virus strains/variants that are chosen to represent the most frequency circulating strains in the population. This vaccine strategy was used to successfully develop the current hepatitis B and influenza vaccines (119, 120). Recently, several groups have developed such polyvalent/mosaic vaccines that have shown promise in increasing the immune system's ability to cross-recognize circulating variants (121-123).

Additionally, several groups have developed vaccines that contain only highly conserved regions of the HIV genome. Preliminary work with these vaccines has demonstrated that a strong CTL response targeting only these conserved regions can be effectively induced, thus decreasing the odds that an individual will be infected with an unrecognized variant (124-126).

These new vaccine strategies have already shown some clinical promise. In one recent trial, HIV infected individuals on ART were vaccinated with a conserved region HIV vaccine and given several doses of romidepsin (a histone deacetylase inhibitor that can reactivate latent HIV in an attempt to flush out the HIV reservoir). When ART treatment was interrupted in these individuals 40% were able to suppress viremia for several weeks to months (Mothe *et al.* CROI 2017). While these individuals were not cured of HIV, this trial demonstrated a proof of concept that an effective CTL response may be sufficient to control or prevent HIV infections.

Overview of the dissertation

The goal of this project was to design a system that would allow a thorough examination of all possible mutational pathways that HIV-1 could utilize to escape CTL pressure and to use this system to investigate how effectively CTL clones from chronically infected and vaccinated individuals can block off viral escape routes.

In **Chapter 2** I describe a novel method to create replication competent libraries of HIV that can be utilized to examine the full range of sequence variation that the virus can tolerate within any given epitope.

In **Chapter 3** I utilize these epitope libraries of HIV to examine how tolerant HIV is to mutation at three immunodominant epitopes presented by either protective or non-protective HLA types. Then in an attempt to understand the basis of immune control by CTL in individuals with protective HLA types, I utilize these libraries to determine which of these live variants can be effectively recognized by CTL targeting these epitopes.

I expand upon this work in **Chapter 4** by examining how a CTL clone induced by the MRK/Ad5 vaccine differs from clones induced by natural infection in its ability to cross recognize epitope variants. And in **Appendix A** I utilize this library system to demonstrate that highly conserved epitopes presented by non-protective HLA types can be restricted in their ability to mutate and thus represent potential targets for immunotherapy and vaccine design.

Finally, in **Chapter 5** I expand the focus to examine the constraints of the nef protein's ability to allow evasion from the CTL response. I utilize use a saturation virus library of nef mutants to identify regions of the protein that are necessary for its ability to downregulate HLA I and minimize CTL recognition.

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Chapter 2: Construction of HIV-1 Saturation Libraries with Minimal Lentiviral Vector Recombination

Introduction

In a Nobel prize-winning experiment in 1978 Michael Smith first demonstrated the ability to mutate a specific sequence of the phage genome and to create virus that carried this mutation (1). Today, in large part thanks to the discoveries of Dr. Smith and the invention of the polymerase chain reaction (PCR) in the 1990s, much of modern molecular biology is based on the ability to disrupt alter a sequence of DNA and then determine the effect of this mutation on the resulting RNA, protein, or organism. Recent advances in cloning, DNA synthesis, and high throughput sequencing have now made it possible to investigate the effects of thousands of such mutations in a single experiment. These advances have made it possible to rapidly assess the effect of mutations throughout the genomes of several viruses on their ability to replicate.

Multiple strategies for high throughput mutagenesis of genes and genomes have been developed. The first of these is insertional mutagenesis, in which short stretched of DNA are inserted randomly throughout a genome to disrupt the original sequence. This technique has been adapted to virology to assess the ability of several viruses to tolerate mutations throughout their genome (2-4). However, insertional mutagenesis has the intrinsic limitation that it inserts stretches of non-native DNA sequence that would not naturally appear in a virus through transcriptional error, thus while it can identify important functional domains it cannot determine the effect of single amino acid substitutions on the functions of the protein or growth of the virus.

Another high throughput site-directed mutagenesis approach that overcomes this constraint is saturation mutagenesis. In this approach a library of mutants representing every possible mutation within a given codon (or stretch of codons) is synthesized to examine the effects of any of these mutations on a protein of interest. This technique, which has been utilized for several years by biochemists studying enzyme function (5), has recently adopted by several

groups to identify the mutational landscape of HIV(6, 7), influenza (8, 9), and to identify putative drug resistance mutants in influenza (10) and hepatitis C (11). In this work, we describe a method to adopt this system to understanding the fitness profile of HIV-1 escape from CD8+ Cytotoxic T-Lymphocytes (CTLs).

Typically, putative HIV-1 escape variants are evaluated by sequencing patient samples, identifying potential escape variants, synthesizing the few identified variants, and determining their replicative capacity and ability to escape CTLs one by one. This technique is time consuming, and can only determine the fitness of a very limited number of possible mutations due to time and financial constraints. To overcome this issue, we have developed saturation mutagenesis libraries of commonly targeted HIV-1 CTL epitopes that can be used to interrogate the full mutational landscape of HIV-1 escape from CTL. Herein we describe the creation of such libraries, representing every possible single and double amino acid mutation within a given epitope, as well as an approach we have developed to simplify large scale cloning into lentiviral vectors.

Methods

Design of a full-length HIV vector with increased stability upon bacterial transformation

Lentiviral vector recombination upon transformation into bacterial cells is a common problem complicating cloning of lentiviral variants (12). This recombination is presumed to be driven by high self-complementarity within the vectors as a result of identical LTRs flanking the virus genome (13). We reasoned that we could reduce bacterial recombination of the full-length HIV plasmid by minimizing self-complementarity of within the plasmid by reducing the length of the LTR. Since HIV-1 only requires an RNA transcript from the 5'R to 3'R region for packaging and reverse transcription of its genome, we removed the 5' U3 region and 3' U5 region of the LTR from the NL4-3 genome and cloned this truncated genome into the FG12 vector (14). This resulted in the HIV genome being placed under control of the CMV-IE promoter and terminally flanked by a BGH polyA sequence (**Figure 2-1A**) to yield what we called the FG-NL4-3 vector. To demonstrate that this vector could create replication competent HIV, we transfected the plasmid into 293T cells and used the resulting supernatant to infect HIV-1 permissive cells for multiple rounds (**Figure 2-1B**).

We next sought to determine whether this vector was less prone to recombination upon transformation into bacterial cells. Since electroporation of the plasmid into bacterial competent cells induced maximal recombination of HIV vectors in our hands, we electroporated the FG-NL4-3 vector alongside the standard NL4-3 into electrically competent stellar cells (Clontech) per manufacturer protocol. 100uL of the transformed bacteria was plated onto LB/ampicillin plates incubated at either 30C or 37C. The colonies from these plates were pool by washing the plate off with LB media and then miniprepped (Plasmid miniprep kit, Biopioneer). Plasmid recombination was detected by digesting the miniprepped vectors (pNL4-3 was digested with

SphI and BssHII, expected product size = 832 and 11,263 nt; FG-NL4-3 vector with SpeI, expected product size = 1.636 and 12,386 nt) and running the digest on an agarose gel. Any DNA bands outside of these expected sizes were considered recombined plasmid. We observed that while the NL4-3 containing full length LTRs exhibited large amounts of recombination, no such recombination was detected in the FG-NL4-3 vector (**Figure 2-1C**).

Construction of HIV epitope library DNA fragments utilizing synthetic double stranded DNA

Several companies now offer the ability to synthesize large double stranded fragments of DNA with randomized codons; examples include GeneArt from thermofisher scientific and gBlocks from Integrated DNA technologies. We utilized IDT gBlocks to synthesize libraries encoding all possible single and double amino acid variants within four epitopes and their immediately flanking residues: SLYNTVATL (SL9, Gag 77-85, A*02-restricted), KAFSPEVIPMF (KF11, Gag 162-172, B*57-restricted), KRWIILGLNK (KK10, Gag 263-272, B*27-restricted), and GLNKIVRMY (GY9, Gag 269-277, B*15-restricted).

gBlocks were ordered to encode a 550-700 stretch of DNA encoding the epitope and surrounding NL4-3 sequence. The gBlocks were randomized so as to synthesize a library encoding every possible single and double amino acid variant within the epitope and its flanking codons. The randomization was facilitated by using degenerate codons (NNK, where N is any nucleotide and K is guanine or thymidine, which includes every amino acid while reducing stop codons) as demonstrated in **Figure 2-2**.

The gBlock libraries were reconstituted in TE buffer upon receipt to a concentration of 10ng/mL. 1uL of the gBlock library was PCR amplified using the KOD hotstart high fidelity polymerase (EMD Millipore) with primer designed to anneal to the ends of the gBlock library.

The PCR product was run on a 1% agarose gel, gel purified (NucleoSpin gel cleanup, Macherey-Nagel) and eluted into 30uL water. Samples were stored at -80C.

Cloning of gBlock library fragments into full-length HIV vector

FG-NL43 vectors were modified so that all but the first and last 15 nucleotides encoded in the gBlock libraries were removed from the plasmid. The junction of this deleted region was designed to be a blunt-cutting restriction enzyme site (SfoI for the SL9 library, and AfeI for the KF11 and KK10 libraries) as shown in **Figure 2-3**. These vectors, missing several-hundred amino acids within gag could not create replication competent HIV, thus even if this vector were to carry through into our cloning as a result of background, it would not create live virus that could contaminated any downstream virus libraries.

The PCR amplified gBlocks library were added to 400ng of the linearized vector in a 2:1 molar ratio and an "infusion" cloning reaction (clontech) was carried out in a total volume of 10uL (per manufacturer recommendations). 1uL of the infusion reaction product was then transformed into 50uL Stellar chemically competent cells (clontech) per manufacturer protocol followed by recovery in a total volume of 1mL SOC at 30C, 160RPM for 1 hour. 10-fold dilutions of 100uL of the transformed cells were plated on LB/ampicillin plates and incubated at 30C for 24 hours. The unused portion of the infusion reaction was stored at -20C.

Colonies from the in-fusion reaction transformed cells were counted, and the number of transformed cells in the transformation reaction was calculated based on the number of colonies and dilution factor. Next, the in-fusion reaction was thawed, and 9-1uL transformation reactions into stellar cells were performed as described above. The transformation reactions were plated onto pre-warmed LB/ampicillin plates so as to yield approximately 20,000 colonies per plate

(based on calculated transformation efficiency). The plates were grown for 24 hours at 30C, at which point colonies were collected from all plates (by washing with LB media) into one tube. These bacteria were midi-prepped (PureLink HiPure midiprep, Invitrogen) to obtain the final plasmid library of HIV-1 epitope variants. Illumina deep sequencing of these libraries revealed that all possible single codon variants, and almost all double codon variants were represented in the plasmid libraries (**Figure 2-4**).

Synthesis of replication competent HIV library stocks

20ug of plasmid libraries were transfected into a 60-70% confluent T75 flask of 293T cells utilizing 30uL of BioT reagent (Bioland Scientific) as described in the manufacturer protocol. However, the supernatant of these transfected cells contained very low titer, despite high concentrations of p24 protein in the supernatant (data not shown). We reasoned that the low titer was a result of a majority of the mutations in library encoding lethal mutations.

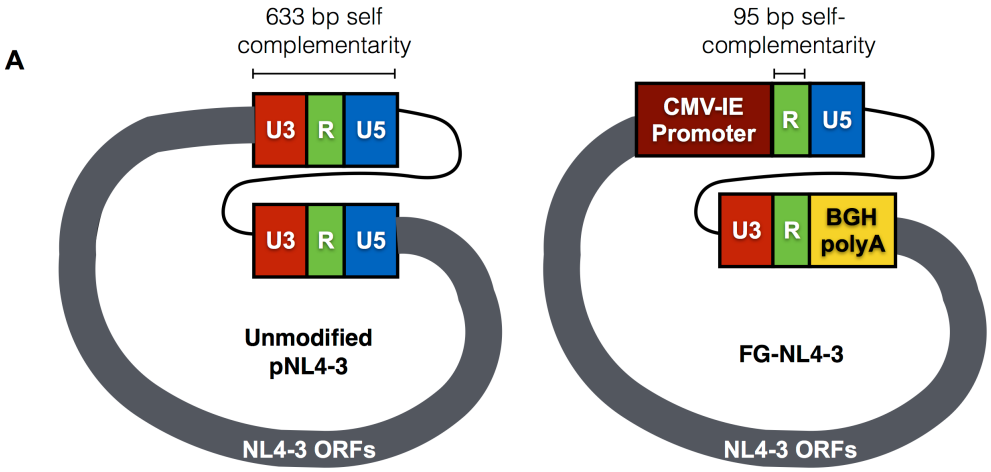
To increase the titer of the virus and increase the growth of live variants, 293T cells were transfected as above, 24 hours after transfection the media was removed from the 293T and replaced with 10 million T1 cells in 20mL RPMI medium supplemented with 10% FBS. These cells were added directly to the flask of transfected 293T cells to facilitate cell-cell infection of the T1 cells. After 24 hours of coculture, nonadherent cells and media were transferred to a new flask, and the infected T1 cells were cultured for 7-8 days to allow expansion of the replication competent HIV variants. The supernatant containing the live HIV variant library was removed, filtered through a 0.45 membrane, and stored at -80C and used for experiments described in Chapter 3, Chapter 4, and Appendix A.

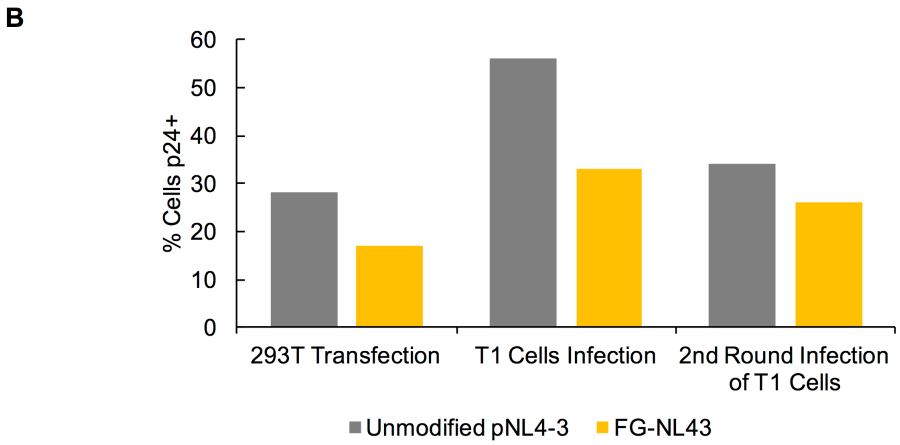
Discussion

We have developed a method that allows for construction of replication-competent HIV-1 libraries containing every single and double amino acid mutation within an epitope. This method can be applied to any stretch of up to 13 codons in the HIV genome. In the following chapters, we utilize these libraries to understand the fitness landscape of HIV-1 escape from CTLs, however, such randomized libraries could be used for a variety of purposes including determining the mutation tolerance of any region of the HIV-1 genome, identification of antibody and drug escape mutations, and protein binding interactions.

Previous work with saturation mutagenesis of virus genomes was limited to either single codon mutations (7-9) or relied on the use of error-prone PCR (6, 9, 10) which does not allow introduction of all amino acid variants. Our method overcomes both of these limitations, allowing for the analysis of almost all single and double amino acid variants within a short stretch of the virus genome.

Additionally, we have developed a full-length HIV-1 vector that minimizes recombination upon bacterial transformation. Methods such as lowering the incubation temperature of lentivirus transformed bacteria have previously been used to reduce recombination, but have limited efficacy. Bacterial strains that reduce lentiviral recombination are commonly used (12), but have significantly lower transformation efficiencies than normal strains, limiting their use for cloning large libraries. By simply replacing the 5' U3 and 3' U5 region of the NL4-3 LTR with a CMV promoter and BGH polyA sequence (respectively) we have managed to greatly minimize lentiviral recombination in normal laboratory strains of chemically competent bacteria. This strategy of truncating the LTR can be applied to any lentiviral vector to simplify cloning work.


A
633 bp self complementarity
95 bp self-complementarity
U3
R
U5
U3
R
U5
Unmodified pNL4-3
NL4-3 ORFs
CMV-IE Promoter
R
U5
U3
R
BGH polyA
FG-NL4-3
NL4-3 ORFs


B
% Cells p24+
60
50
40
30
20
10
0
293T Transfection
T1 Cells Infection
2nd Round Infection of T1 Cells
Unmodified pNL4-3
FG-NL43

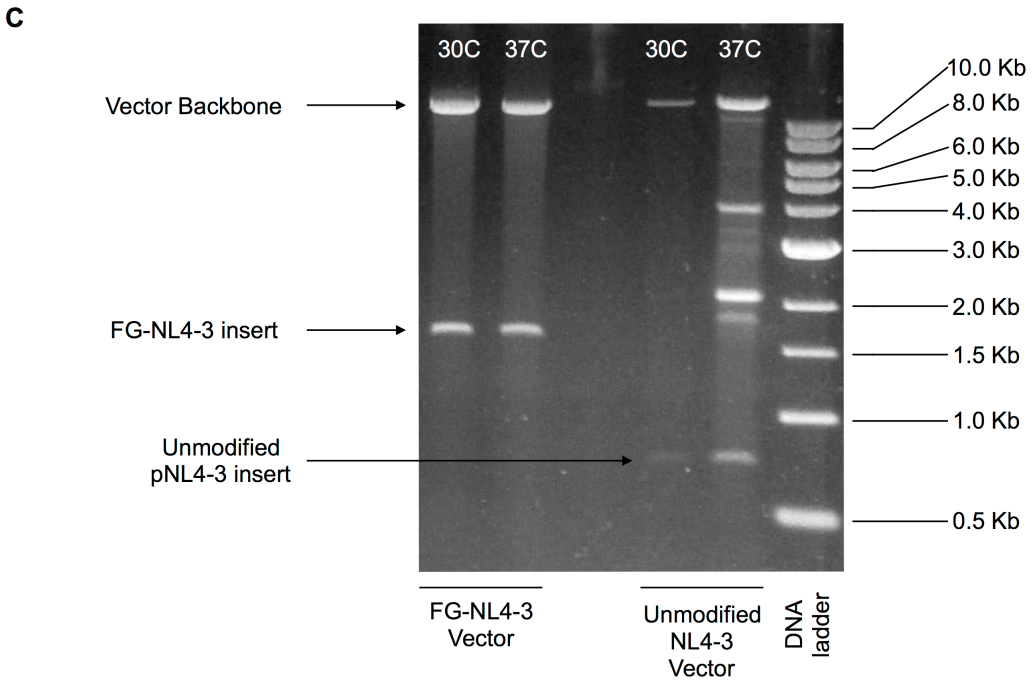

C
30C
37C
30C
37C
Vector Backbone
FG-NL4-3 insert
Unmodified pNL4-3 insert
10.0 Kb
8.0 Kb
6.0 Kb
5.0 Kb
4.0 Kb
3.0 Kb
2.0 Kb
1.5 Kb
1.0 Kb
0.5 Kb
FG-NL4-3 Vector
Unmodified NL4-3 Vector
DNA ladder

Figure 2-1: Shortening of LTRs minimizes bacterial recombination of HIV-1 plasmids. The 5' U3 and 3' U5 regions of the LTR were removed and replaced with the CMV-IE promoter and BGH polyA sequence, respectively, thus reducing self-complementarity in the vector by 538 nt (A). The unmodified pNL4-3 and FG-NL4-3 vectors were transfected into 293T cells. Supernatant from these cells was used to infected T1 cells for 2 successive rounds of 3-4 days. Transfection/infection efficiency was determined with flow cytometry by intracellular staining of cells with KC57-FITC antibody specific for HIV p24 (B). Pooled minipreps obtained from bacterial transformations (grown at either 30 or 37C) of the FG-NL4-3 and unmodified pNL4-3 vectors were digested with SpeI and BssHI/SphI respectively. Expected sizes of the digests are indicated. Any DNA fragments other than those of the expected size were considered to be recombined plasmid (C).

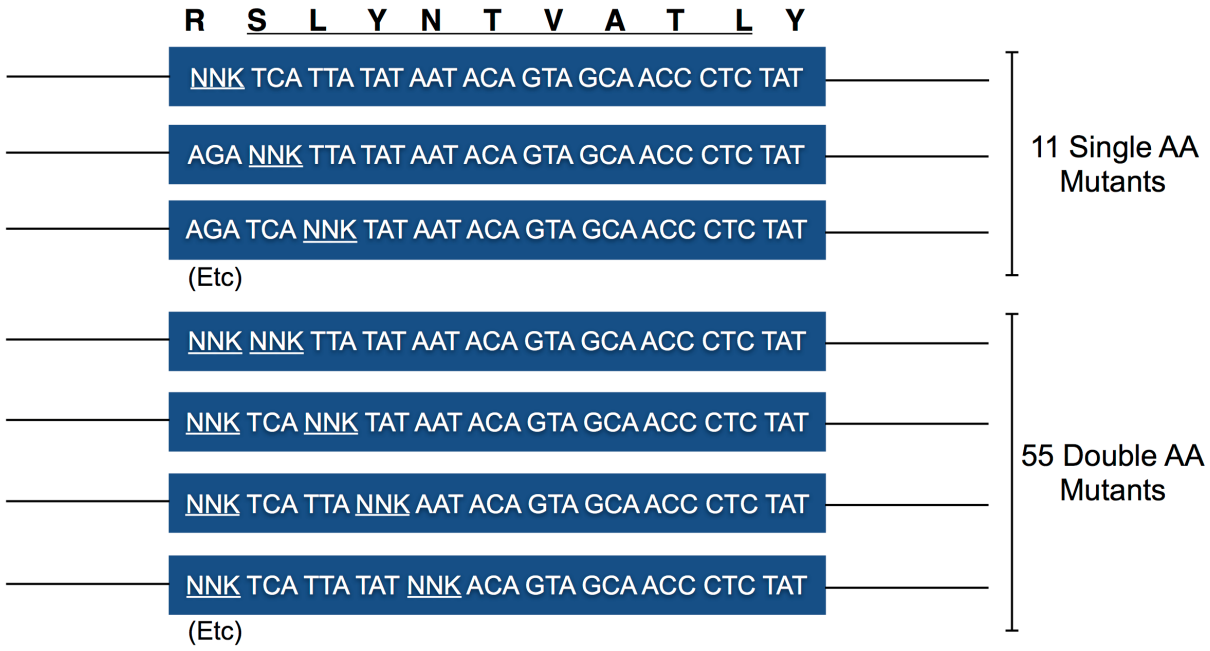


Figure 2-2: Strategy for saturation mutagenesis of HIV-1 epitopes. Double-stranded DNA gblocks encoding an HIV-1 genomic region including an epitope of interest were synthesized to encode every possible single and double amino acid variant within the epitope and its flanking codons, using degenerate codons (NNK, where N is any nucleotide and K is guanine or thymidine, which includes every amino acid while reducing stop codons). Shown above is the sequence utilized for saturation mutagenesis of the SL9 epitope.

A

Overlap With Vector

ATCTCTAGCAGTGGCGCCCGAACAGGGACTTGAAAGCGAAAGTAAAGCCAGAGGAGATCTCTCGACGCAG

GACTCGGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGGTGAGTACGCCAAAAATTTTG

ACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCGGTATTAAGCGGGGGAGAATTAGATAAA

TGGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAACAATATAAACTAAAACATATAGTATGGGCAAGCA

GGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTTTTAGAGACATCAGAAGGCTGTAGACAAATACTGGG

ACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATATAATACAATAGCAGTCCTC

SL9 Sequence

TATTGTGTGCATCAAAGGATAGATGTAAAAGACACCAAGGAAGCCTTAGATAAGATAGAGGAAGAGCAAA

ACAAAAGTAAGAAAAAGGCACAGCAAGCAGCAGCTGACACAGGAAACAACAGCCAGGTCAGCCAAA

Overlap With Vector

B

SfoI Cut Site

ATCTCTAGCAGTGGCGCCAGGTCAGCCAAA

FG-NL4-3 Vector
with SL9 gBlock region deleted

C

Overlap With Vector

AGACACCAAGGAAGCCTTAGATAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAGGCACAGCAAGCA

GCAGCTGACACAGGAAACAACAGCCAGGTCAGCCAAAATTACCCTATAGTGCAGAACCTCCAGGGGCAAA

TGGTACATCAGGCCATATCACCTAGAACTTTAAATGCATGGGTAAAAGTAGTAGAAGAGAAGGCTTTCAG

CCCAGAAGTAATACCCATGTTTTCAGCATTATCAGAAGGAGCCACCCCACAAGATTTAAATACCATGCTA

KF11 Sequence

AACACAGTGGGGGGACATCAAGCAGCCATGCAAATGTTAAAAGAGACCATCAATGAGGAAGCTGCAGAAT

GGGATAGATTGCATCCAGTGCATGCAGGGCCTATTGCACCAGGCCAGATGAGAGAACCAAGGGGAAGTGA

CATAGCAGGAACTACTAGTACCCTTCAGGAACAAATAGGATGGATGACACATAATCCACCTATCCCAGTA

GY9 Sequence

GGAGAAATCTATAAAAGATGGATAATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTACCAGCA

KK10 Sequence

TTCTGGACATAAGACAAGGACCAAAGGAACCCTTTAGAGACTATGTAGACCGATTCTATAAAACTCTAAG

AGCCGAGCAAGCTTCACAAGAGGTA

Overlap With Vector

AfeI Cut Site

D

AGACACCAAGGAAGCGCTTCACAAGAGGTA

FG-NL4-3 Vector
with KF11/KK10/GY9 gBlock region deleted

Figure 2-3: Strategy for cloning gBlock libraries into the FG-NL4-3 vector with minimal background virus contamination. gBlock sequences for the SL9 (A), KF11, KK10, and GY9 (C) libraries are indicated. Sequences corresponding to the first and last 15nt of the respective gBlock were removed from the FG-NL4-3 vector in such a way that the junction of the deleted region would yield a cut site for a blunt cutting restriction enzyme (B and D).

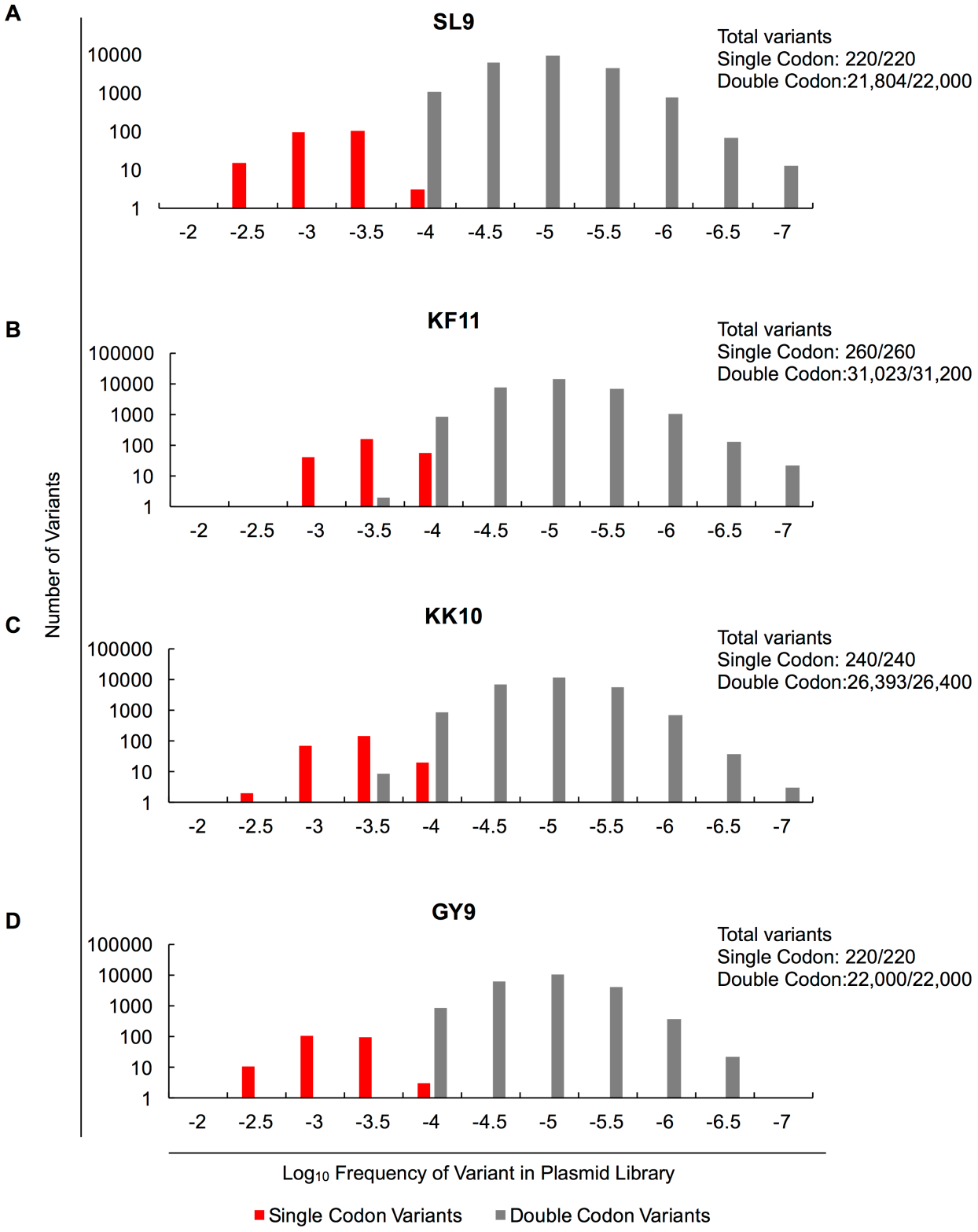

A
SL9
Total variants
Single Codon: 220/220
Double Codon:21,804/22,000
B
KF11
Total variants
Single Codon: 260/260
Double Codon:31,023/31,200
C
KK10
Total variants
Single Codon: 240/240
Double Codon:26,393/26,400
D
GY9
Total variants
Single Codon: 220/220
Double Codon:22,000/22,000
Number of Variants
10000
1000
100
10
1
100000
-2
-2.5
-3
-3.5
-4
-4.5
-5
-5.5
-6
-6.5
-7
Log10 Frequency of Variant in Plasmid Library
Single Codon Variants
Double Codon Variants

Figure 2-4: Frequency of variants in HIV-1 plasmid libraries. The frequency breakdown of all single (red) and double (grey) codon variants within the plasmid SL9 (A), KF11 (B), KK10 (C), and GY9 (D) libraries detected by deep sequencing are indicated. Also indicated are total number of single and double codon variants that were detected in each library as a fraction of the total number of possible single and double codon NNK variants within each library.

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Chapter 3: HIV-1 Epitopes Presented by Protective MHC Class I Types Have Highly Constrained Fitness Landscapes

Introduction

HIV-1-specific $CD8^+$ cytotoxic T-lymphocytes (CTLs) play a significant protective role in the pathogenesis of HIV-1 infection(1-3), but ultimately fail to prevent disease progression in most persons. Myriad failure mechanisms have been proposed, but given the remarkable mutation rate and sequence plasticity of HIV-1(4, 5), the major factor is viral epitope escape mutation resulting in a cascade of viral persistence, CTL exhaustion, dysfunction, and senescence in chronic infection(6). Indeed, evasion of CTLs is the major determinant of viral evolution *in vivo* (7-10). Moreover, the major histocompatibility complex class I (MHC-I) locus is the best defined genetic determinant of disease progression rate in genome-wide association(11-13) and epidemiologic studies(14, 15), indicating that MHC-I-associated properties of CTLs are important determinants of their efficacy.

Several studies of persons with "protective" MHC-I types who contain viremia without treatment have shown limited variation in targeted epitopes. Some have suggested that these are limited escape mutations with high fitness costs, based on examination of a few epitope variants observed *in vivo*(16-24). However, the generality and mechanisms behind this observation are unclear, and the contributions of viral versus immune constraints for HIV-1 escape from CTLs are incompletely understood. Properties of the targeted epitope could be important; HIV-1 sequence plasticity is not uniform and epitopes likely vary in their constraints for mutation(25). Alternatively, properties of the CTLs could differ; it has been proposed that the T cell receptors (TCRs) associated with protective MHC-I types either have greater cross-reactivity for epitope mutants and thus better limit possibilities for escape(26-28), or rather are better matched to common epitope variants(29). Thus it is unresolved whether the limited escape is due to properties of the epitopes versus CTLs.

Finally, CTL responses against a given epitope are generally comprised of multiple clones with differing TCRs(30, 31). Because individual clones recognizing the same epitope can vary in the recognition of different variants(32-34), it has been proposed that clonal breadth may be important for preventing escape(30), but protective MHC-I types do not appear to yield greater TCR breadth overall(31). This suggests qualitative differences in the composition or function of TCRs, and it is unclear to what degree the constraints for HIV-1 to escape CTLs are shared ("public escape") versus specific for each clone ("private escape").

Such issues are difficult to address *in vivo*, where the CTL response is polyclonal and the starting sequences of HIV-1 are typically undefined. Here we assess the effect of HIV-1-specific CTLs on the fitness landscape of viral epitope mutation at clonal resolution. Libraries of HIV-1 epitope mutants are propagated under selective pressure to define the options for immune escape for multiple CTL clones associated with protective and non-protective MHC-I types, addressing these issues.

Results

Construction of HIV-1 epitope libraries.

Saturation mutagenesis was applied to three immunodominant HIV-1 epitopes in Gag (**Table 3-1**): SLYNTVATL (SL9, Gag 77-85, A*02-restricted), KAFSPEVIPMF (KF11, Gag 162-172, B*57-restricted), and KRWIILGLNK (KK10, Gag 263-272, B*27-restricted). Degenerate nucleotide DNA synthesis was utilized for each codon encoding the epitope and its flanking amino acids, as well as every combination of two codons, followed by substitution into the whole proviral genome of HIV-1 strain NL4-3 (**Figure 3-1A**). The resulting plasmid libraries were found by deep sequencing to contain a full representation (100% for each epitope) of single amino acid variants and partial representation of double amino acid variants (38 to

43%) achieving the threshold frequency of $2.5x10^{-5}$ that was considered adequate for virus production after transfection (**Figure 3-1B**). As expected, the consensus epitope sequence was overrepresented in each library because consensus amino acids were included in every degenerate codon (**Figure 3-2A**). These proviral DNA libraries were transfected into producer cells to yield starting virus libraries after a week of expansion. Deep sequencing of viral RNA in these libraries revealed again that the consensus epitope variant was predominant, but also demonstrated that only a minority of the adequately represented variants in the plasmid library persisted as replication-competent variants (**Figure 3-1B** and **Figure 3-2A**), suggesting that most epitope mutations were deleterious (36.4 to 86.6% of single codon mutants, 99.12 to 99.97% of double codon mutants). Epitope variants with a threshold frequency $\geq 10^{-4}$ in two experimental replicates of virus libraries were considered as poorly replicating in the following analyses, because those with lower frequencies tended to decay if present in only one library, suggesting insufficient replicative capacity (not shown).

Assessing differential selection of epitope variants by CTL clones.

The HIV-1 epitope variant virus libraries were passaged in permissive cells in the absence and presence of CTLs (**Table 3-1**), which had been confirmed to have antiviral activity in virus suppression assays (**Figure 3-S1**). Epitope sequences were obtained by deep sequencing after each of two serial passages of one week (**Figure 3-S2**). Shifts in the frequencies of epitope variants within a library occurred after passaging in the absence of CTLs, suggesting evolution of the population due to fitness differences between variants (**Figure 3-2A**). Library propagation with the addition of CTLs targeting the epitope yielded distinctly different profiles of epitope variants, suggesting superimposed selection by the CTLs (**Figure 3-2B**). The specificity of this

CTL effect was evident because control CTLs targeting an irrelevant epitope did not induce a profile distinct from passaging without CTLs, and the magnitude of the epitope-specific CTL-induced change was dose-dependent (**Figure 3-2C**). The small minority of variants containing stop codons that achieved the detectable threshold in the initial virus libraries generally showed sharply decaying frequencies under these conditions (**Figure 3-S3**), confirming the reflection of replicative capacity in these conditions. The outcome for each epitope variant was quantified as a relative enrichment value (RE) compared to the subtype B consensus epitope sequence, calculated as the $\log_{10}$ transformed ratio of frequencies normalized to subtype B consensus variant (**Figure 3-2D**) in the absence or presence of CTLs (RE_{-CTL} and RE_{+CTL} respectively). The REs between experimental replicates were highly correlated (**Figure 3-2E and 3-F**), demonstrating the robustness of this measurement of epitope variation effects on viral replication.

Consistent patterns of amino acid substitutions are seen in the absence and presence of CTL selection.

The effect of single amino acid polymorphisms was assessed for each epitope. For the SL9 epitope (**Figure 3-3A**), substitutions at multiple positions were associated with RE_{-CTL} values ≥ 0 in the absence of CTL selection, indicating that multiple substitutions had neutral to positive impacts on intrinsic viral replication compared to consensus. Under selective pressure from CTLs, there were several substitutions that yielded RE_{+CTL} values ≥ 0, suggesting these to be options to reduce CTL recognition compared to consensus. For KF11 epitope variants (**Figure 3-3B**), there were few substitutions associated with RE_{-CTL} values ≥ 0, suggesting few options for mutations without loss of replicative capacity, but several substitutions yielding increased

RE_{+CTL}. For KK10 epitope variants (**Figure 3-3C**), again there were few substitutions associated with RE_{-CTL} values ≥0, but several substitutions yielding increased RE_{+CTL}. Despite varying T cell receptors (**Table 3-1**), different CTL clones yielded mostly similar patterns of substitutions associated with increased RE_{+CTL} for all three epitopes, although there were also some clone-specific differences. Examining the net effect of CTL selection (difference between RE_{+CTL} and RE_{-CTL}, defined as ΔRE) further confirmed similarity between different CTLs targeting each epitope, and showed that the majority of single substitution mutants viable in the starting libraries conferred a benefit for persistence under CTL selection (**Figure 3-4**). The major exception to this pattern was the N-terminal flanking amino acid of the SL9 epitope, where substitutions were deleterious under CTL selection, suggesting that the consensus sequence represents an escape mutation for this epitope. Overall, these data suggest that the fitness landscape for SL9 mutation is vaster than for KF11 or KK10, that multiple mutations for each epitope can disrupt CTL recognition, and that most escape mutations are shared across different CTL clones (representing "public" options).

CTL evasion via epitope mutation necessitates a greater fitness cost for KF11 and KK10 epitopes versus the SL9 epitope.

All epitope variants in the libraries, including double substitutions relative to consensus, were analyzed for SL9, KF11, and KK10. First examining RE_{-CTL} (**Figure 3-5 and Figures 3-S4, 3-S5, 3-S6** first columns), the KF11 library contained the fewest variants within 2 RE_{-CTL} units of consensus, followed by the KK10 and SL9 libraries (39 versus 70 and 120 respectively). Among those, however, the SL9 library possessed more variants with RE_{-CTL} values above -0.5 than the KK10 and KF11 libraries; 44 and 30 SL9 variants had RE values above -0.5 and 0,

respectively, while 10 KF11 and 8 KK10 variants had RE values above -0.5, and 2 KF11 and 3 KK10 variants had RE values above 0. Comparing changes in epitope frequencies under CTL selection (RE_{+CTL}, **Figure 3-5** and **Figures 3-S4, 3-S5, 3-S6** second columns), many variants were enriched ($RE_{+CTL}>0$) for all three epitopes. Again examining the net effect of CTLs, there were many variants with ΔRE >0 (**Figure 3-5** third columns). Considering those with ≥5-fold net advantage (ΔRE ≥0.70) as being CTL-resistant (**Figure** 3-5 fourth columns and **Figure 3-6**), it was apparent that there were many SL9 variants resistant to all three clones tested that had intrinsically high RE_{-CTL}, whereas there were fewer resistant variants for KF11 and KK10 with high intrinsic RE_{-CTL}. This pattern held true across varying ΔRE thresholds (**Figures 3-S4, 3-S5, 3-S6**), suggesting there are more epitope mutation options to escape CTLs while maintaining fitness for SL9 compared to KF11 and KK10.

Library epitope variants selected by CTLs represent potential escape mutants.

To validate the above determination of CTL resistance of epitope variants, HIV-1 NL4-3 provirus was mutagenized to contain the consensus SL9 sequence, an SL9 variant observed to have reduced RE by CTLs (ΔRE = -1.42 and -1.43 with CTL clones 3.23T and 10.11T respectively), and several SL9 variants with increased RE values by CTLs (ΔRE = 1.39-1.66 and 1.37-2.32 for CTL clones 3.23T and 10.11T respectively). These viruses were tested for susceptibility to suppression of replication by the SL9-specific CTL clones 3.23T and 10.11T (**Figure 3-7**). Viruses with consensus and control variant SL9 epitopes were suppressed by both clones, but viruses with the SL9 variants associated with higher ΔRE values were uniformly unaffected by the CTLs. These results confirm that ΔRE values reflect the relative sensitivity or resistance of epitope variants to CTLs as options for immune escape.

Discussion

This study addresses the fitness landscape for mutational variation of three HIV-1 epitopes and the restrictions imposed by CTLs. While *in vivo* observations have revealed the effects of CTL on viral evolution to escape, our data dissect this process in greater detail, resolving the interaction at the level of individual CTL clones and defined starting virus quasispecies populations. For each epitope, the effect of every single amino acid polymorphism (as well as about a third of all double amino acid polymorphisms) versus the subtype B consensus sequence is assessed by frequency change as a reflection of fitness during serial passaging, as well as the impact of clonal CTL selection on these variants. Two epitopes presented by protective MHC-I types B*57 (KF11) and B*27 (KK10) and an epitope presented by the non-protective type A*02 (SL9) are examined in detail.

The quantities of mutation options in the absence of CTL selection markedly differ between these epitopes. The SL9 epitope exhibits many variants with similar or higher fitness compared to consensus, whereas KF11 and KK10 epitopes appear to have very few. This finding indicates that the SL9 epitope is much less constrained for mutation than KF11 and KK10, suggesting that HIV-1 generally has fewer options for mutational escape in KF11 and KK10 than SL9 epitopes.

The epitope variants that were enriched under CTL selection further illuminate the constraints for escape mutation. For SL9, there are several highly CTL-enriched variants with intrinsic fitness near the consensus epitope. In contrast, KF11 and KK10 both exhibit few CTL-enriched variants with preserved fitness, in agreement with prior studies showing that CTL escape mutations for these epitopes require high fitness costs(16-19, 21-24). Moreover, the variants enriched by CTL selection recapitulate several previously reported escape variants *in*

vivo, such as Y79F in SL9(16) and A163G in KF11(18), although some other reported escape variants such as KF11 A163G/S165N(18) were present in the initial plasmid library but appeared replication incompetent (not shown). As a whole, these data support the concept that protective MHC-I types such as B*27 and B*57 are beneficial through generating CTL responses against epitopes for which escape occurs only at a high fitness cost to HIV-1.

Regarding an alternative hypothesis that protective MHC-I types yield TCRs with greater promiscuity for epitope variation (26-28), our findings do not provide definitive evidence. While KK10-specific CTLs do appear to recognize more variants than SL9-specific CTLs, those targeting KF11 seem to recognize fewer variants. However, our assessments are limited to CTL interactions only with viable variants, and are thus not a comprehensive evaluation of promiscuity across all epitope variation. Within the subset of viable mutants, there is no clear difference in coverage by CTLs across the three epitopes, and the findings are consistent with a study suggesting that better immune containment of HIV-1 is mediated by CTL responses that are more focused on viable epitope variants despite recognizing fewer epitope variants overall(29).

An unexpected finding is that CTL recognition of SL9 is enhanced by various substitutions at the N-terminus flanking amino acid. This suggests that these substitutions increase epitope presentation compared to the consensus sequence. Although the influence of various mutations within the SL9 epitope reducing its proteasomal processing and presentation have been demonstrated(35) , the impairment of processing associated with the N-terminus flanking residue in the consensus sequence has not been reported. Given the high prevalence of A*02 and the capacity of other MHC-I types such as B*40 to present the SL9 epitope, it is plausible that the consensus sequence represents escape adaptation across the human population.

Also unexpected is the observation that several SL9 epitope variants had apparently higher fitness than the consensus sequence. Both these findings support the proposal that HIV-1 can accumulate escape mutations in the consensus sequence for circulating strains, as has been suggested specifically for SL9(36) and more generally across the HIV-1 genome(7, 8).

We previously reported the differential ability of CTL clones targeting the same epitope to cross-recognize escape variants(32-34). Here we confirm such differences between clones, but find that the overall options for escape are strikingly similar even between TCRs with entirely different variable chains. For each epitope, the amino acid substitutions resulting in CTL evasion follow stereotypic patterns mostly sparing the main MHC-I anchor-binding residues. Although such substitutions could affect proteasomal processing, epitope stability, or MHC-I binding, this suggests convergent shared motifs for binding of sequence-divergent TCRs, and that "public escape" pathways may predominate for these epitopes, as indicated by population-based studies of HIV-1 escape from these epitopes *in vivo*(8, 37).

Several caveats must be considered for the interpretation of our data. Our libraries provide complete coverage for single amino acid polymorphisms in the epitopes, but incomplete coverage for double amino acid polymorphisms, and no coverage for three or more changes (although most reported escape mutations are single or double polymorphisms compared to consensus). The RE values for epitope variants are semiquantitative reflections of HIV-1 fitness, given the saturating conditions for viral growth that can exaggerate the competitive advantage of the most fit variants. Moreover, the selective pressure exerted by CTLs is dependent on the experimental conditions, i.e. the number of added cells and functional activity of the cells. While these parameters are kept as constant as possible between experiments, there is biologic variability that is difficult to control entirely; thus setting RE values based on consensus

sequence epitopes provides a frame of reference for comparisons between different experiments and SL9, KF11, and KK10 epitopes, because HIV-1 with consensus sequences in all three epitopes is shared between all libraries. Finally, fitness costs for sequence polymorphisms can vary considerably in different genomic contexts, and our results in HIV-1 strain NL4-3 using single epitope targeting may not reflect the outcome for different virus with CTL pressure on multiple epitopes simultaneously. Related to this point is the inability to assess for compensatory mutations. However, the general patterns we observe are striking, and provide insight into the overall levels of constraints for these epitopes.

In summary, our findings indicate that two immunodominant epitopes associated with protective MHC-I types have highly restricted fitness landscapes for mutation compared to one that is not associated with protection, and that this carries through to very limited options for escape from CTLs. Additionally, most escape pathways appear to be public and shared between different clones recognizing these epitopes. These results have implications for harnessing CTL responses as vaccines and/or immunotherapies. An early attempt at therapeutic adoptive transfer of CTLs resulted in rapid viral escape(38), and analysis of the failed Step trial demonstrated a "sieve" effect in infected individuals, reflecting viral escape from vaccine-induced CTLs(39). Thus, a successful CTL-based approach will require understanding of the constraints for escape and strategies to block HIV-1 escape routes through reducing HIV-1 options for mutational escape and/or increasing CTL coverage of mutation options.

Methods

Epitope mutational libraries of plasmid HIV-1 NL4-3.

Double-stranded DNA spanning the Gag epitope regions of interest were commercially synthesized (gBlock, Integrated DNA Technologies, Coralville, IA) using NNK degenerate codons (where "N" is any nucleotide, and "K" is guanine or thymidine) at each single or double codon position for the epitope and its flanking codons. These gBlock DNA fragments were then PCR amplified using primers 5'-ATCTCTAGCAGTGGCGCCC-3' with 5'-TTTGGCTGACCTGGCTGTTG-3' for the fragment containing the SLYNTVATL (Gag 77-85, SL9) epitope, and 5'-AGACACCAAGGAAGCCTTAGATAAGA-3' with 5'-TACCTCTTGTGAAGCTTGCTCG-3' for the fragments containing the KAFSPEVIPMF (Gag 162-172, KF11) and KRWIILGLNK (Gag 263-272, KK10) epitopes.

These primer sequences corresponded to the start and end sequences of the synthesized DNA fragments. A modified HIV-1 NL4-3 provirus plasmid was created to reduce LTR-driven recombination during cloning, with 5' U3 and 3' U5 regions of the HIV LTR removed (to reduce LTR homology), flanked by the CMV immediate-early promoter and the BGH polyA sequence (**Figure 1**). Additionally, this vector was modified to delete the synthesized epitope regions except the first and last 15 nucleotides; the junction of the deleted regions were modified to have blunt cutting restriction enzyme sites: SfoI for the region containing SL9, AfeI for the region containing KF11 and KK10. After linearizing each plasmid vector with the appropriate enzyme, the PCR-amplified gBlock DNA fragments were inserted via the 15 nucleotide homology by "Infusion" (Clontech, Mountain View, CA) to created whole genome plasmid libraries. The resulting plasmids were then transformed into Stellar chemocompetent *E. coli* (Clontech,

Mountain View, CA), plated onto 100mm LB/ampicillin plates at ~$2x10^4$ colonies/plate and grown for 24 hours at 30˚C. Colonies were collected by washing the bacteria from the plates with Luria broth with ampicillin. The plasmid DNA isolated from these bacteria served as the initial "plasmid libraries" for each epitope.

Creation of HIV-1 epitope libraries.

The plasmid libraries of each epitope were lipofected into two T75 flasks of 70% confluent HEK 293T cells using 20µg DNA with BioT (Bioland Scientific, Paramount, CA). After 24 hours the media was removed, and 10^7 T1 cells(40) in 20mL RPMI 1640 medium supplemented with 10% FCS, L-glutamine, HEPES, and penicillin-streptomycin (R10) were added to each flask to promote cell-cell infection of the T1 cells. After 24 hours, the nonadherent cells were removed from the flask and transferred to a new flask. These cells were then cultured for 6-8 days in R10 media until at least 50% of the cells were infected with HIV-1 (determined by expression of p24 antigen in the cells by intracellular staining and flow cytometry). The supernatant was then filtered through a 0.45 micron filter and cryopreserved to be utilized as the "starting virus library." All virus libraries were produced in duplicate, and all experiments utilized both libraries in parallel, with duplicates for cultures without CTLs (two replicates for each library, four total) and singles for cultures with CTLs (one replicate for each library, two total).

HIV-1-permissive cell lines.

Cell lines utilized for passaging of HIV-1 included T1(40) (expressing A*02 for the SL9 library and A*02-restricted CTLs), 1CC4.14 cells (41) (expressing B*57 for the KF11 library

and B*57-restricted CTLs), and Subject 00076 EBV-transformed B-cells that were transduced with human CD4 (expressing B*27 for the KK10 library and B*27-restricted CTLs).

HIV-1-specific CTLs.

CTL clones (**Table 1**) were isolated from chronically HIV-1-infected persons and maintained as previously described(42-44) from blood obtained under a University of California, Los Angeles Institutional Review Board-approved protocol, with the exception of 68A62 provided by Dr. Bruce Walker. In brief, peripheral blood mononuclear cells (PBMCs) were enriched for the CTLs of interest by culture with the appropriate epitope, followed by cloning at limiting dilution. Some experiments utilized KK10-specific CTLs previously produced by stable lentiviral transduction of allogeneic $CD8^+$ T-cells with a KK10-specific T cell receptor (TCR) sequence identified by quantitative spectratyping (31) (TCR5) that had been cloned into a lentiviral vector as previously described (34). CTLs were maintained by periodic stimulation with 200ng/mL of the monoclonal anti-CD3 12F6 antibody(45) with irradiated allogeneic PBMCs in R10 media supplemented with recombinant human interleukin-2 (NIH AIDS Reference and Reagent Repository) at 50IU/mL (R10-50). For the CTL clones, TCR beta variable (BV) chain sequences were determined after RNA isolation using Trizol reagent (ThermoFisher Scientific, Waltham, MA), amplification and cloning of the BV gene using the SMARTER 5' RACE kit (Clontech, Mountain View, CA) with a constant region primer (5'-CTTCTGATGGCTCAAACAC-3'), and sequencing using the same primer.

Passaging of virus libraries. $5x10^6$ permissive cells (10^6 cells for the SL9 library passaged with the 1.9 CTL) were infected with the starting virus library, yielding about 10-20% infected cells after 72-96 hours (determined by intracellular staining for p24). The cells were

then washed twice and resuspended at $5x10^5$ cells/mL in R10-50. CTLs were added at effector:target ratios of 1:8 (except 1:2 for the SL9 library with CTL 1.9), with parallel no-CTL controls. These cultures were fed every 3 days by removing and replacing half of the media. After 7 days the supernatant was filtered through a 0.45 micron filter and cryopreserved; virus in the supernatant was quantified via p24 ELISA (Xpress Bio, Frederick, MD). This virus was utilized to infect cells for a second passage in the same manner using $5x10^3$ pg p24 per 10^6 target cells (10^3 pg p24 per 10^6 target cells for the KK10 library), followed by collection and cryopreservation as before. All passaging with CTLs was performed with duplicate virus libraries, and passaging without CTLs was done in quadruplicate (2 replicates for each virus library).

Deep sequencing of passaged virus libraries.

The passaged virus supernatant was treated with DNAse I (New England Biolabs, Ipswich, MA) to remove residual plasmid DNA. HIV-1 RNA was isolated with the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany), and reverse-transcribed with the high capacity cDNA reverse transcription kit (ThermoFisher Scientific, Waltham, MA) and quantified by real-time PCR with ssoFast EvaGreen supermix on a CFX96 (Bio-Rad, Hercules, CA) with gag-specific primers (5'-ATCTCTAGCAGTGGCGCCC-3' and 5'-TTTGGCTGACCTGGCTGTTG-3') compared to NL4-3 plasmid standard to ensure $\geq 5x10^5$ copies/μL of cDNA per specimen. This cDNA and the starting plasmid libraries were prepared for deep sequencing by PCR amplification using primers tagged with 6 base-pair customized barcodes. The gene specific portions of the primers were:

5’-CCATCCCTTCAGACAGGATCAGA-3’ and 5’-AAGGCTTCCTTGGTGTCTTTTAC-3’ for the SL9 libraries,

5’- AGGCCATATCACCTAGAACTTTA-3’ and 5’- CCCACTGTGTTTAGCATGGTATT-3’ for the KF11 libraries, and

5’-TCCACCTATCCCAGTAGGAGAAA-3’ and 5’-GTCCTTGTCTTATGTCCAGAATGC-3’ for the KK10 libraries.

Deep sequencing was performed with Hiseq PE150 sequencing (Illumina, San Diego, CA).

Deep sequencing analyses.

The sequence data were parsed using the SeqIO function of open source BioPython software (http://biopython.org/). Sequences from different samples were de-multiplexed by the barcodes and mapped to the corresponding region in the HIV-1 genome. Since both forward and reverse reads covered the mutated region, paired reads were used to compensate for sequencing errors. A polymorphism was accepted as valid only if observed in both reads and with a quality score ≥30. Further filtering for errors was done by comparison to control deep sequencing of the index NL4-3 plasmid; variants present at a frequency $<10^{-4}$ were only accepted if their frequencies in duplicate virus libraries exceeded 10-fold the observed frequency of the variant in the control plasmid sequences (due to background error). The sequencing depth was $>6x10^5$ and $>4x10^6$ for the virus and plasmid libraries respectively. All the data processing and analysis was performed with customized python scripts, which are available upon request. Variants above threshold in initial virus libraries whose frequencies decayed to 0 after passaging were assigned a frequency of 10^{-6} for calculation of RE values. All sequences will be available through GenBank (accession numbers pending).

HIV-1 clonal mutagenesis of the SL9 epitope.

Site-directed mutagenesis was performed with the Q5 mutagenesis kit (New England Biolabs, Ipswich, MA) on the modified pNL4-3 vector described above, which had been further modified to contain the M20A mutation that ablates Nef-mediated MHC-I downregulation(46, 47). The SL9 epitope was modified to create variants SLYNAVAVL (codon 4 = GCT, codon 7 = GTG), SLYNTVACL (codon 8 = TGT), SLYITVATL (codon 4 = ATA), SLYNCVACL (codon 5 = TGT, codon 8 = TGT), SLYCTVATL (codon 4 = TGT), and the resulting plasmids were lipofected into HEK 293T cells as above to produce virus.

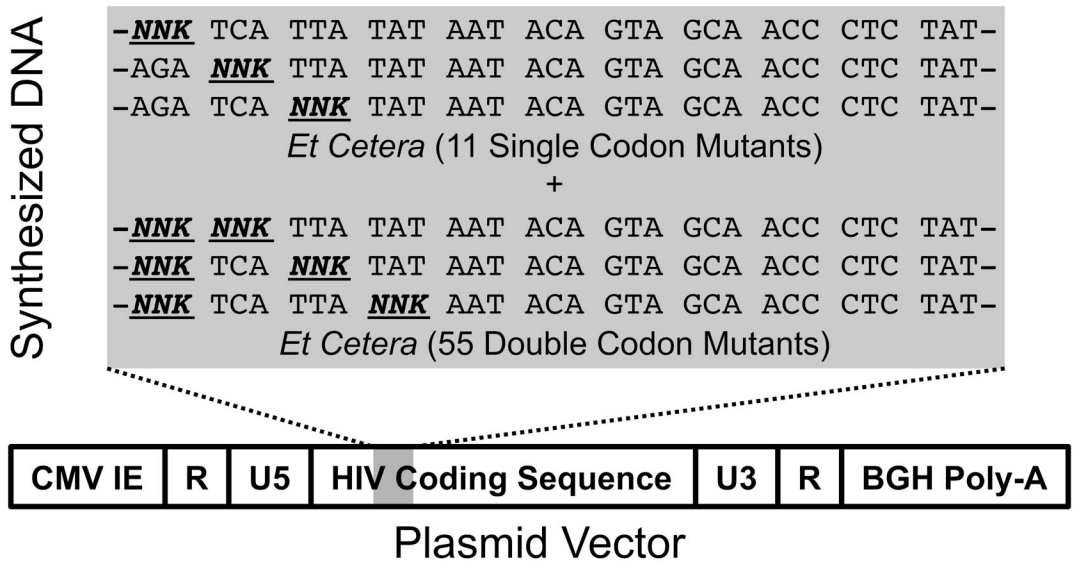
Virus suppression assays.

Evaluation of HIV-1 susceptibility to CTL suppression was performed as previously described(32, 44). Briefly, T1 cells(40) were infected with 500pg p24/10^6 cells of the indicated viruses, and $5x10^4$ infected cells with $5x10^4$ CTL (**Supplemental Fig. S2**) or $1.25x10^4$ CTL (**Fig. 7**) were cultured in 200µL R10-50 U/ml IL-2 in a 96 well flat-bottom plate, with monitoring of supernatant p24 antigen by ELISA (Xpress bio, Frederick, MD).

Epitope (Abbreviation)	Gag Amino Acids (HXB2 Numbering)	MHC-I Restriction	CTL Clone Designation	TRBV	CDR3	TRBJ
SLYNTVATL (SL9)	77-85	A*0201	S00001-SL9-**3.23T**	11-2	CASSLEHEQYF	2-7
			S00031-SL9-**10.11T**	12-3*01	CASSWEISDGYTF	1-2*01
			S00036-SL9-**1.9**	5-1*01	CASSFDSEQYF	2-7*01
KAFSPEVIPMF (KF11)	162-172	B*5701	S00014-KF11-**10.6**	15*02	CATSGTEYGYTF	1-2*01
			S00094-KF11-**3.4**	5-8*01	CASSVGFGANVLTF	2-6*01
KRWIILGLNK (KK10)	263-272	B*2705	S00076-KK10-**10.37**	27*01	CASREGQGALEQYF	2-7*01
			S00048-KK10-**TCR5**	7-9*03	CASSFDAGEQFF	2-1*01

Table 3-1: HIV-1 epitopes and CTL clones. The underlined portion of the CTL clone designation is the abbreviated name used throughout this work.

A



B

Epitope	Codon Mutants	Possible	In Plasmid Library	In Virus Library	Replicating
SL9	Single	209	209	77	36.8%
	Double	19,855	8,505	75	0.88%
KF11	Single	247	247	33	13.4%
	Double	28,158	10,754	18	0.17%
KK10	Single	228	228	145	63.6%
	Double	23,826	9,557	3	0.03%

Figure 3-1: Production of HIV-1 epitope mutant virus libraries. (A) Double-stranded DNA encoding an HIV-1 genomic region including an epitope of interest was synthesized to represent every possible single and double amino acid variant within the epitope and its immediately flanking amino acids, using degenerate codons (NNK, where N is any nucleotide and K is guanine or thymidine, which includes every amino acid while reducing stop codons). This DNA was then substituted into the full length HIV-1 NL4-3 genome to create a plasmid HIV-1 library for virus production. (B) The number of possible single and double amino acid variants within each library (excluding stop codons) is given, as well as the number of these variants detected

above threshold in the plasmid library ($\geq 2.5x10^{-5}$ for consideration in our analyses), and the number that carried forward above threshold ($\geq 10^{-4}$ in both biological replicates) in the virus libraries. If a variant was present above threshold in the plasmid library but not in the virus library, it was considered to be a deleterious mutation.

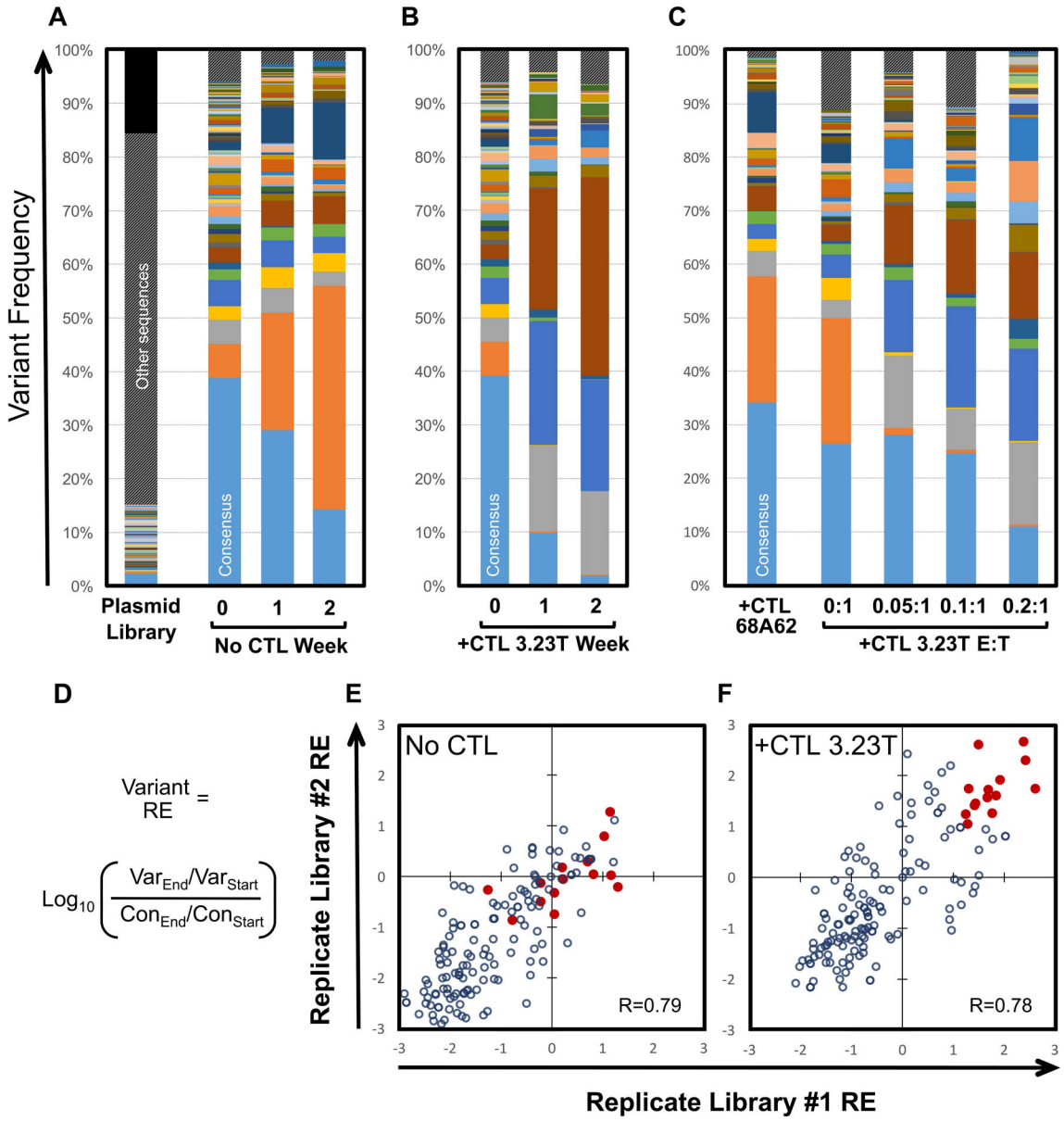


Figure 3-2: CTL selective pressure results in altered HIV-1 epitope variant frequencies.

The frequencies of each SL9 epitope variant are plotted for the plasmid library, initial virus library, and virus populations after one or two weeks of passage (in the absence of CTL selection) (A). All sequences below a frequency threshold of 2.5×10^{-5} in the plasmid library are represented in black. The frequencies of epitope variants are plotted again for the initial virus library, in comparison to their frequencies after one and two weeks of passaging in the presence of the SL9-specific CTL clone 3.23T (B). The frequencies are plotted after a week of passaging

in the presence of a control CTL clone 68A62 recognizing an A*02-restricted epitope in reverse transcriptase, or different effector to target (E:T) ratios of clone 3.23T (C). The subtype B consensus variant of the SL9 epitope is indicated by the bottom light blue bars in all bar graph panels, and color coding of each variant is consistent across panels; variants not achieving the frequency cutoff of $1x10^{-4}$ in both replicates of the starting virus library are labeled "other sequences" and indicated by hatched grey. Using the frequency data, the relative enrichment (RE) of each variant compared to the subtype B consensus epitope variant was calculated (D). The REs between two biological replicate experiments with the SL9 variant library passaged in the absence (E) or presence (F) of clone 3.23T (RE_{-CTL} values without CTLs and RE_{+CTL} values with CTLs respectively) are plotted. Red dots in both panels E and F indicate variants with high RE_{+CTL} values, demonstrating their locations in the RE_{-CTL} plot. All panels are representative of individual replicate experiments. All further determinations of RE_{-CTL} and RE_{+CTL} values were calculated as averages of quadruplicates (duplicate virus libraries each passaged in duplicate without CTLs) and duplicates (duplicate virus libraries each passaged singly with CTLs) respectively.

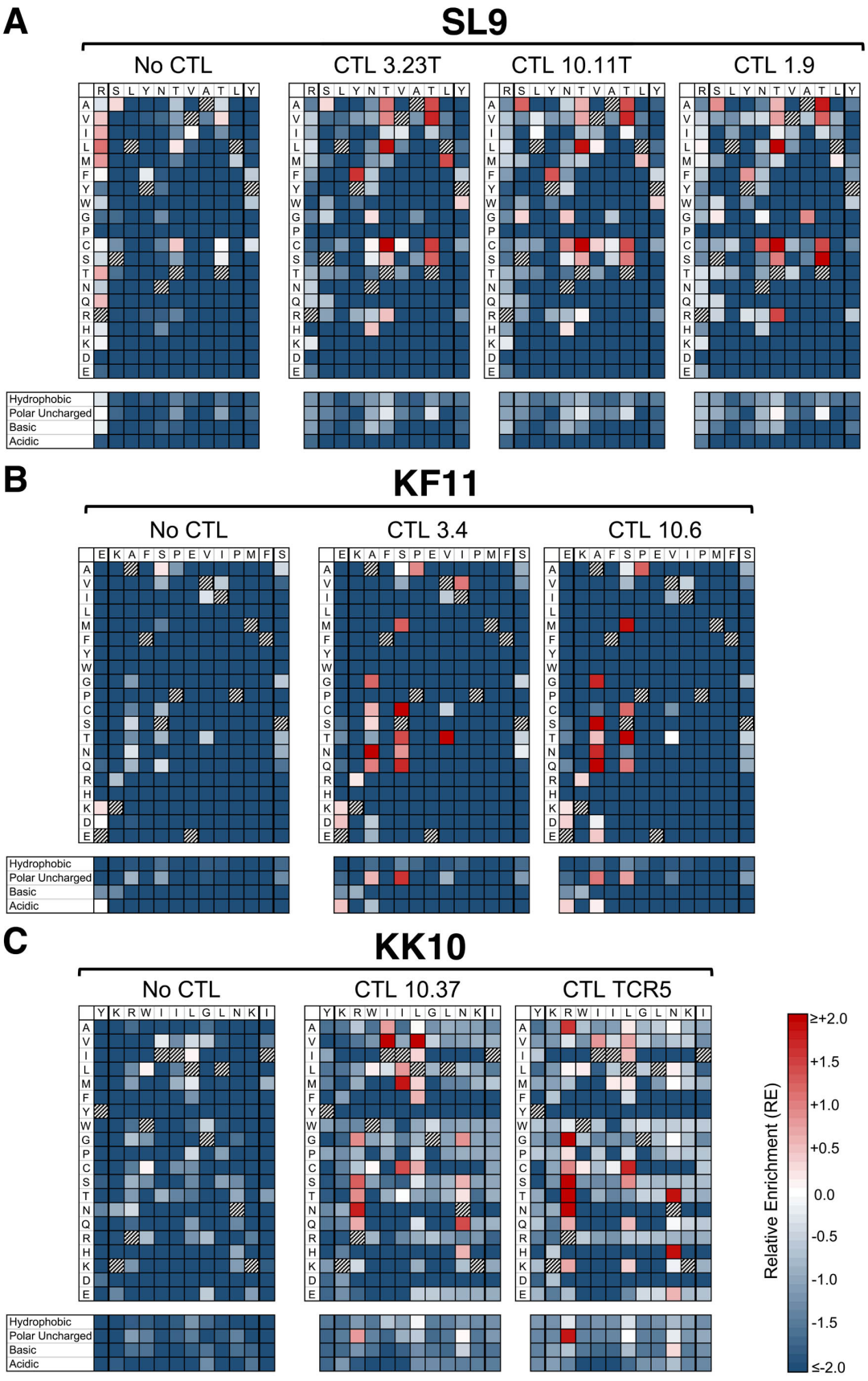

A
SL9
No CTL
CTL 3.23T
CTL 10.11T
CTL 1.9
B
KF11
No CTL
CTL 3.4
CTL 10.6
C
KK10
No CTL
CTL 10.37
CTL TCR5
Hydrophobic
Polar Uncharged
Basic
Acidic
Relative Enrichment (RE)
≥+2.0
+1.5
+1.0
+0.5
0.0
-0.5
-1.0
-1.5
≤-2.0

Figure 3-3: Effects of single amino acid variation within HIV-1 epitopes. The RE values of all single amino acid variants with or without addition of the indicated CTL clones are displayed as color-scaled boxes for epitopes SL9 (A), KF11 (B), and KK10 (C). The horizontal axis indicates each subtype B consensus amino acid of each epitope and its immediately flanking amino acids, and the vertical axis indicates substituting amino acids. Hatched boxes indicate consensus amino acids. Additionally, the mean REs for substitutions of amino acids that are hydrophobic (A, V, I, L, M, F, Y, W, G, and P), polar-uncharged (C, S, T, N, and Q), basic (R, H, K), or acidic (D and E) are indicated below each plot. Variants that were detected above threshold in the plasmid library but not in the virus library were considered non-replicating and assigned a RE_{-CTL} value of -2.0 for these analyses. RE_{-CTL} and RE_{+CTL} values were calculated as averages of quadruplicates (duplicate virus libraries each passaged in duplicate without CTLs) and duplicates (duplicate virus libraries each passaged singly with CTLs) respectively.

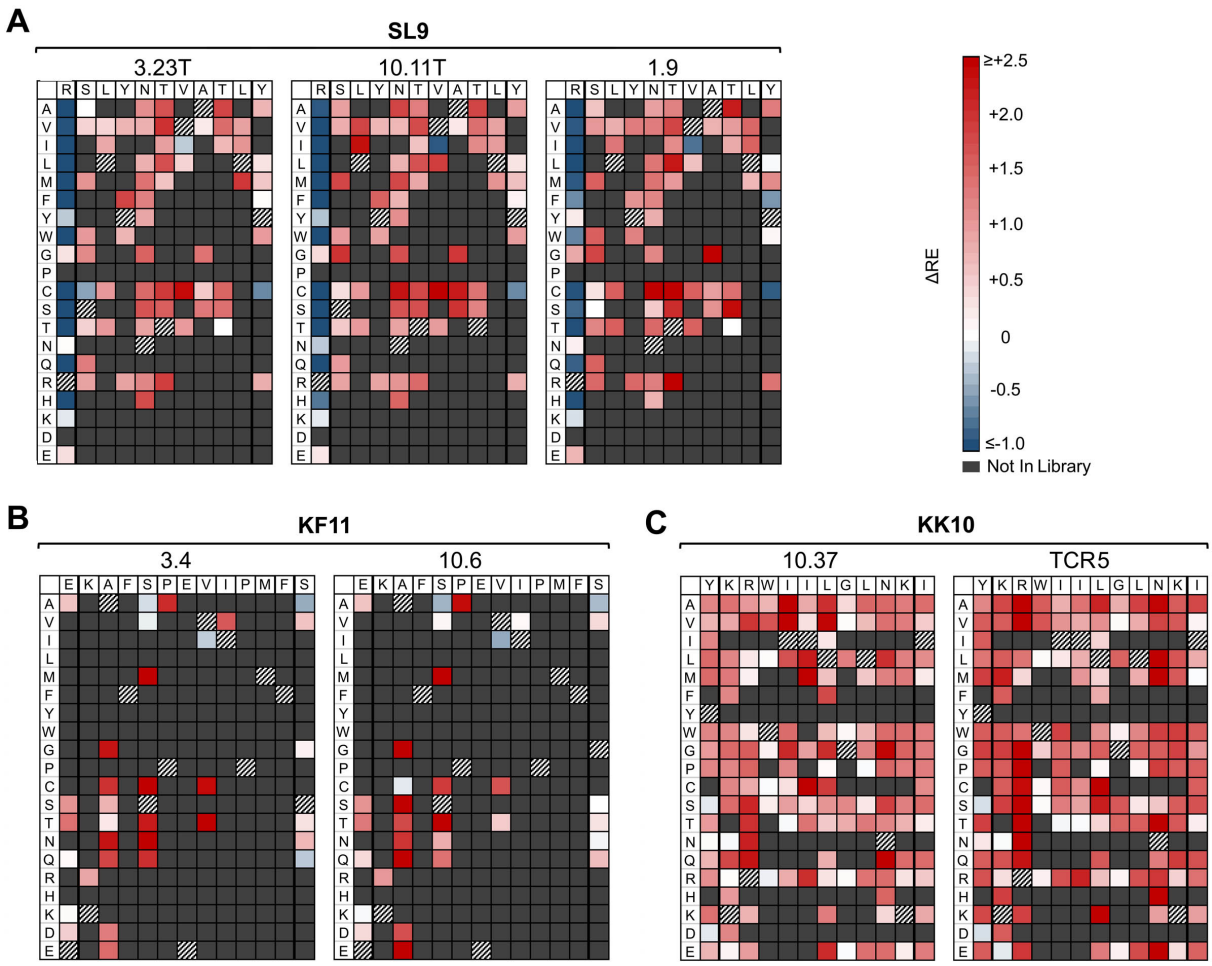


Figure 3-4: CTL selection of single amino acid variants. The changes in RE values due to CTL selection ($\Delta RE = RE_{+CTL} - RE_{-CTL}$) are displayed as color-scaled boxes for all single amino acid variants of epitopes SL9 (A), KF11 (B), and KK10 (C). The horizontal axis indicates each subtype B consensus amino acid of each epitope and its immediately flanking amino acids, and the vertical axis indicates substituting amino acids. Variants that were detected above threshold in the plasmid library but not in the virus library are considered non-replicating and coded by dark grey. Unchanged amino acids are coded by hatched boxes. RE_{-CTL} and RE_{+CTL} values were calculated as averages of quadruplicates (duplicate virus libraries each passaged in duplicate without CTLs) and duplicates (duplicate virus libraries each passaged singly with CTLs) respectively.

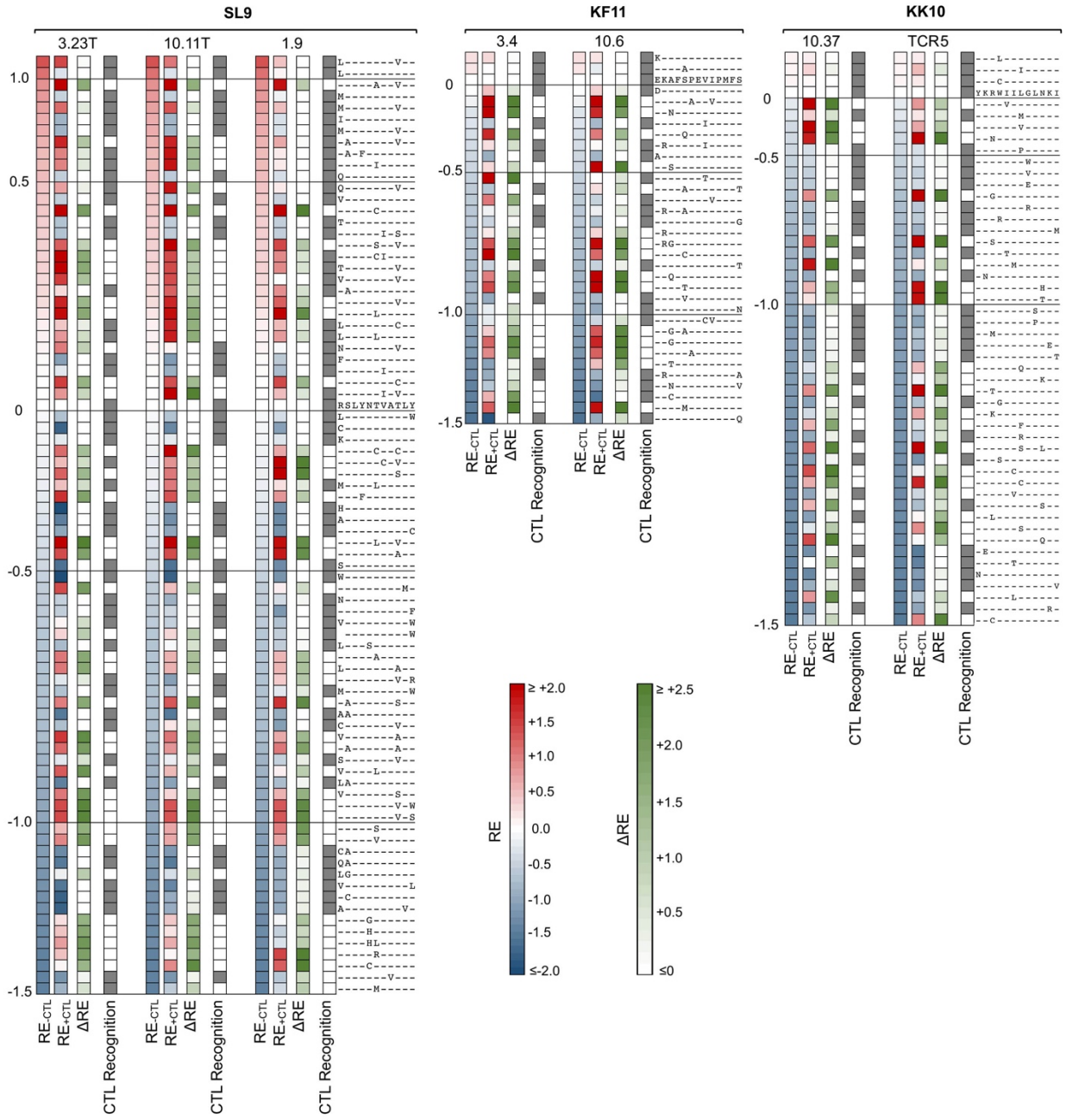


Figure 3-5: CTL selection of all library epitope variants. The RE values of all epitope variants with or without addition of the indicated CTL clones are displayed as color-scaled boxes for epitopes SL9 (A), KF11 (B), and KK10 (C). The horizontal axis indicates each subtype B consensus amino acid of each epitope and its immediately flanking amino acids, and the vertical axis indicates substituting amino acids. First columns indicate relative enrichment versus subtype

B consensus variant without CTLs (RE_{-CTL}), second columns indicate relative enrichment versus subtype B consensus variant with added CTLs (RE_{+CTL}), third columns indicate the difference between values in the presence versus absence of added CTLs (ΔRE), and fourth columns indicate variants with net CTL enrichment (ΔRE) ≥5-fold (white boxes) or <5-fold (gray boxes). Variants with RE_{-CTL} < -1.5 and stop codons are not shown. RE_{-CTL} and RE_{+CTL} values were calculated as averages of quadruplicates (duplicate virus libraries each passaged in duplicate without CTLs) and duplicates (duplicate virus libraries each passaged singly with CTLs) respectively.

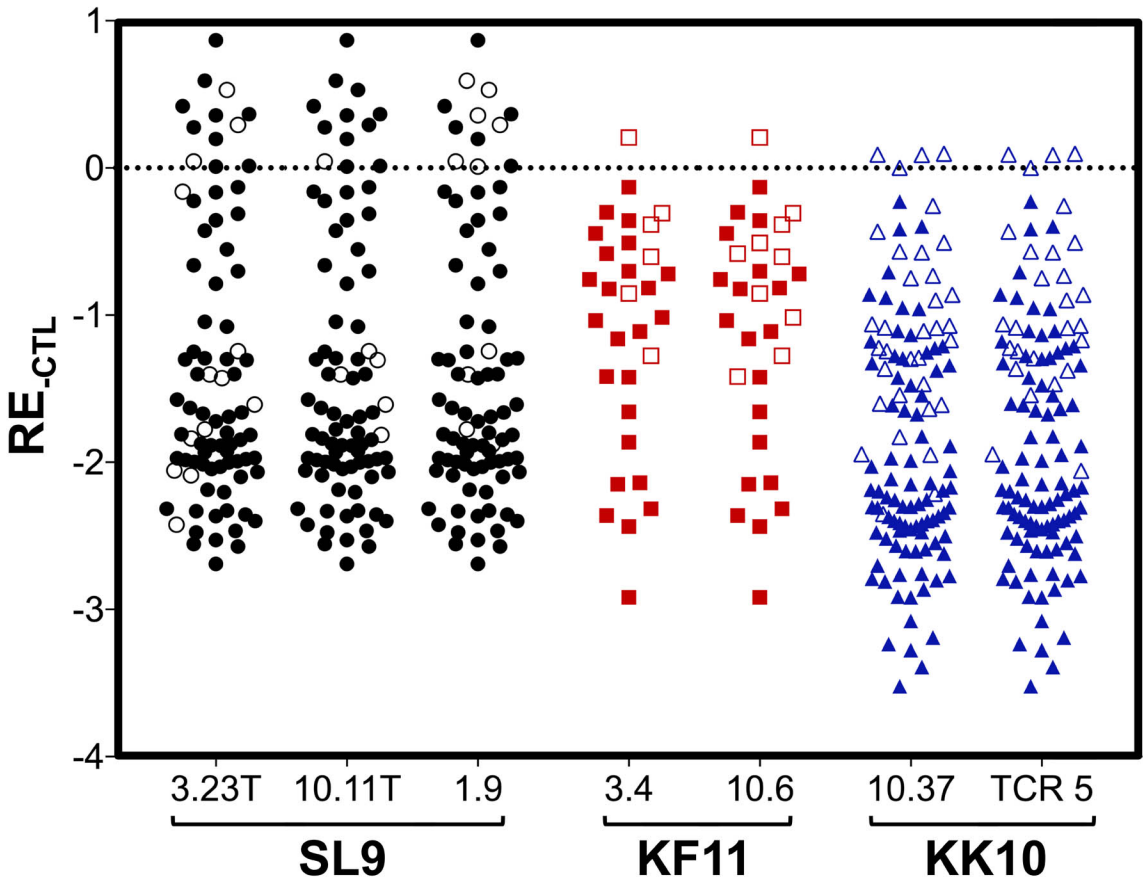


Figure 3-6: Comparison of RE values for SL9, KF11, and KK10 epitope variants that are enriched by CTLs. RE_{-CTL} values are plotted for all SL9, KF11, and KK10 epitope variants (excluding those with substitutions in flanking residues) in the libraries. Variants that were enriched at least 5-fold by added CTLs versus no CTLs ($\Delta RE > 0.7$) are indicated by filled symbols, and the remainder are open symbols. RE_{-CTL} and RE_{+CTL} values were calculated as averages of quadruplicates (duplicate virus libraries each passaged in duplicate without CTLs) and duplicates (duplicate virus libraries each passaged singly with CTLs) respectively.

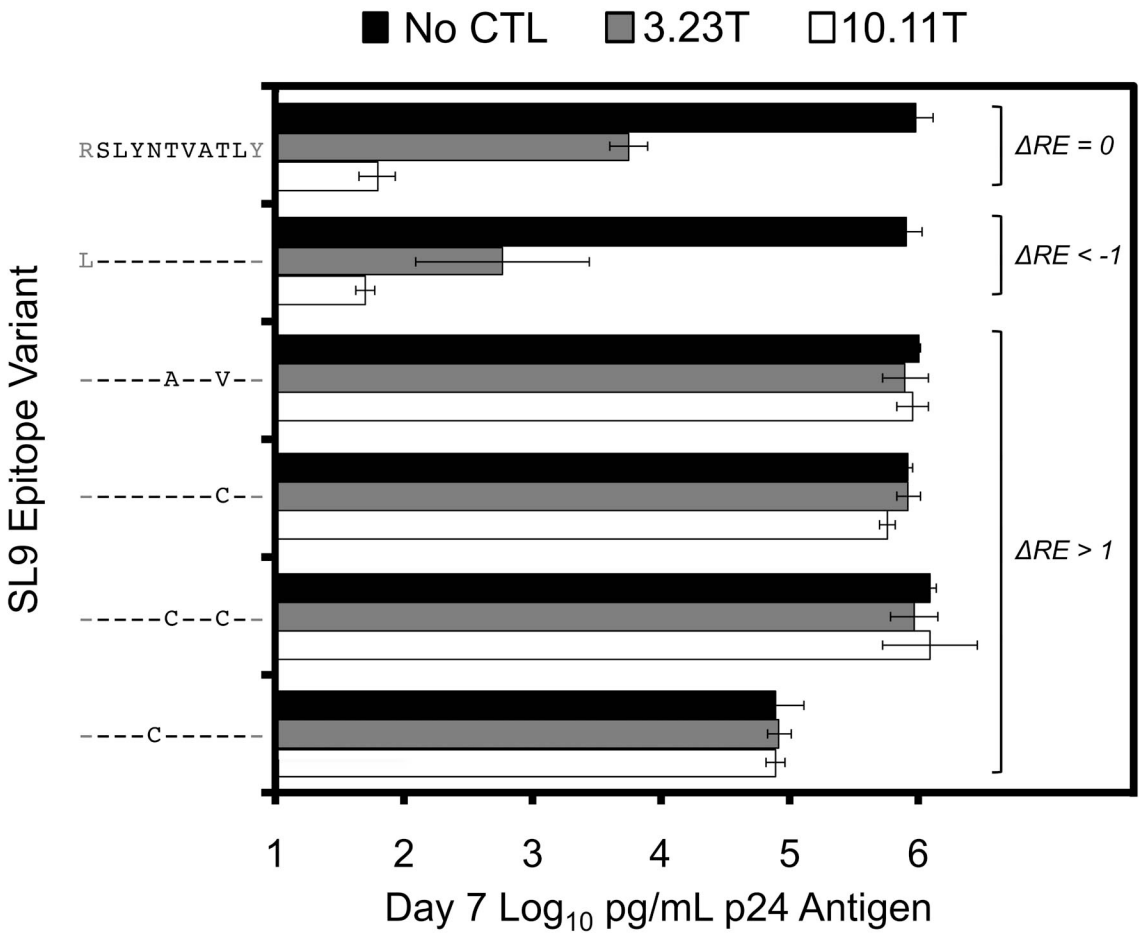


Figure 3-7: Confirmation that epitope variants enriched by passaging with SL9-specific CTLs represent potential escape options. Five SL9 epitope variants associated with increased (> +1) and one with decreased (< -1) ΔRE in the presence of SL9-specific CTLs were individually tested for effect on susceptibility for suppression by CTLs, as well as the subtype B consensus epitope. T1 cells were acutely infected with HIV-1 NL4-3 containing each variant, and cultured in the presence or absence of CTL clones 3.23T or 10.11T; supernatant p24 antigen values after 7 days are plotted for each indicated variant. Each value is the mean of triplicates, and error bars represent standard deviations.

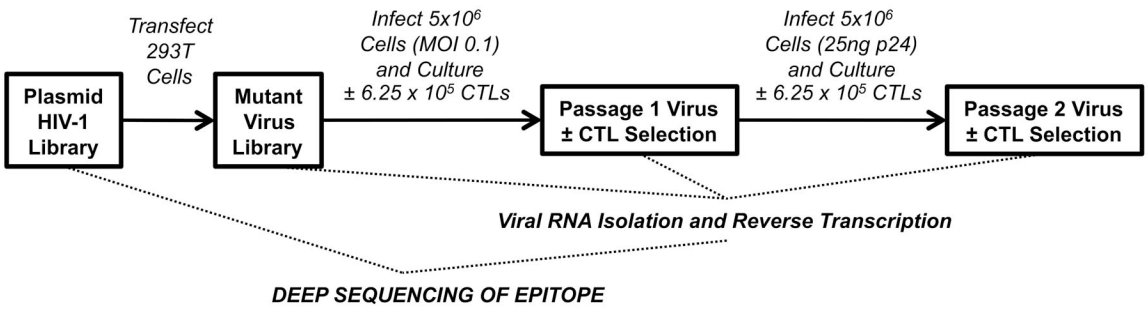


Figure 3-S1: Schematic describing the passaging of HIV-1 epitope mutant virus libraries under selective pressure from CTLs. Plasmid libraries created as described in Figure 1 were transfected into 293T cells to produce starting virus libraries, which were then passaged in the presence or absence of CTLs for two consecutive rounds of 7 days each. Deep sequencing of the epitope region was performed for the initial plasmid library and the virus libraries before and after selective passaging.

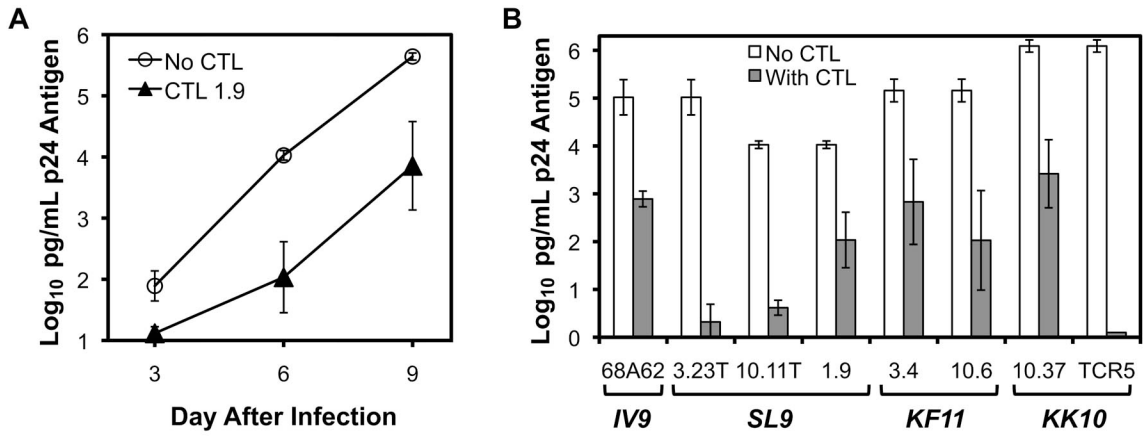


Figure 3-S2: CTLs utilized in this study suppress HIV-1 replication. T1 cells were infected with HIV-1 NL4-3.1 (with the subtype B consensus SL9 epitope sequence) and co-cultured with CTL clone 1.9, followed by monitoring of supernatant p24 antigen. Results are plotted for viral replication in the presence (closed triangles) or absence (open circles) of CTL co-culture (A). Results for day 6 or 7 are shown for all CTLs utilized in this study (B). Each value is the mean of triplicates, and error bars represent standard deviations.

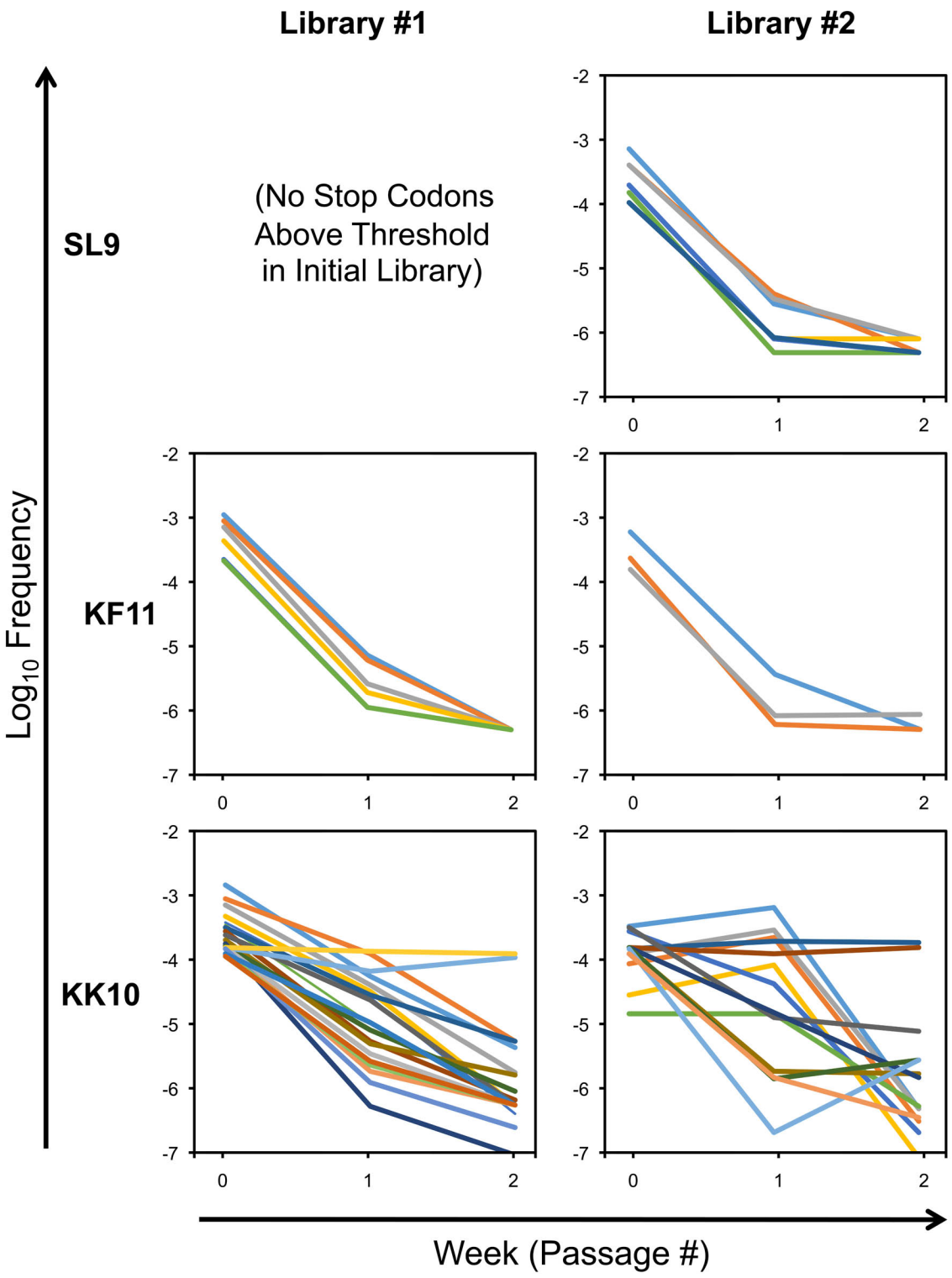


Figure 3-S3: HIV-1 epitope variants with stop codons generally decay during passaging of the epitope variant libraries. For each stop codon present above a threshold frequency of 10^{-4}

in the starting virus library, the frequency over time after passaging in the absence of CTLs is plotted. Each value represents the average of experimental duplicates for each library.

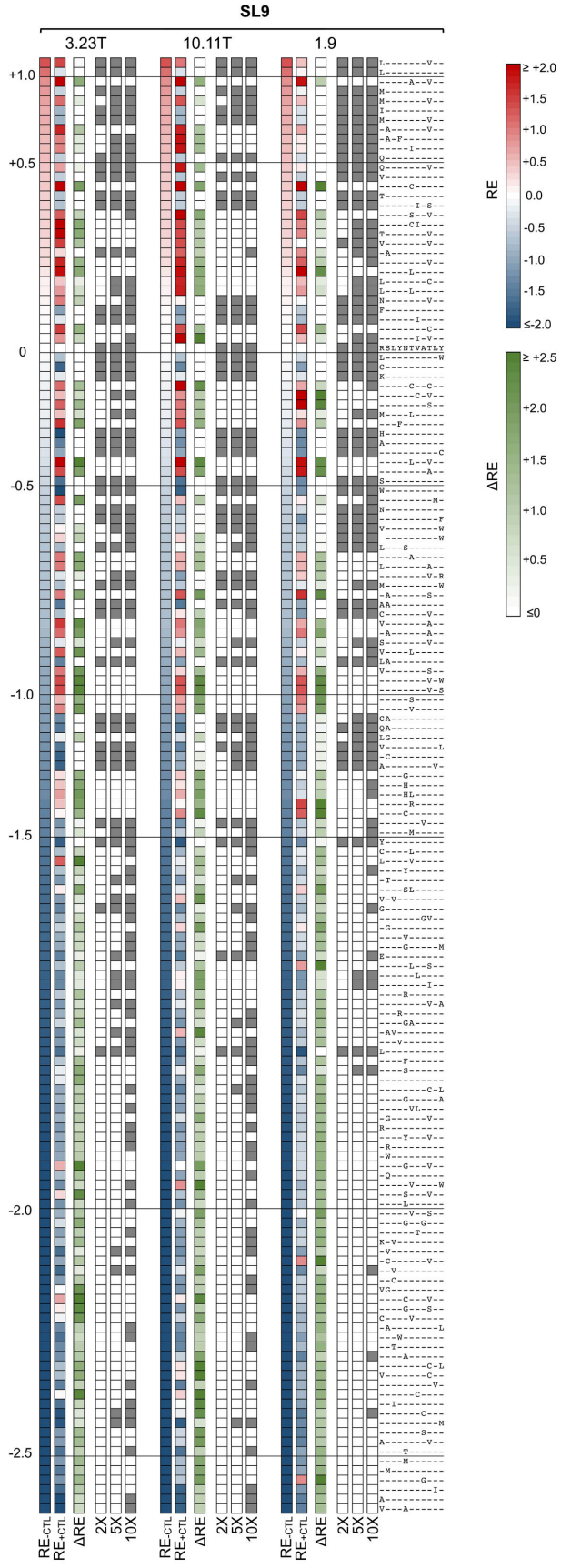

SL9
3.23T
10.11T
1.9
+1.0
+0.5
0
-0.5
-1.0
-1.5
-2.0
-2.5
RE
≥ +2.0
+1.5
+1.0
+0.5
0.0
-0.5
-1.0
-1.5
≤-2.0
ΔRE
≥ +2.5
+2.0
+1.5
+1.0
+0.5
≤0
RE-CTL
RE+CTL
ΔRE
2X
5X
10X

Figure 3-S4: Number of SL9 epitope variants attaining various thresholds for enrichment by CTLs. For each epitope variant (including immediately flanking residues) with initial library frequencies above 10^{-4} in both replicates, first columns indicate relative enrichment versus consensus without CTLs (RE_{-CTL}), second columns indicate relative enrichment with added CTLs (RE_{+CTL}), and third columns indicate the difference (ΔRE) as in Figure 4. Fourth, fifth, and sixth columns indicate variants achieving two-fold, five-fold, and ten-fold enrichment ($\Delta RE >$ 0.30, 0.70, or 1.0, respectively) by CTLs (open squares) or not (shaded squares). RE_{-CTL} and RE_{+CTL} values were calculated as averages of quadruplicates (duplicate virus libraries each passaged in duplicate without CTLs) and duplicates (duplicate virus libraries each passaged singly with CTLs) respectively.

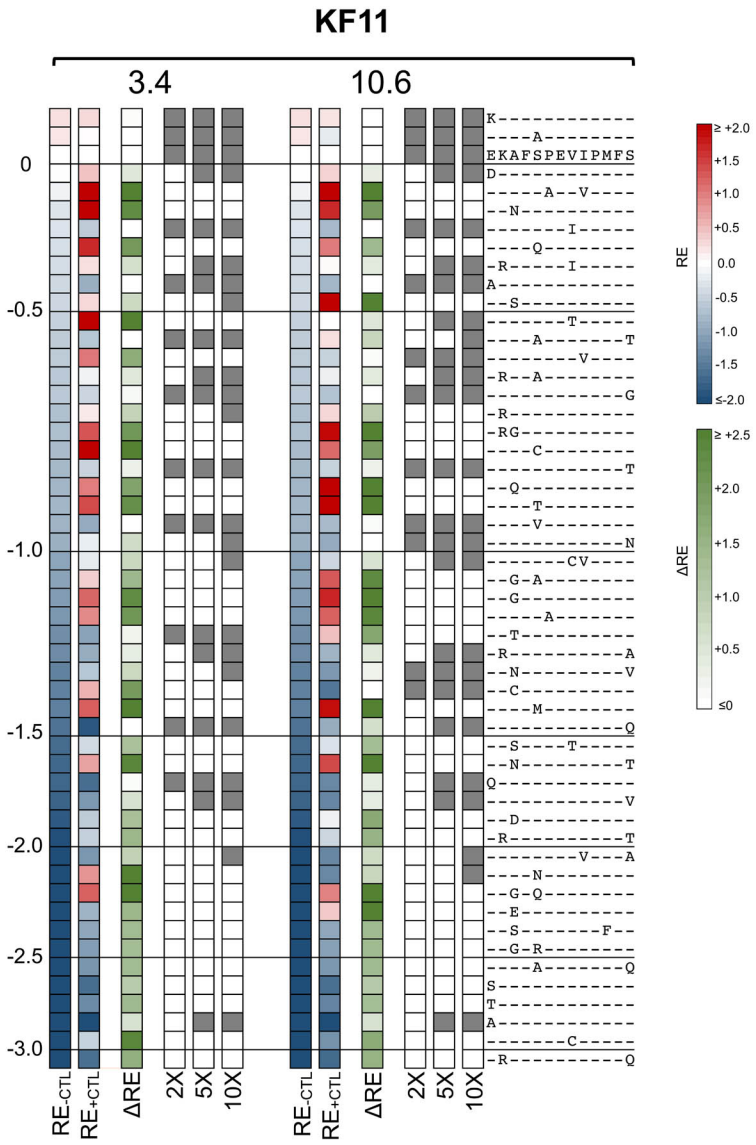


Figure 3-S5: Number of KF11 epitope variants attaining various thresholds for enrichment by CTLs. For each epitope variant (including immediately flanking residues) with initial library frequencies above 10^{-4} in both replicates, first columns indicate relative enrichment versus consensus without CTLs (RE_{-CTL}), second columns indicate relative enrichment with added CTLs (RE_{+CTL}), and third columns indicate the difference (ΔRE) as in Figure 4. Fourth, fifth, and sixth columns indicate variants achieving two-fold, five-fold, and ten-fold enrichment (ΔRE > 0.30, 0.70, or 1.0, respectively) by CTLs (open squares) or not (shaded squares). RE_{-CTL} and

RE_{+CTL} values were calculated as averages of quadruplicates (duplicate virus libraries each passaged in duplicate without CTLs) and duplicates (duplicate virus libraries each passaged singly with CTLs) respectively.

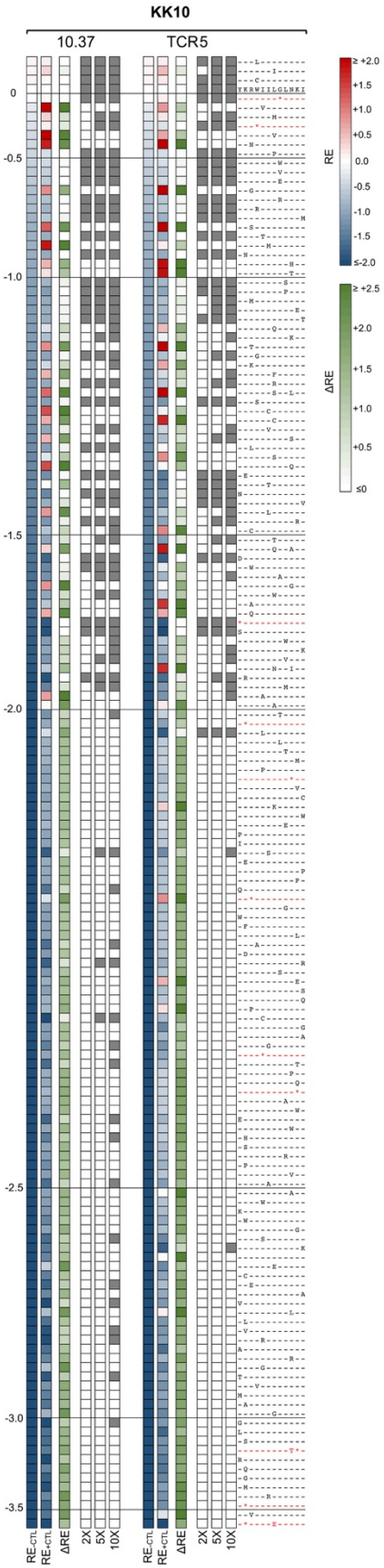

KK10
10.37
TCR5
RE
ΔRE
0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
2X
5X
10X

Figure 3-S6: Number of KK10 epitope variants attaining various thresholds for enrichment by CTLs. For each epitope variant (including immediately flanking residues) with initial library frequencies above 10^{-4} in both replicates, first columns indicate relative enrichment versus consensus without CTLs (RE_{-CTL}), second columns indicate relative enrichment with added CTLs (RE_{+CTL}), and third columns indicate the difference (ΔRE) as in Figure 4. Fourth, fifth, and sixth columns indicate variants achieving two-fold, five-fold, and ten-fold enrichment ($\Delta RE >$ 0.30, 0.70, or 1.0, respectively) by CTLs (open squares) or not (shaded squares). RE_{-CTL} and RE_{+CTL} values were calculated as averages of quadruplicates (duplicate virus libraries each passaged in duplicate without CTLs) and duplicates (duplicate virus libraries each passaged singly with CTLs) respectively.

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Chapter 4: Decreased Cross-Recognition of Escape Variants by a CTL Clone Generated in Response to the Mrk/Ad5 (STEP Trial) Vaccine

Introduction

The HVTN502/STEP trial was a clinical trial assessing the efficacy of the recombinant Mrk/Ad5 HIV vaccine. This vaccine was similar to adenovirus based vaccines that successfully induced a CTL response in nonhuman primate and lead to a decreased viral load at set point following HIV infection (1, 2). However, despite inducing a CTL response in 77% of vaccinated individuals (3), the STEP trial ultimately failed, with no difference being observed between the placebo and control groups in infection rate or viral set point (4, 5).

Several theories have been proposed for the failure of this vaccine. First, it has been suggested that individuals previously infected with adenovirus may have mounted a strong response to the ad5 vector rather than HIV. This is consistent with the observation that vaccine may actually have increased risk of HIV infection in Ad5 seropositive individuals (4). However, the Ad5 response still does not explain why the vaccine was not effective in Ad5 seronegative individuals. To address this issue, some groups have pointed out that although the vaccine did elicit a CTL response, responding CTL typically recognized only one or two HIV epitopes and that such a narrow response may not have been sufficient to recognize infected variants, or to effectively suppress virus replication (6).

Others have proposed that the vaccine-induced CTL response was incapable of recognizing common variants of targeted of targeted epitopes, thus leading to rapid escape from immune recognition (3, 7, 8). Previous work has demonstrated that the ability of a CTL to cross-recognize variants of an epitope depends at least partially on exposing the immune system to these epitope variants (9, 10). Since the Mrk/Ad5 vaccine contained only a single consensus strain of HIV, the immune response may have been inadequately primed to cross-recognize frequent escape variants of the virus. This idea is supported by the finding that the vaccine did

appear induce a CTL response capable to driving viral evolution, but that the response lead to rapid escape and evolution at targeted T-cell epitopes (11).

In this project, we set out to determine whether CTL clones generated in response to the MRK/Ad5 vaccine lacked the ability to cross recognize frequent escape variants. We assessed the ability of an HIV-specific CTL clone isolated from a vaccinated individual to suppress HIV replication and cross recognize all single and double amino acid variants of the targeted epitope. We then compare the cross-recognition capability of this clone with two clones isolated from individuals with chronic HIV infection to demonstrate that the vaccine-induced CTL fails to recognize some of the most fit escape variants within the targeted epitope.

Results

An HIV-specific clone derived from Mrd/Ad5 vaccinated subject suppresses HIV replication

The HVTN7 1.2 CTL clone specific for the HLA B*57 KRWIILGLNK (KK10, Gag 263-272) epitope was isolated from an HLA B*57 positive individual who received the Mrk/Ad5 vaccine as part of the STEP/HVTN502 trial. The TCR sequence of the clone is provided in **Table 3-1**.

We assessed the ability of this clone to suppress HIV replication. HLA B*57-expressing HIV-permissive cells were infected with NL4-3 virus and cultured in the presence or absence of the HVTN7 1.2 CTL. In the presence of the CTL, no p24 antigen could be detected by ELISA even after 7 days of growth, whereas when cultured without CTL over 10^6 pg/mL of p24 antigen could be detected (**Figure 1)**.

The HVTN7 1.2 clone targets the KK10 (Gag263-272) epitope

Next we sought to confirm that the clone was indeed recognizing the HLA B*57-restricted KK10 epitope. We utilized a library of HIV-1 containing every possible single and double amino acid variant within the KK10 epitope and immediately flanking residues as described in **Chapters 2 & 3**. HIV-permissive HLA B*5701-positive cells were infected with this KK10 library and cultured for two rounds of 7-days in the presence and absence of the HVTN7 1.2 clone. After each round the supernatant virus was collected and the region of the HIV genome encoding the KK10 epitope was deep sequenced.

Deep sequencing analysis showed that the consensus KK10 sequence composed over 63.4% of all virus sequences in the starting virus library. When the virus library was passaged in the absence of the CTL clone, the consensus virus expanded with each passage, so that after the

second passage it made up over 83% of the entire library (**Figure 2A**). However, in the presence of the HVTN7 1.2 clone, the frequency of consensus plummeted; the frequency of consensus dropped to 7.9% after one passage, and to 2.9% after the second passage under CTL pressure (**Figure 2B**). Thus, this HVTN7 1.2 clone could specifically suppress the growth of the virus possessing the consensus KK10 epitope.

Single substitution epitope variants demonstrate patterns of enrichment in the absence or presence of CTL selection

The effect of single amino acid substitutions (compared to consensus) was assessed for the KK10 epitope in the absence and presence of the HVTN7 1.2 clone. As described in **Chapter 3**, the outcome for each epitope variant was quantified as a relative enrichment value (RE), calculated as the $\log_{10}$ transformed ratio of change in frequency over two weeks in culture (see **Figure 3-2D**). We observed previously that very few amino acid substitutions in the KK10 region were associated with RE values ≥0 in the absence of CTL (defined as RE_{-CTL}) (**Figure 4-3A**, same as **Figure 3-3C**). In the presence of the HVTN7 1.2 CTL several substitutions yielded greatly increased RE values (RE_{+CTL}) (**Figure 4-3B**), indicating this CTL was driving the enrichment of certain variants.

We examined the net effect of CTL selection (change in RE_{+CTL} and RE_{-CTL}, defined as ΔRE) on single substitution mutants persisting in the starting libraries. We observed that most single amino acid mutants lead to an increased enrichment under CTL pressure (**Figure 4-3C**). We then compared these data to those obtained from passaging the KK10 library under the pressure of CTL clones isolated from chronically HIV-infected individuals (described in **Chapter 3**). While the overall pattern of enrichment was similar for the clones obtained from

vaccinated and infected subjects, slight differences were could be detected. Notably, hydrophobic variants of the leucine at position 6 within KK10 performed better under pressure of the HVTN clone than those from infected individuals. In particular, the ΔRE for the KRWIIMGLNK (L6M) variant was 0.24 and 0.44 for the two clones isolated from infected individuals and 2.33 for the HVTN7 1.2 clone and for the KRWIIIGLNK (L6I) variant the ΔRE was 0.61 and 0.45 for clones isolated from infected individuals while it was 1.99 for the HVTN7 1.2 clone.

Identification of variants capable of evading the HVTN7 1.2 CTL clone

To further examine the possibility that variant cross-recognition of the vaccine induced HVTN7 1.2 clone differed from those isolated from infected individuals, we examined enrichment of all single and double amino acid variants in the KK10 library. Epitope variant RE_{-CTL} (**Figure 4-4A**) and RE_{+CTL} in the presence of the HVTN7 1.2 clone (**Figure 4-4B**) were evaluated, and the ΔRE was calculated (**Figure 4-4C**). We identified variants that increased in growth rate at least 2, 5, and 10-fold (ΔRE > 0.3, 0.7, or 1.0, respectively) under CTL pressure (**Figure 4-4D**) to understand how the enrichment of each variant changed under CTL pressure.

We examined all variants that were enriched at least 5-fold under CTL pressure and compared these to the results obtained with CTL clones from chronically infected individuals (data can be seen in **Figure 3-S6**). Although the behavior of the variants under pressure from these different CTLs was overall similar, as noted above, there was a difference in recognition of the L6M and L6I variants (**Figure 4-5)**. These two variants were highly enriched by pressure the HVTN7 1.2 clone, but not the clones from chronically infected individuals. Notably, these two variants had some of the highest RE_{-CTL} values of all KK10 variants.

Discussion

We have isolated a CTL clone capable of suppressing HIV replication that targets the HIV Gag KK10 epitope from an HIV seronegative individual vaccinated with the Mrk/Ad5 vaccine. We found that this CTL can cross-recognize several KK10 variants and that general pattern of cross-recognition is similar to that seen for CTL clones targeting the same epitope isolated from chronically HIV-infected individuals with the exception of the L6I and L6M variant. The L6I and L6M variants were highly enriched under pressure of the HVTN7 1.2 CTL, while they were well suppressed by CTLs targeting KK10 isolated from two different chronically infected HLA B*57-positive individuals. These two variants had some of the highest RE_{-CTL} values, and therefore likely represented some of the most fit variants of KK10. Thus, the CTL clone generated in response to the Mrk/Ad5 vaccine failed to recognize hydrophobic variants at position 6 of KK10 providing high fitness escape routes for the virus that are typically blocked off in individuals with chronic infection.

Previous work has shown that in chronically infected individuals, KK10-specific CTLs do not cross-recognize the L6M variant until after this variant arises in the virus population (9, 10), suggesting that the immune system needs to be exposed to the variant virus to stimulate the expansion of CTL clones capable to recognizing this sequence. Our results indicate that a vaccine consisting of only the consensus sequence may produce a similar response wherein the responding CTL can effectively target the consensus sequence, but cannot cross-recognize frequent escape variants. Whereas CTLs in chronically infected individuals are exposed to a swarm of viruses, the individual vaccinate with the Mrk/Ad5 vaccine was only exposed to the consensus KK10 sequence and not the L6M or L6I variant. This likely lead to the expansion of CTL clones capable of recognizing only the consensus sequence and not the variants.

The results of this study are highly limited since we were only able to examine one CTL clone against a single epitope from a single vaccinated individual. However, these results do warrant a larger study to examine the ability of vaccine-elicited CTLs to cross-recognize frequent variants and to compare this to the "maximal" possible cross-recognition of CTLs arising naturally in chronically-infected individuals.

Currently, several approaches are being examined to develop a vaccine capable of eliciting CTL responses that can limit virus escape. First, several groups have developed vaccines consist of only highly conserved regions of the virus with the hope that the virus will be inherently limited in options for escape if targeted at these regions (12-15). A second approach, called mosaic (or polyvalent) vaccines, directly addresses the gaps in cross-recognition that we have detected in CTL responding to vaccines containing only a consensus virus sequence. Such mosaic vaccines consist of multiple variants of a virus corresponding to the most frequent circulating variants and have been successfully developed for influenza (16) and hepatitis B (17). These HIV vaccines have shown promising results by generating a CTL response capable of recognizing a broad array of HIV epitope variants(18-20) and are currently being adapted for clinical trials. Our methods of identifying all recognized variants within an epitope can be expanded to evaluate CTLs isolated from subjects vaccinated with these new vaccines to understand how effective these new strategies are for preventing viral escape.

Methods

All methods and analysis were performed identical to those described in **Chapter 3** which the exception of the following:

HIV-permissive cell line

All virus library growth and virus suppression assays were performed in EBV-immortalized B-cells from subject 76 (HLA B*27 positive) that were transduced with CD4.

Isolation and Culture of HVTN7 1.2 CTL clone

The HVTN7 1.2 CTL clones was obtained from a vaccinated subject in the HVTN502/STEP trial by cloning of PBMC at limiting dilution. The specificity of the clone was determined as previously described(21). The CTL clone was maintained by periodic stimulation with 200ng/mL of 12F6 anti-CD3 antibody and irradiated allogeneic peripheral blood mononuclear cells in R10 media supplemented with 50IU interleukin-2/mL (NIH AIDS Reference and Reagent Repository).

TRAV	TRAJ	CDR3	TRBV	TRBJ	TRBD	CDR3
21*01	53*01	CAVTPLYSGGSNYKLTF	20-1*02	2-3*01	2*01	CSAKVPPWGLARDTQYF

Table 4-1: T-cell receptor sequence of the HVTN7 1.2 CTL clone.

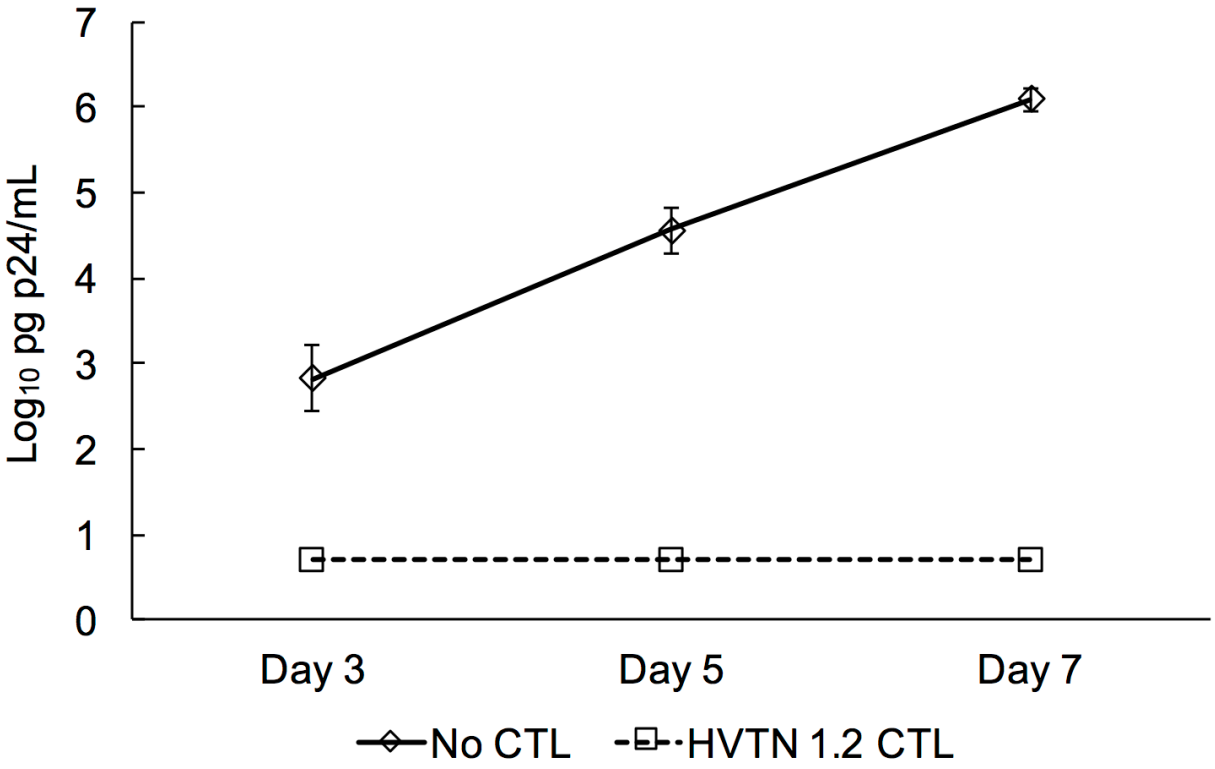

Figure 4-1: The HVTN 7 1.2 CTL clone suppresses HIV-1 replication. HLA B*57 positive, HIV-permissive cells were infected with NL4-3 virus and cultured in the presence and absence of the HVTN7 1.2 CTL at an effector:target ratio of 1:1. Virus production was monitored by assessed by quantifying supernatant HIV-1 p24 protein levels.

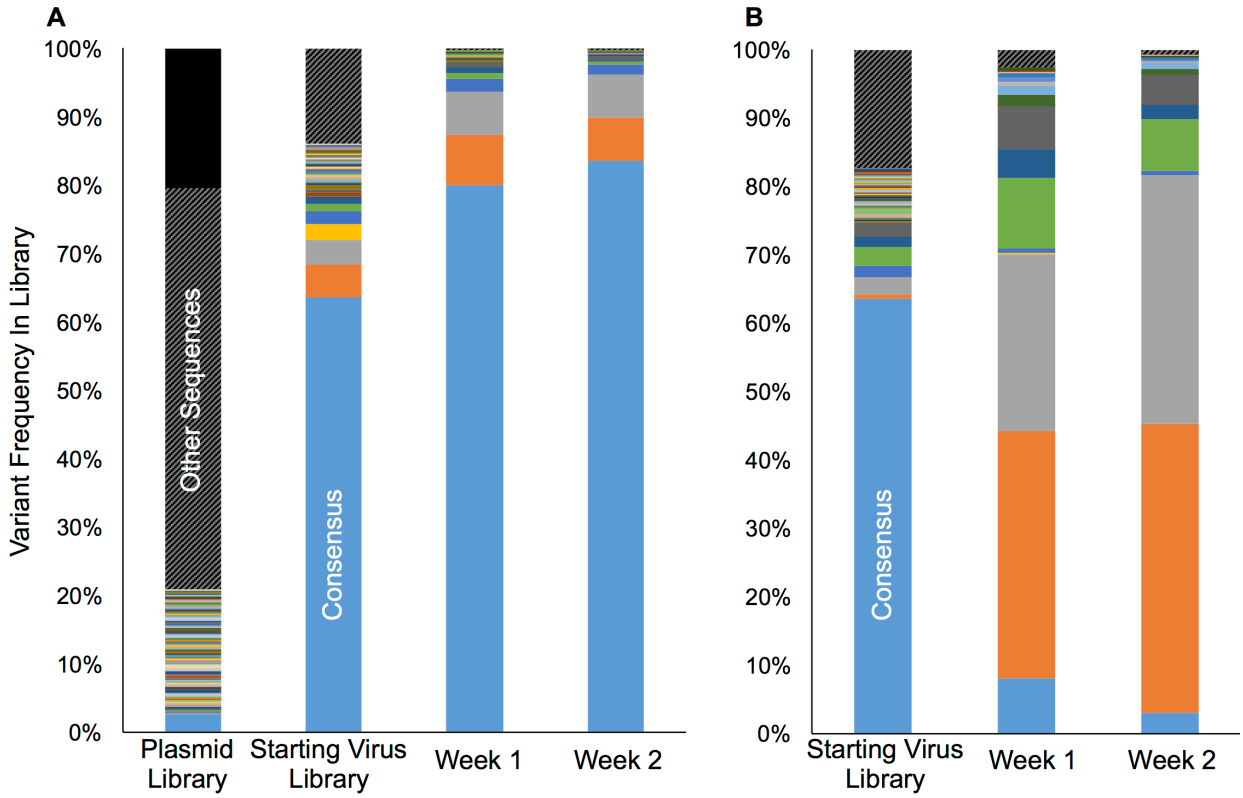

Figure 4-2: The HVTN7 1.2 CTL clone recognizes the KK10 epitope and selects for escape variants. The frequencies of each KK10 epitope variant are plotted for the plasmid library, initial virus library, and virus populations after one or two weeks of passage in the absence of CTL selection (A) and in the presence of the HVTN7 1.2 CTL clone (B). Sequences below the plasmid library frequency threshold of 2.5×10^{-5} are represented in black in bar graphs. Variants other than those above the frequency cutoff of 1×10^{-4} in both replicates of the starting virus library are labeled "other sequences" and indicated by hatched grey in bar graphs.

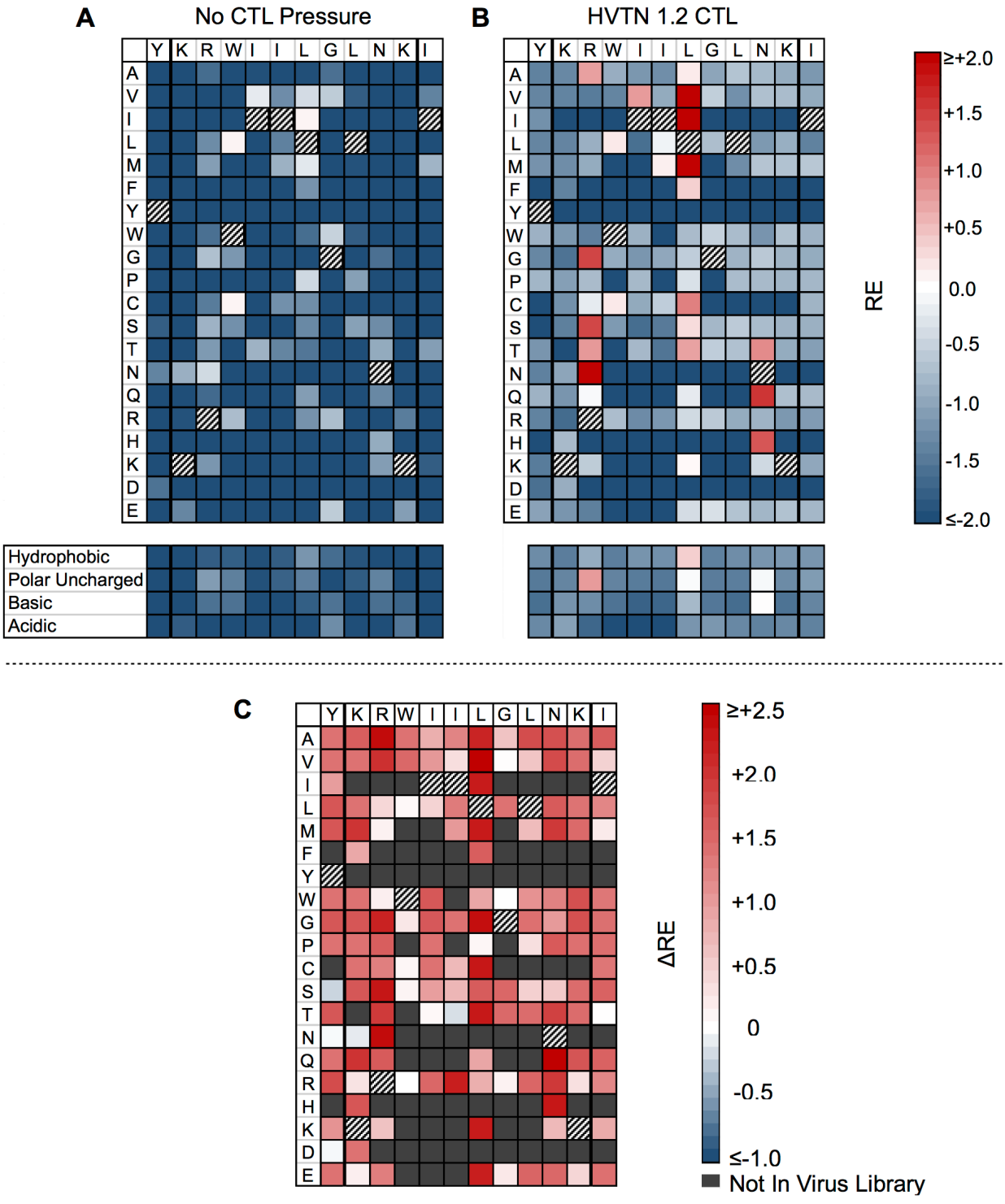

Figure 4-3: HVTN7 1.2 CTL recognition of single amino acid KK10 variants. The RE values of all single amino acid variants in the absence (A) or presence (B) or the HVTN7 1.2 CTL are shown for KK10. The top horizontal axis indicates the consensus sequence of each epitope and

the immediately flanking amino acids. Each box indicates the RE for the substituting amino acid indicated on the vertical axis. The average REs for substitutions of amino acids that are hydrophobic (A, V, I L, M, F, Y, W, G, and P), polar-uncharged (C, S, T, N, and Q), basic (R, H, and K), or acidic (D and E) are shown. The RE of each variant is indicated by the color of each box with red denoting more fit and blue less fit. Variants that were detected above threshold in the plasmid library but not in the virus library were considered unfit and assigned an RE of -2. The effect of HVTN7 1.2 CTL pressure on the RE of all single amino acid variants is shown in (C). Each box indicates the change between RE_{-CTL} and RE_{+CTL} (ΔRE) for the substituting amino acid indicated on the vertical axis. The ΔRE is indicated by the color of each box with green denoting an increase in RE under CTL pressure and red a decrease. Variants represented in the plasmid library but not present in the starting virus libraries above a frequency of 10^{-4} are denoted in dark grey.

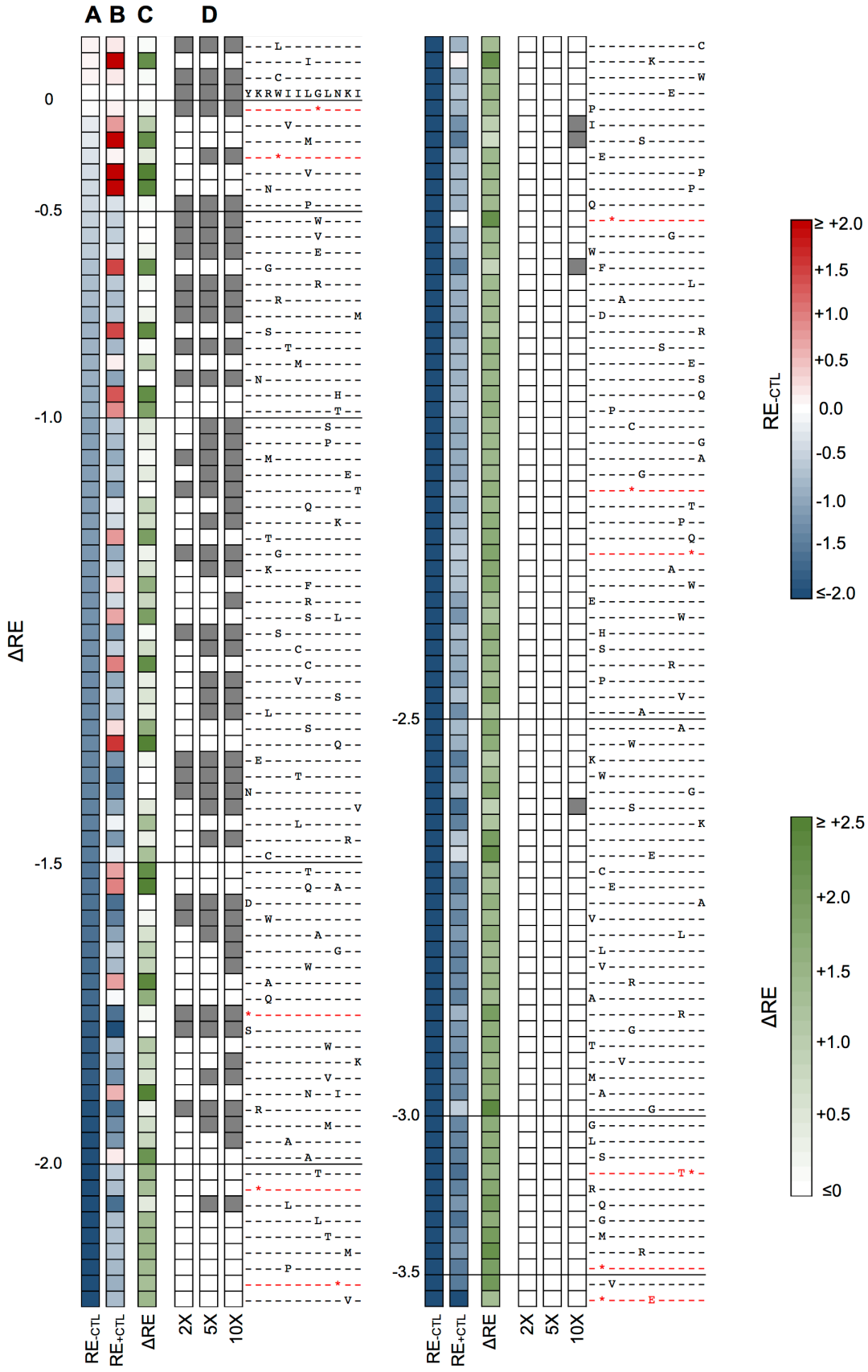
A
B
C
D
0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
ΔRE
RE-CTL
RE+CTL
ΔRE
2X
5X
10X
≥ +2.0
+1.5
+1.0
+0.5
0.0
-0.5
-1.0
-1.5
≤-2.0
≥ +2.5
+2.0
+1.5
+1.0
+0.5
≤0

Figure 4-4: The effect of HVTN7 1.2 CTL pressure on the relative enrichment of all KK10 variants. For epitope variants of KK10 with initial library frequencies above 10^{-4} in two replicates, RE_{-CTL} (A), RE_{+CTL} values for the HVTN7 1.2 CTL (B), the ΔRE for the HVTN7 1.2 CTL (C) are represented. The three columns in (D) indicate variants achieving two-fold, five-fold, and ten-fold enrichment (ΔRE < 0.30, 0.70, and 1.0, respectively) by CTLs (open squares) or not (shaded squares).

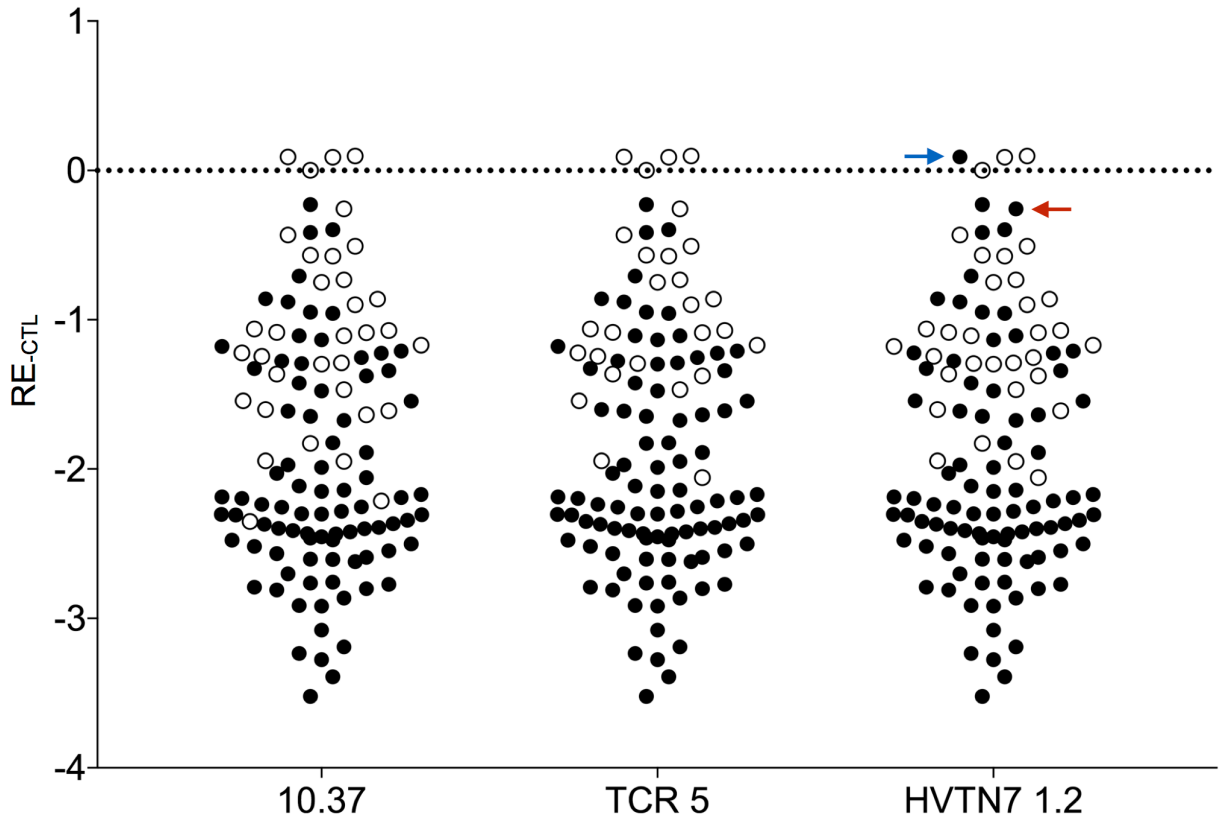

Figure 4-5: Comparison of RE-CTL values for KK10 epitope variants that are enriched by CTLs from infected and vaccinated individuals. The RE_{-CTL} values are plotted for all KK10 epitope variants (excluding mutations in flanking residues) with a frequency of at least 10^{-4} in the starting virus library. Variants that were enriched at least 5-fold ($\Delta RE > -0.7$) with added CTLs versus no CTLs are indicated by filled symbols, and the remainder are open symbols. The 10.37 and TCR5 CTLs were isolated from subjects with chronic HIV infection (as described in **Chapter 3**), the HVTN7 1.2 CTL came from an individual who received the MRK/Ad5 vaccine as part of the STEP trial. The L6I variant is indicated by a blue arrow, the L6M variant is indicated by a red arrow.

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Chapter 5: Systemic Characterization of HIV-1 Nef Residues Necessary for Mediating Evasion from CD8+ Cytotoxic T-Lymphocytes

Introduction

HIV-1 Nef is a 27-kDa myristoylated accessory HIV protein that despite being disposable for virus replication plays an essential role in maintaining HIV virulence *in vivo.* Nef promotes virulence through many distinct pathways, the best defined of which are its effect on lymphocyte activation to promote virus replication (1-4), its ability to increase the infectivity of budding virus particles (5-7), to downregulate CD4 on the surface of infected cells (8-10), and to downregulate expression of MHC-I on the surface of infected cells (11, 12). Clinically the importance of nef has demonstrated in cohorts infected with nef-defective HIV. Such individuals suppress viremia to low or undetectable for prolonged periods of times (13, 14). Additionally, nonhuman primates challenged with nef-deficient SIV do not progress to AIDS and develop immunity to more pathogenic strains of SIV, demonstrating that nef is necessary for complete virulence and evasion from the immune response (15).

HIV viremia during chronic infection is largely controlled by HIV-specific CD8+ Cytotoxic T-Lymphocytes (CTL) that recognize and kill infected cells (16-19). CTLs recognize infected cells via interaction of their T-Cell Receptor (TCR) interaction with a viral epitope presented on the MHC-I molecules on the surface of virus-infected cells. By downregulating expression of the MHC-I complex on the surface of infected cells, Nef diminishes the ability of CTLs to suppress viral replication (20, 21), and likely contributes to viral persistence and immune evasion. Individuals infected with nef-deficient virus mount a strong CTL response against the virus, suggesting that the absence of nef may result in an enhanced CTL response which yields improved clinical outcomes (22-24).

Several Nef residues and domains critical to MHC-downregulation and CTL escape have been identified. Nef downregulates the MHC-I by rerouting the MHC to lysosomes and

preventing recycling of the complex to the cell surface. This process is accomplished at least in part by nef binding to the MHC and the clathrin adaptor protein complex I (AP-1) (25, 26). The nef $EEEE_{65}$ acidic cluster is necessary for direct binding to AP-1, while the proline rich PxxP repeats (residues 68-78) mediate MHC-I binding (27). Additionally, the methionine at position 20 in the N-terminal alpha-helix is necessary for positioning nef close to the lipid membrane, and mutations at this positions abrogate Nef's ability to downregulate the MHC (28, 29).

Outside of these motifs, little is known about residues or motifs necessary nef's ability to evade the CTL response. Additionally, nef is a highly variable gene, with little conservation across most of its sequence across patient samples, with some virus isolates containing nef with decreased ability to downregulate the MHC (30). This raises the question of how conserved regions associated with MHC downregulation are, and whether there is population-wide evolutionary drive to maintain a nef protein capable to downregulating the MHC.

In this project, we culture an HIV saturation mutagenesis library of nef in the presence of HIV-1 specific CTL to identify regions of the protein that are necessary for evading the CTL response. We demonstrate that while some of the identified regions map to already known motifs necessary for MHC-I and AP-1 interaction, several of these lay outside of previously characterized regions necessary for MHC-downregulation. Finally, we utilize our results to examine the population-wide conservation of residues necessary for CTL-evasion.

Results

HIV-1 Nef decreases the ability of CTL to inhibit HIV replication

We first confirmed the ability of nef to diminish the effect of HIV-specific CTL on virus replication in our system. NL4-3 virus bearing either the wildtype NL4-3 nef or nef with an alanine substitution at positon 20 (M20A), which has disrupts nef's ability to downregulate the MHC-I (28, 29), was used to acutely infect 1CC4.14 cells. The cells were cultured in the presence or absence of the S14-KF11-10.6 CTL clone specific for the HLA B*5701-restricted KF11 epitope (Gag 162-172, KAFSPEVIPMF). In agreement with previously reported results, the CTL clone suppressed the replication of both viruses, however virus bearing the M20A nef was greatly increased in its sensitivity to suppression (**Figure 5-1**). At day 7 post-infection the CTL clone suppressed M20A virus production by 3.9 $\log_{10}$ units of pg p24/mL, while NL4-3 virus production was suppressed by only 2.5 $\log_{10}$ units.

Nef library screen identifies residues necessary for facilitating evasion from CTL recognition

Having established that nef mutations that decrease the ability of the protein to downregulate the MHC-I increase CTL-mediated suppression of virus growth in an *in vitro* system, we utilized this approach to identify positions within nef that are required for CTL evasion. A library of HIV-1 possessing point mutations in residues 1-88 in nef was cultured in the presence of an HIV-specific CTL to determine the effect of each of these mutations on the ability of nef to facilitate evasion from the HIV-specific CTLs.

1CC4.14 cells were infected with the nef virus library and cultured for 7-days in the presence and absence of the S14-KF11-10.6 CTL clone. After 7 days, the virus was collected, reverse transcribed, and the region encoding nef was deep sequenced. To understand the impact

of every mutation on virus growth, we calculated the relative fitness (RF) of each variant defined as the $\log_{10}$ transformed value of ratio of the frequency of a variant at the end of the passage to its frequency at the start. In the absence of CTL pressure, silent, missense, and most nonsense mutations had minimal effect on the RF of the variant (**Figure 5-2A**). However, in the presence of CTL pressure, nonsense mutations resulted in decreased RF, indicating increased suppression of these variants by the CTL **(Figure 5-2B**). Several missense mutants also decreased RF, thus indicating that these mutations were found in regions critical for nef-mediated evasion of CTL recognition (**Figure 5-2B-D**). Two replicates of this experiment demonstrated that these results were reproducible as indicated by a high correlation between biological replicates (**Figure 5-2E-F**).

Identification of well-established and novel residues critical for Nef function

To determine the effect of each mutation in the library on evasion from CTLs, we calculated the "relative CTL resistance score" for each variant, which represented the difference between the RF for each variant with and without CTL pressure. As expected, several mutations at M20 lead to decreased CTL resistance scores (**Figure 5-3A**). Mutations in the $EEEE_{65}$ acidic cluster, which is critical for nef binding to AP-1, and the proline rich PxxP repeats (residues 68-78), which mediates MHC-I binding, also had a large effect on nef resistance (**Figure 5-3B-C**). However, we observed that the mutations with the consistently strongest effects on CTL resistance were either in residues that have not been reported as necessary for nef-mediated MHC-downregulation or CTL evasion or at the G2 position which is necessary for myristoylation of the protein (**Figure 5-3D**).

The average resistance of all variants at a given residue was calculated and plotted across the nef sequence (**Figure 5-4A**). Additionally, to identify the maximal effect of mutating a given residue, the value for the variant yielding the lowest CTL resistance score at each position was plotted (**Figure 5-4B**). To examine a potential functional significance of each mutant, the calculated CTL resistance scores were aligned to the previously determined nef structure (**Figure 5-4C-D**). As expected, mutations in the MHC-I and AP-1 binding motifs of nef had large effects on CTL resistance. However, clusters of mutations in other regions of the protein, not previously characterized as functionally important for MHC-downregulation also showed greatly decreased CTL resistance scores.

Nef residues required for CTL evasion are highly conserved *in vivo*

The CTL response to HIV is largely responsible for controlling viremia and has been shown to be one of the main driving factors of HIV evolution in an infected individual. Given Nef's ability to dampen the effect of HIV-specific CTLS, we wanted to examine whether there was evolutionary pressure on the virus to maintain nef sequences capable of driving escape from CTL recognition. The CTL resistance values for all of the mutated residues in the nef library were compared to the Shannon entropy of these residues reported in Clade B sequences in the Los Alamos HIV Sequence Database (**Figure 5-5**). There was a positive correlation between the average CTL resistance score of a residue and its conservation in the Los Alamos database ($R=0.46$, $p=9.47\times10^{-6}$), with residues critical for CTL evasion (those with CTL resistance values below -0.5) highly conserved in the Los Alamos database.

Discussion

The HIV-1 nef protein is a highly variable protein that is not required for virus viability, and has little effect on virus growth rates *in vitro*. However, *in vivo*, nef is required for enhancing virus infectivity and increasing immune evasion. In this study, we utilized a library of viruses containing nucleotide mutations throughout the first 88 codons of nef to identify amino acid residues critical for nef's ability to facilitate CTL evasion.

Nef's contribution to immune evasion from the CTL response is largely attributed to its ability to downregulate expression of the peptide-MHC-I complex on the surface of infected cells. The PxxP (31, 32) and $EEEE_{65}$ motifs (33) in nef were previously reported to allow direct binding of nef to AP-1 and MHC-I, respectively (27). Therefore, mutations within these regions likely disrupt the ability of nef to sequester MHC-I and redirect the protein to the lysosome. Our successfully validated that mutations within these regions as well as at M20 increase CTL recognition of infected cells. Additionally, we report the identification of new sites within the protein that play a role in facilitating viral escape from CTLs. Studies to confirm the importance of these sites are currently underway.

Given the ability of the CTLs to control viremia in infected individuals, it makes evolutionary sense that the virus has evolved a mechanism to dampen this immune response. However, nef is a very poorly conserved gene, with sequences varying greatly in different individuals. Previously, Münch, *et al* found an evolutionary pressure to maintain functional nef during acute SIV infection (34) while Ali *et al*. demonstrated that *in vitro* there is evolutionary pressure to preserve the nef ORF in the presence of HIV-specific CTLs (29). Further along these lines, Lewis *et al.* demonstrated that the ability of nef to downregulate the MHC-I is directly correlated to the breadth of the CTL against HIV within an individual, suggesting that the

stronger the CTL response against the virus, the more important it is for the virus to maintain functional nef (30). Still this previous study found that several virus isolates lacked the ability to downregulate the MHC-I, suggesting that there may only be an evolutionary drive to preserve nef functionality only in individuals with strong CTL responses. In this study, we observed that residues necessary for mediating evasion from the CTL response are highly conserved in the Los Alamos Database of HIV Sequences. These data suggest that although nef is highly tolerant of mutations in most of its genome, there is an evolutionary drive at the population level to preserve the parts of the protein necessary for mediating immune escape.

Out study is limited by two major constraints. First, we were only able to analyze the first 88 amino acid residues within nef. Our approach of utilizing saturation mutagenesis across the gene allowed for rapid identification of positions necessary for nef function by deep sequencing the nef gene. However, approximately the second half of the nef ORF overlaps with the 3' LTR in HIV. Since the LTRs are copied from the 3' end to the 5' end of the genome during the process of reverse transcription, mutations in the latter portion of the nef sequence would also be copied into the 5' LTR, thus making it impossible for us to distinguish whether the effects of these mutations were a result of alteration of nef activity, or rather the alteration in the function of the LTR. Additionally, despite successfully mutating every codon in nef, our library did not encode every possible amino acid mutation at every position, thus limiting our analysis to less than all 20 possible amino acid variants at each position. However, despite these limitations, we have performed, what is to our knowledge, the most comprehensive screen for identifying residues within nef that are necessary for evading CTLs.

Overall these results validate previously identified motifs necessary for nef-mediated CTL escape, and identify new regions of the protein that are necessary for maintaining this

function. Further studies will be needed to investigate whether these newly identified residues are necessary for nef binding to the MHC, whether they act through another novel mechanism, or whether they simply disrupt the folding or stability of the nef protein.

Methods

Library construction

We used error-prone PCR strategy to introduce single nucleotide mutations onto HIV-1 genome. Nef was divided into two sub-libraries to control mutation rate. The pNL4-3 plasmid was digested by NheI and NcoI to generate the PCR template. Then we used two sets of primers (Table S1) and Mutazyme II DNA polymerase (Stratagene) to do error-prone PCR and got two fragments F2 and F3. The mutation rate of ~1 mutation per fragment was obtained by adjusting template amount and cycle number of PCR. We used KOD hot start polymerase (Novagen) to generate a fragment F1, which was upstream of F2. Fragments F1 and F2 were digested by restriction enzyme BsmI (NEB) and ligated together by T4 ligase (NEB) to get fragment F12. Then we PCR amplified F12 by KOD hot start polymerase (Novagen) and digested it by restriction enzyme NheI and XhoI (NEB). And fragment F3 was digested using XhoI and NcoI. The pNL43 vector was digested by the same enzymes, respectively. We used T4 ligase (NEB) to ligate the mutagenized fragments and the corresponding vector. The ligation products were electroporated into MegaX DH10B electro-competent cells (Thermo) and plate to LB agar plates. More than 10,000 colonies were collected for each sub-library to ensure the sufficient coverage of all possible single nucleotide mutations. We used Hipure midi-prep kit to extract DNA (Thermo).

HIV-specific CTL clone

CTL clones were obtained from HIV-infected subjects by cloning of PBMC at limiting dilution. Clones were cultured in RPMI supplemented with 50IU/mL IL-2 (NIH AIDS reagent program). Clones were intermittently stimulated with anti-CD3 antibody as previously described

(35).

Virus Suppression Assay

ICC4.14 cells (36) were acutely infected with 500pg p24/10^6 cells of NL4-3 virus bearing either the NL4-3 or M20A mutant nef gene. $5x10^4$ cells were cultured in 200µL of RPMI supplemented with 50IU IL-2 (NIH AIDS reagents program) of a 96-well flat bottomed plate. Cell were cultured in the absence or presence of $1.25x10^4$ cells of the S14-KF11-10.6 CTL clone. Every 2-3 days 100µL of supernatant was removed, preserved at -20C, and replaced with fresh media. P24 production was determined by ELISA of collected supernatant against a standard dilution (Xpress Bio).

Virus recovery and Titer

Nef virus libraries were created by transfecting the nef plasmid libraries into ~70% confluent T75 flasks of 293T cells utilizing the BioT Reagent (BioLand). 48 hours after transfection, the supernatant was collected, filtered through a 0.45 micron filter, and stored at -80C. Virus titers were approximated by infecting ICC4.14 cells with dilutions of virus, culturing for 72 hours, and intracellularly staining (Flow Cytometry Fixation and Flow Cytometry permeabilization/was buffer from R&D Systems) for expression of the HIV p24 antigen (KC57-FITC antibody from Beckman Coulter). The titer was calculated based on the percentage of FITC+ cells as determined by flow cytometry on a BD FACScan machine.

Virus screen in presence and absence of CTLs

10^7 ICC4.14 cells were acutely infected with nef virus libraries. The infected cells were

cultured in 20mL RPMI supplemented with 50IU IL-2/mL in T75 flasks for 7 days. Cells were cultured either in the presence or absence of $2.5x10^6$ cells of the S14-KF11-10.6 CTL clone. Cells were fed every 3 days by replacing half of the supernatant with fresh media. After 7 days, the supernatant was collected, filtered through a 0.45 micron filter, and stored at -80C.

Virus RNA extraction and next generation sequencing

Virus supernatant was treated with DNAse I (NEB). Virus RNA was extracted from viral supernatant using viral RNA mini kit (Qiagen) and then reverse transcribed into cDNA using high-capacity reverse transcription kit (Thermo). The Nef region was amplified from two sub-libraries respectively using KOD hot start polymerase (Novagen). Every sub-library was separated into small amplicons, each spanned ~70bp of mutated regions. Table S2 listed the amplicon primers. The amplicons from the same sample were pooled together and purified. We used T4 PNK (NEB) to phosphorylate the products and used home-made Taq polymerase to append a dATP at 3' end of the amplicons. A Y-shape sequencing adaptor with T-sticky end was ligated to the product using T4 ligase (NEB). A 6-nt barcode is in the end of the adaptor to distinguish different samples. Table S3 listed the barcode sequences. The ligated products were amplified using the Illumina sequencing specific flow cell primer pair (5-FC: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG, 3-FC: CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCC). The PCR product was purified and deep sequencing was performed with the Illumina Hiseq PE150 sequencing platform.

Data Processing

Sequences were mapped to HIV-1 genome using primer region as a bait. Mutations were called if both reads of pair-end sequencing were different from NL4-3 sequence. The relative fitness of a mutation was defined by the $\log_{10}$ ratio of mutation frequency in output viral population and that in plasmids. Every amplicon had a coverage of ~1 million reads. Mutations were discarded if their frequency is lower than 10^{-5} in plasmid library. The essentialness of a residue is defined as the average of all observed mutations' fitness. The resistance score of a mutation is calculated by subtracting relative fitness with and without the CTL.

The structure of Nef was modeled using SWISS-MODEL (37). The reference for modeling is HIV-1 Nef in complex with MHC-1 cytoplasmic domain and Mu1 adaptin (PDB id: 4EN2) (27).

The Shannon entropy was calculated by Entropy-One tool on Los Alamos Database. All type B HIV-1 sequences with complete Nef gene were aligned to calculate Shannon entropy.

All custom scripts were deposited at https://github.com/Tian-hao/Nef.

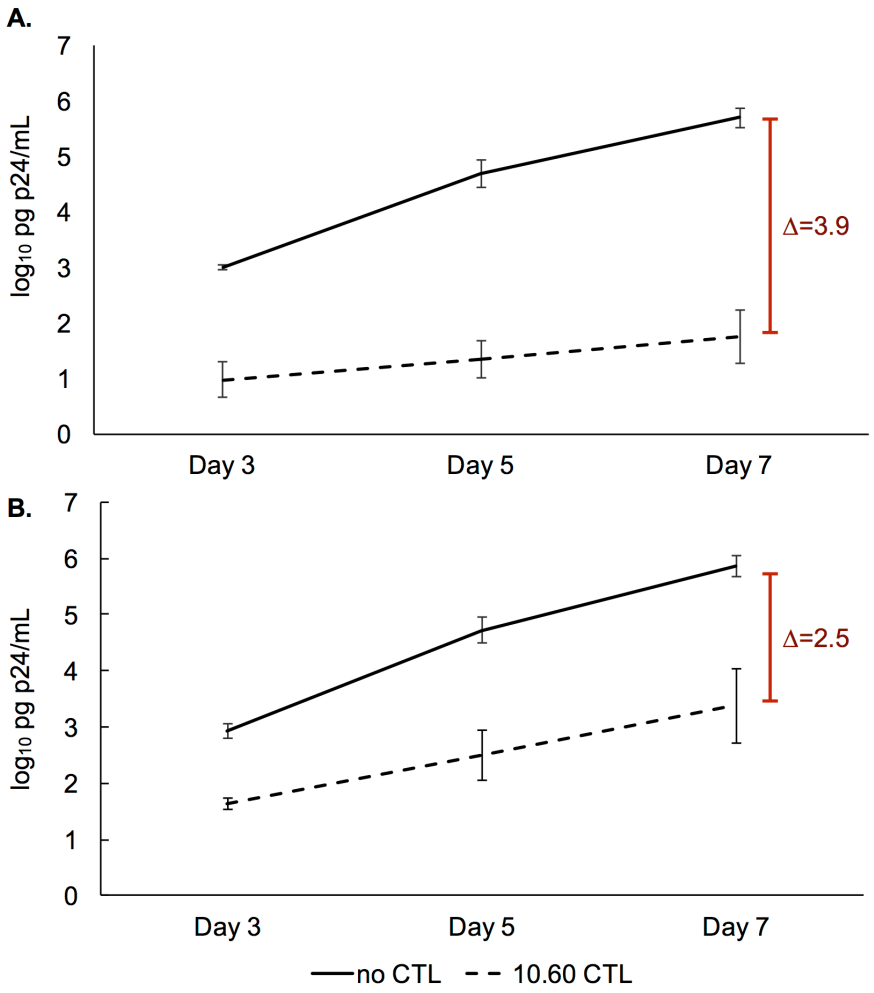

Figure 5-1: The M20A mutation in nef increases CTL-mediated suppression of HIV replication. ICC4.14 cells were infected with HIV and cultured in the presence of the S14-KF11-10.6 CTL clone, followed by monitoring of supernatant p24 antigen. Results for infection with NL4-3 bearing the M20A mutation in nef (A) and bearing the "wildtype" NL4-3 nef (B) are shown.

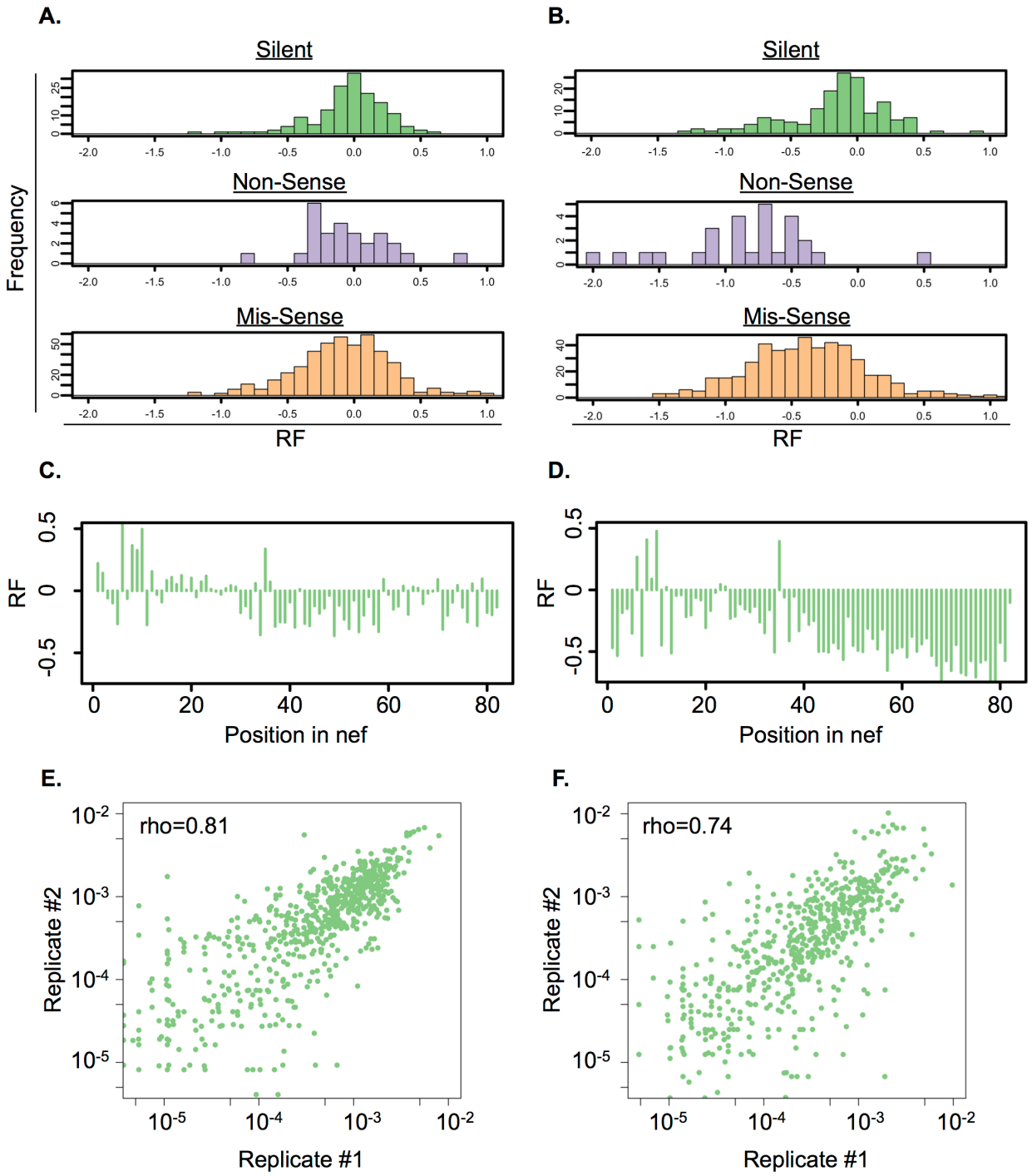

Figure 5-2: CTL pressure decreases fitness of nef mutants. The frequency of silent, nonsense, and missense nef mutants achieving indicated RF values is shown for virus grown in the absence (A) and presence (B) of the S14-KF11-10.6 CTL clone. The RF values of missense mutants, aligned by position in the nef gene is given for virus grown in the absence (C) and presence (D)

or CTLs. The correlation for RF values between two biological replicates is shown for virus grown in the absence (E) and presence of CTLs (F).

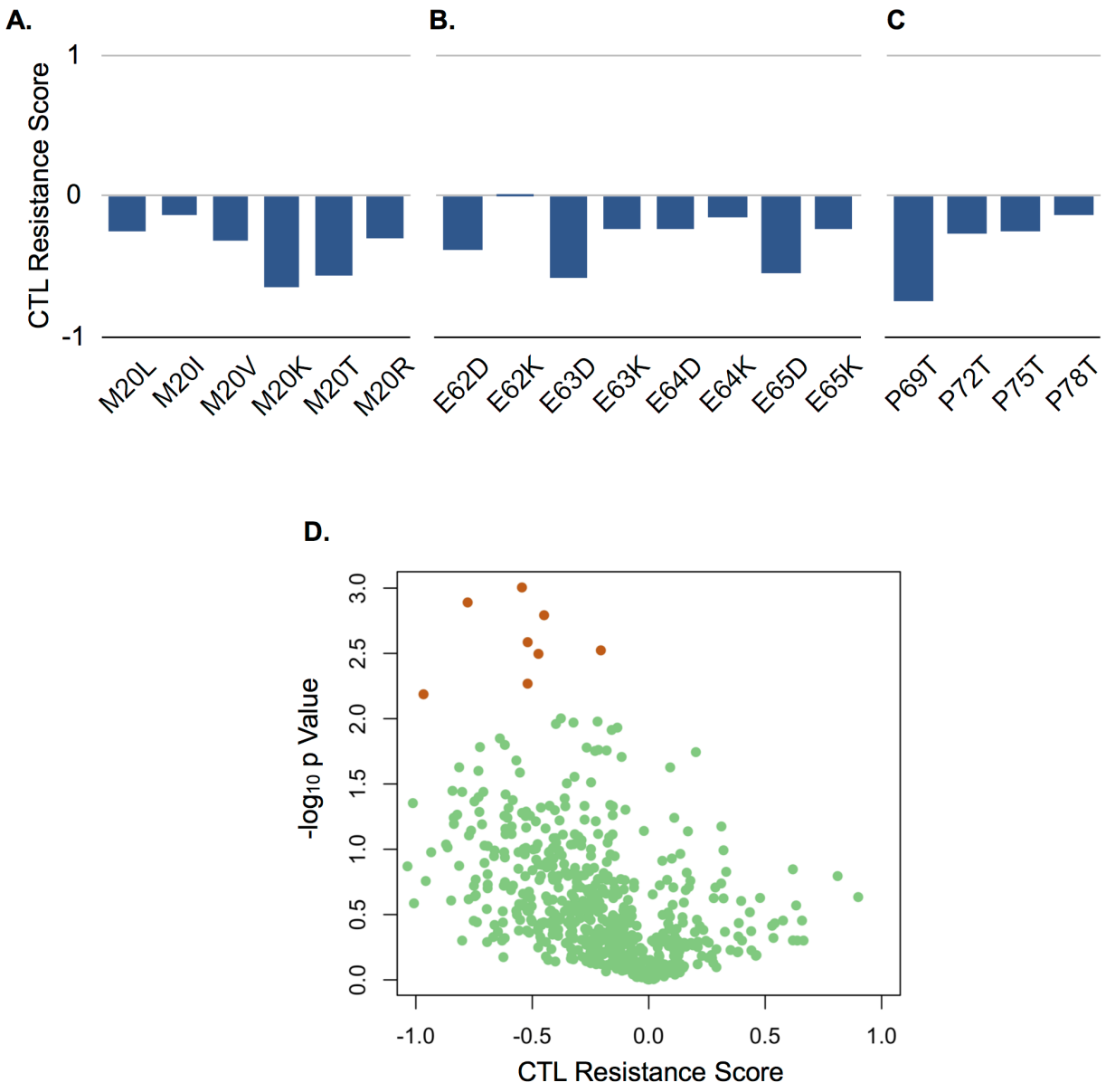

Figure 5-3: Identification of nef mutants that increase CTL recognition. The nef resistance score of mutations at all codons was calculated. The CTL resistance scores of mutations in residues previously identified as necessary for MHC-downregulation were examined. CTL resistance scores for mutations in M20 (A), the $EEEE_{65}$ motif, (B) and the PxxP repeat: residues 68-68 (C) are plotted. A volcano plot of all mutants in the nef library under CTL pressure (D) identified mutations that significantly decrease the CTL resistance score. Red dots indicate

mutants with most statistically decreased CTL resistance scores (the mutants were G2S, W13R, G41R, T44A, T48R, and E59Q).

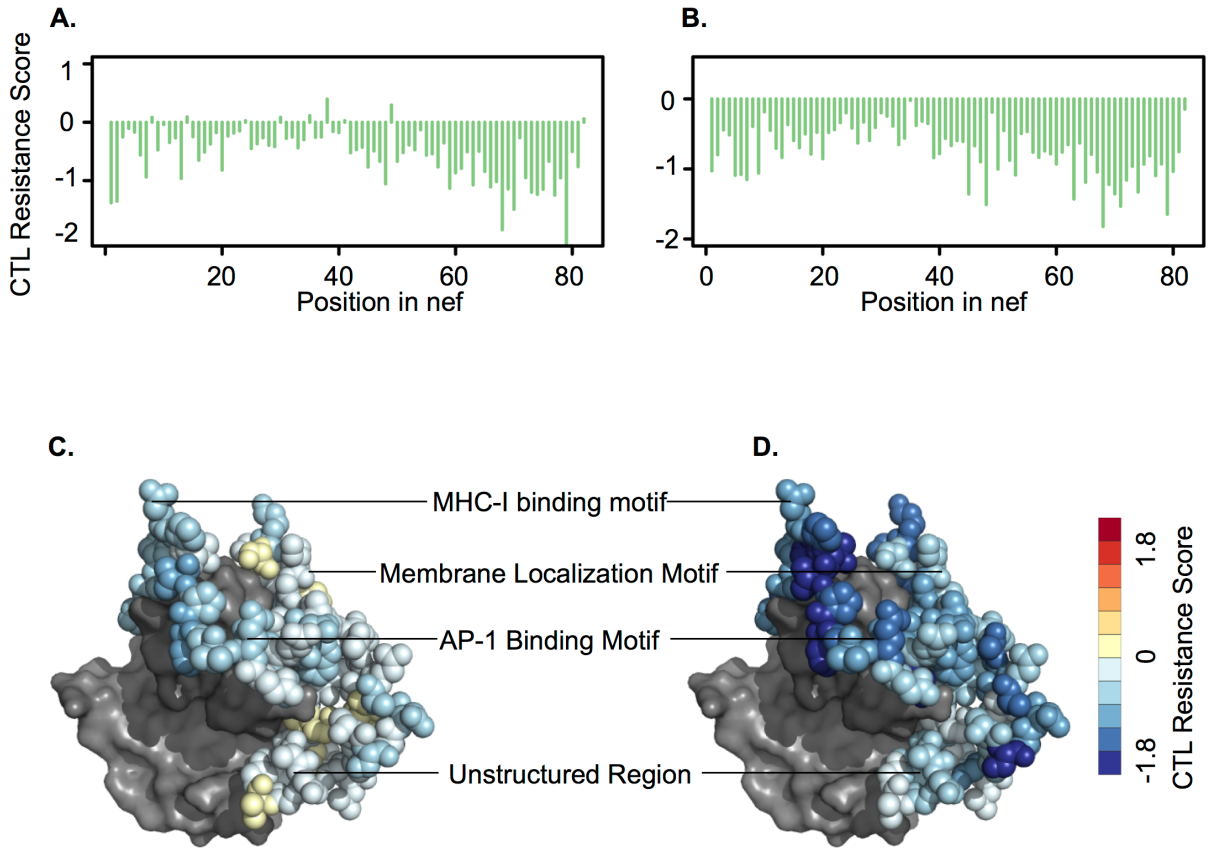

Figure 5-4: Mutations that increase CTL resistance map to structurally important nef motifs. The average resistance score of all mutations at a given residue (A, C) and the score for the single variant with the lowest resistance score at a given residue (B, D) is plotted by residue in the Nef protein (top) and superimposed onto the structure of the Nef protein (bottom).

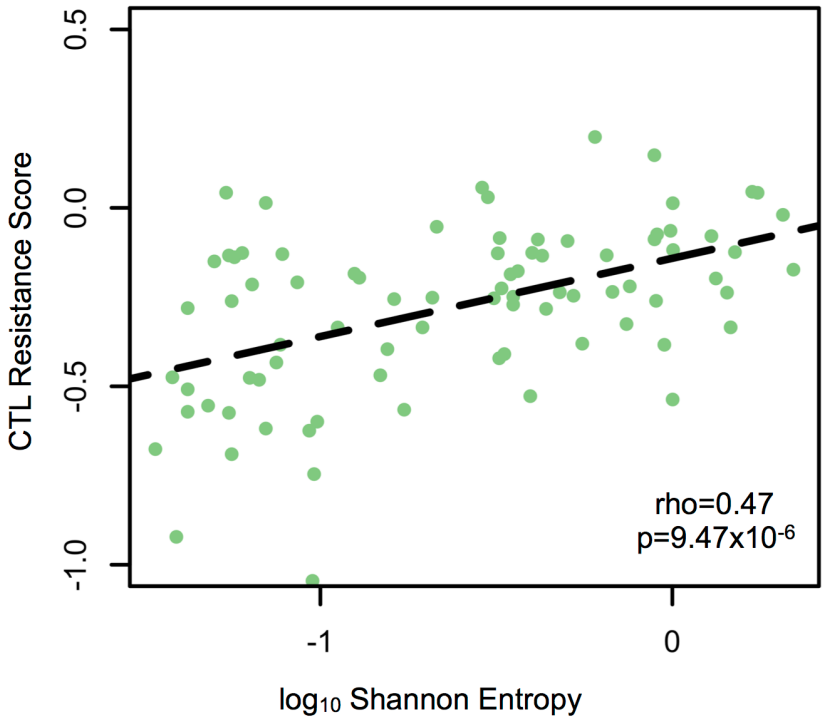

Figure 5-5: Nef residues necessary for CTL evasion are conserved at the population level. The average resistance score of all mutations at a given residue is plotted against the Shannon entropy of the residue amongst Clade B sequences in the Los Alamos HIV Sequence Database.

F1_Forward	CAGATAGCTAGCAAATTAAGAGAAC
F1_Reverse	ATTACTCCAACTAGCATTCCAAGGCACAGC
F2_Forward	GCTGTGCCTTGGAATGCTAGTTGGAGTAAT
F2_Reverse	CTAGGTCTCGAGATACTGCTC
F3_Forward	GAGCAGTATCTCGAGACCTAG
F3_Reverse	CCTGCACTCCATGGATCAGC

Table 5-1: Primers for constructing mutant nef libraries.

F2_A1_F	GTATTACAAGCAGCTTATAGAGC
F2_A1_R	ATCCAATCACACTACTTTTTGAC
F2_A2_F	TGCTATAAGATGGGTGGCAAGTG
F2_A2_R	TCTCGAGATACTGCTCCCACCCC
F2_A3_F	CAAGTGGTCAAAAAGTAGTGTGA
F2_A3_R	CTCCATGTTTTTCTAGGTCTCGA
F3_A1_F	CAGATGGGGTGGGAGCAGTATCT
F3_A1_R	CTCTTCCTCCTCTTGTGCTTCTA
F3_A2_F	TAACAATGCTGCTTGTGCCTGGC
F3_A2_R	GGCTAAGATCTACAGCTGCCTTG
F3_A3_F	AGGTACCTTTAAGACCAATGACTTA
F3_A3_R	ACAGATCAAGGATATCTTGTCTTCT

Table 5-2: Primers for amplifying sequencing amplicons

F2 plasmid	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCACAGT
	/5Phos/CTGTGCAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
F3 plasmid	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGAGCGT
	/5Phos/CGCTCTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
F2 +KF11 (replicate1)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTCAGT
	/5Phos/CTGACTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
F2 +KF11 (replicate2)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGCGTT
	/5Phos/ACGCGTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
F2 –KF11 (replicate1)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATGTT
	/5Phos/ACATCGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
F2 –KF11 (replicate2)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTGACT
	/5Phos/GTCAGCAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
F3 +KF11 (replicate1)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGCGTT
	/5Phos/ACGCGTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
F3 +KF11 (replicate2)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGTAGT
	/5Phos/CTACGTAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
F3 –KF11 (replicate1)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTGACT
	/5Phos/GTCAGCAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
F3 –KF11 (replicate2)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCACAGT
	/5Phos/CTGTGCAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG

Table 5-3: Y-shape adaptor sequences for different samples

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Chapter 6: Conclusions and Discussion

Summary of the dissertation

In the first section of this dissertation, we defined the fitness landscape of HIV-1 escape from CD8+ Cytotoxic T-Lymphocytes at several commonly targeted epitopes. We created a saturation mutagenesis library of four commonly targeted CTL epitopes (Chapter 2), which were then passaged in the presence and absence of CTL clones specific for these epitopes (Chapter 3). The results demonstrated that HIV can escape CTL recognition at the non-protective HLA A*02 restricted SLYNTVATL (SL9) with little or no loss in fitness, however, the protective HLA B*57-restricted KAFSPEVIPMF (KF11) and B*27-restricted KRWIILGLNK (KK10), have few fit variants, which translated into an inability to escape CTL recognition without a loss in fitness. Additionally, we demonstrated that the subtype B consensus SL9 epitope, which is targeted by over 70% of HLA A*02-expressing individuals (1, 2), appears to be evolved to minimize CTL recognition.

Next, utilizing the KK10 library described above, we showed CTLs generated in response chronic infection versus vaccination the Mrk/Ad5 vaccine differ in their ability to cross recognize epitope variants. The Mrk/Ad5 HIV vaccine utilized in the failed HVTN502/STEP trial contained only the consensus sequence of the *gag*, *pol*, and *nef* genes, begging the question of whether such vaccination can result in the selection of CTLs capable of cross-recognizing escape variants with the same efficiency as CTLs arising in response to the swarm of viruses present during chronic infection. The results (Chapter 4), while limited to only one KK10-specific CTL isolated from a vaccinated individual, demonstrated that these vaccine-induced CTLs are in fact inferior at cross-recognizing fit variants of the epitope.

Finally, we identified sites with the HIV-1 Nef protein that are necessary for its ability to downregulated the MHC-I complex (Chapter 5). By passaging an HIV-1 library containing point

mutations throughout the first 88 codons in *nef* in the presence and absence of HIV-1 specific CTLs, we identified several regions of the protein whose mutation results in increased suppression of the virus by CTLs. This screen detected several motifs previously shown to be necessary for Nef-mediated MHC-I downregulation as well as potentially novel sites within the protein that may mediate this process. Validation of these novel hits is currently ongoing. Furthermore, we found that residues necessary for downregulation of the MHC-I complex by nef are highly conserved among circulating virus strains, suggesting an evolutionary pressure for conserving this Nef function at the population level.

Relevance for pathogenesis

CTLs are largely responsible for the control of viremia during the course of HIV infection (3-6). Several groups have long sought to understand why CTLs fail to fully suppress viremia in most individuals and to identify the factors that mediate effective viral suppression in individuals with protective HLA types. These studies have shown that individuals who efficiently suppress viremia have CTLs that are less exhausted and more polyfunctional (7, 8), however it is still unclear if such markers mediate efficient viremic control, or rather are a result of it. Some studies have suggested that the mechanism of efficient viral suppression by CTLs is mediated by CTLs with higher antigen sensitivity and cross-reactivity of epitope variants (9-12), while others have pointed to the efficient targeting of conserved epitopes as a mechanism for preventing viral escape and maintaining viral suppression (13-15).

In this thesis we directly address the contribution of both CTL cross recognition of escape variants and fitness constraints on viral escape in three commonly targeted HIV epitopes. By utilizing a library of viruses comprising thousands of variants within epitopes (including every

possible single amino acid substitution), we were able to examine the fitness landscape of mutations within these epitopes at the highest resolution yet reported. In agreement with previous studies, we demonstrated that epitopes presented by protective HLA types are in fact highly restricted in their ability to mutate. What was even more striking, was that the few fit variants within these epitopes were all efficiently cross-recognized by CTLs, thus blocking off fit options for escape. Alternatively, the SL9 epitope, presented by a non-protective HLA type, had multiple fit variants, and CTLs recognizing this epitope could not cross-recognize the most fit variants (likely due at least in part to the high number of fit variants), thus allowing for escape with little or no fitness loss. Our results are in agreement with previous work suggesting that elite controllers' CTLs block viral escape by efficiently recognizing common escape mutants, rather than possessing highly promiscuous TCRs (16).

Thus, we propose a model wherein viremic control in individuals with protective HLA types is mediated at least in part by the inability to escape CTL recognition without sacrificing replicative capacity. While we only analyzed the fitness costs of escape at three commonly targeted epitopes, we expect to see a similar pattern at other conserved and variable epitopes. Since infected individuals target multiple CTL epitopes, it is easy to imagine that escape at multiple conserved epitopes would bring about an additive loss in fitness. For example, this has already been demonstrated for the HLA B*57-restricted epitopes where escape mutations in the TW10 epitope further decrease the fitness of KF11 mutant viruses (17). If protective epitopes can as a general rule present multiple conserved epitopes, then the combined fitness loss of escaping CTL recognition at multiple conserved sites might be enough to prevent viral escape and mediate control of viremia.

Evidence of HIV adaptation to CTLs at the population level

There has been increasing evidence over the past decade that HIV is evolving to adapt to frequent HLA types found within the population, so as to minimize the effect of CTLs at a population level (18-21). We find evidence to support this conclusion in our own results investigating escape at the SL9 epitope, which is targeted in a majority of individuals expressing the very common HLA A*02. We show that several mutations in the residue directly preceding the epitope increased viral fitness, but also increased recognition by CTLs targeting SL9. These results suggest that the consensus SL9 sequence represents a slightly less fit variant that is preadapted to minimize CTL in individuals expressing the common HLA A*02.

In addition to mutational escape, HIV evades CTLs through the ability of the Nef protein to downregulate expression of the MHC-I complex on the surface of infected cell. Our screen for Nef residues necessary for MHC-I downregulation further suggested that HIV is adapted at the population level to maximize CTL evasion. Nef residues necessary for the downregulation of the MHC were highly conserved in the Los Alamos Database of HIV Sequences, suggesting a population level pressure to preserve Nef's ability to dampen the effect of antiviral CTLs.

Implications for vaccine design

Development of a CTL-based HIV vaccine will require a thorough understanding of effective CTL responses against HIV and virus's ability to escape these CTLs. As described in the Introduction and Chapter 4, CTL-based HIV vaccines encountered a massive setback with the failure of the HVTN502/STEP and HVTN 503 trials (22, 23). These failed vaccines successfully induced a CTL response in most individuals, yet these responses targeted only a few epitopes within each individual, and were not focused on highly conserved regions. Our results

demonstrate that CTL responses to highly variable region may be minimally effective at containing virus replication as the virus may be able to mutate such epitopes with minimal loss in fitness. Furthermore, the use of a single consensus sequence of virus genes in these vaccines may have induced a CTL repertoire that was unable to recognize common variants of targeted epitopes, further contributing to the ease of viral escape from CTLs induced by these vaccines.

Our results suggest that efficient CTL based vaccines will need to target HIV in a manner that minimizes fit escape options for the virus. Based on these data, we suggest that effective vaccines will need to accomplish two goals. First, vaccines will need to induce CTL responses to multiple highly conserved regions of the virus that are inherently limited in their ability to mutate. Second, the vaccine will need to generate CTLs that are able to specifically cross-recognize the fit variants within these epitopes to further limit escape routes for the virus. Indeed, several groups are currently working on vaccination strategies addressing both of these goals: utilizing vaccines containing only highly conserved regions of virus proteins (24-27), and mosaic/polyvalent vaccines that can induce CTLs capable of cross-recognizing the most frequent escape variants (28-31).

The recent preliminary results of the BCN02 trial (Mothe *et al*. CROI, 2017) provide clinical support for our theory of vaccine design. In this trial, chronically HIV-infected individuals received a Romidepsin (a histone deacetylase inhibitor, which reactivates latent HIV in an attempt to flush out the latent HIV reservoir) in conjunction with a conserved element HIV vaccine (24). Upon interruption of ART, 40% of the vaccinated subjects maintained an undetectable or very low viral load. Furthermore, the study organizers demonstrated that this vaccine successfully shifted the CTL repertoire to one that was directed towards highly conserved regions of the virus, and could presumably control viral replication in the 40% of

individuals who contained viremia. These individuals were not cured, and their viremia may rebound in the coming months. However, this trial demonstrated as a proof of concept, that if we can limit the options that HIV has for escaping CTLs, we may be able to generate a vaccine capable of controlling viral replication.

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Appendix A: The Mutational Fitness Landscape of HIV-1 at The HLA B*15-Restricted GY9 Epitope

Introduction

In addition to the SL9, KK10, and KF11 libraries analyzed in **Chapter 3** of this dissertation, we also constructed an HIV library representing every possible single and double amino acid variant within the HLA B*1501-restricted GY9 epitope (GLNKIVRMY, Gag 269-277), as described in **Chapter 2**. The GY9 epitope partially overlaps with the KK10 (Gag 263-272) epitope and therefore this library offered us the ability to examine how CTLs targeting two overlapping epitopes differed in their ability to cross-recognize variants within these epitopes.

Since we did not have access to any GY9-specific CTL clones, we attempted to passage this library in the presence of CD8+ transduced with GY9-specific TCRs. Unfortunately, throughout the course of this experiment, these cells did not exhibit the ability to suppress HIV infection and therefore, we were unable to analyze escape at this epitope. However, we did successfully passage this library in the absence of CTL pressure to identify fitness constraints of mutation at this highly conserved epitope (as of March 2017, less than 3% of sequences reported in the Los Alamos HIV sequence database are variants from the clade B consensus sequence). The results of this experiment are presented in this appendix.

Results

Assessing the growth of variants in the GY9 library

The GY9 virus library was passaged for two successive 7-day rounds of infection in HIV-permissive cells. After each passage, virus was collected and the region encoding the GY9 epitope was deep sequenced. The results of the deep sequencing demonstrated that the majority starting virus library was composed of the consensus sequence. The consensus sequence continued to expand over the two weeks in culture, while all but two other variants decreased in frequency with every passage (**Figure A-1**).

Visualizing the mutational fitness landscape of the GY9 epitope

The effect of amino acid substitutions was assessed for GY9 epitope and immediately flanking amino acid residues. As described in **Chapter 3**, the outcome for each epitope variant was quantified as a relative enrichment value (RE), calculated as the $\log_{10}$ transformed ratio of change in frequency over two weeks in culture (see **Figure 3-2D**). We first examined the effect of single amino acid substitutions within the epitope (**Figure A-2**). No single amino acid mutants within the epitope had an RE value ≥0, and, only one variant GLNKIVRKY, had an RE approaching 0, indicating that all mutations within the epitope had a detrimental effect on virus growth. The leucine immediately preceding the GY9 epitope was somewhat more tolerant of mutations, with mutations to methionine and isoleucine yielding RE values > 0.

We next examined the RE of all single and double amino acid variants within the GY9 epitope and flanking residues (**Figure A-3**). Only the L→M and L→I mutations in the amino acid preceding the GY9 epitope yielded an RE ≥0. A total of 3 variants within the GY9 epitope

had an RE value ≥ -0.5 (this number increased to 8 if mutations in flanking residues were included).

GY9 is intolerant of mutations within the epitope

We plotted the RE values of single and double amino acid variants within the GY9 epitope and compared these results to those obtained from the SL9, KF11, and KK10 epitopes (**Figure A-4**). No variant within GY9 had an RE ≥0, while at least one variant within every other library did. Furthermore, while 13 SL9, 7 KF11, and 8 KK10, variants had an RE ≥ -0.5, only 3 GY9 variants met this cutoff.

Conclusions

In this appendix we have presented the mutational landscape of the HLA B*1501-restricted GY9 epitope. We observed that this epitope is remarkably constrained as every mutation within the epitope decreased the RE of the virus. These results are not surprising, since the GY9 epitope is extremely conserved, over 97% of sequences isolated from infected individuals in the Los Alamos database encode the consensus at the GY9 epitope.

We were pleased that variants in the region of GY9 that overlapped with the KK10 epitope had similar RE values in both libraries. Both the L-1M and L-1K (L6M and L6K in KK10) had the highest RE values of any variant in the GY9 library, and their values were very close to those obtained in the KK10 library.

Even though we did not passage this library in the presence of GY9-specific CTL, we can conclude that since no variants of the epitope increased fitness, the virus would have to give up some level of fitness to escape any CTL recognizing the consensus sequence of this epitope. This results demonstrates that highly conserved epitopes other than those presented by protective HLA types may be promising targets for vaccine design.

Methods

The GY9 library was synthesized as described in **Chapter 2**. All other experiments and analysis was performed with the same protocol as described in **Chapter 3** with the exception of the following:

HIV-permissive cell lines

The GY9 library was passaged in the 1CC4.14 cell line described in **Chapter 3**. This cell line is an HLA B*1501 positive cell line derived from the fusion of a primary CD4 cell and the T1 cell line.

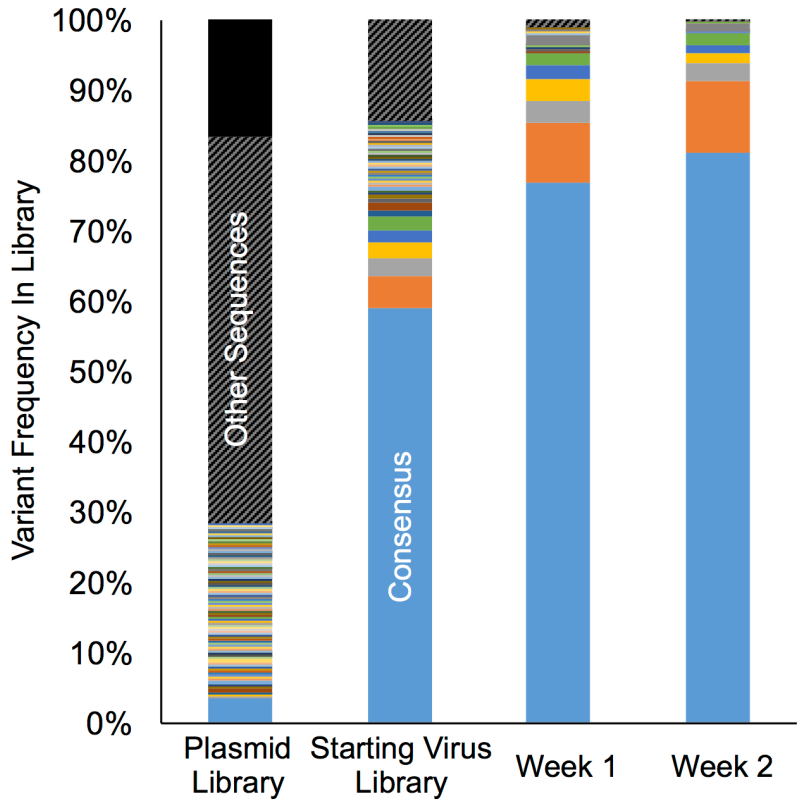

Figure A-1: Frequency of GY9 variants in the passaged virus library. The frequencies of each GY epitope variant are plotted for the plasmid library, initial virus library, and virus populations after one or two weeks of passage. Sequences below the plasmid library frequency threshold of 2.5×10^{-5} are represented in black in bar graphs. Variants other than those above the frequency cutoff of 1×10^{-4} in both replicates of the starting virus library are labeled "other sequences" and indicated by hatched grey in bar graphs.

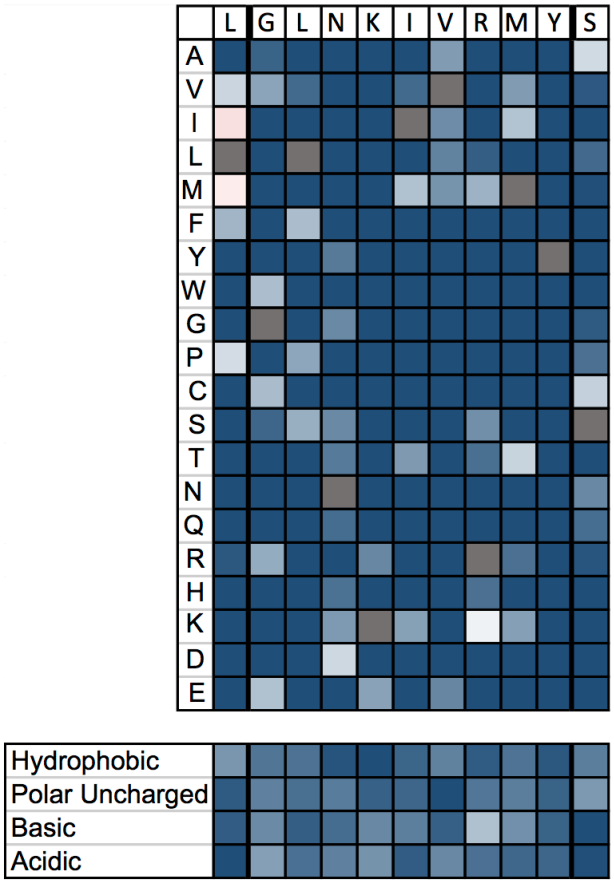

Figure A-2: Effect of single amino acid variants within GY9 on variant enrichment. The RE values of all single amino acid variants are shown. The top horizontal axis indicates the consensus sequence of each epitope and the immediately flanking amino acids. Each box indicates the RE for the substituting amino acid indicated on the vertical axis. The average REs for substitutions of amino acids that are hydrophobic (A, V, I L, M, F, Y, W, G, and P), polar-uncharged (C, S, T, N, and Q), basic (R, H, K), or acidic are shown. The RE of each variant is indicated by the color of each box with red denoting more fit and blue less fit. Variants that were detected above threshold in the plasmid library but not in the virus library were considered unfit and assigned a $\log_{10}$ relative growth rate of -2.

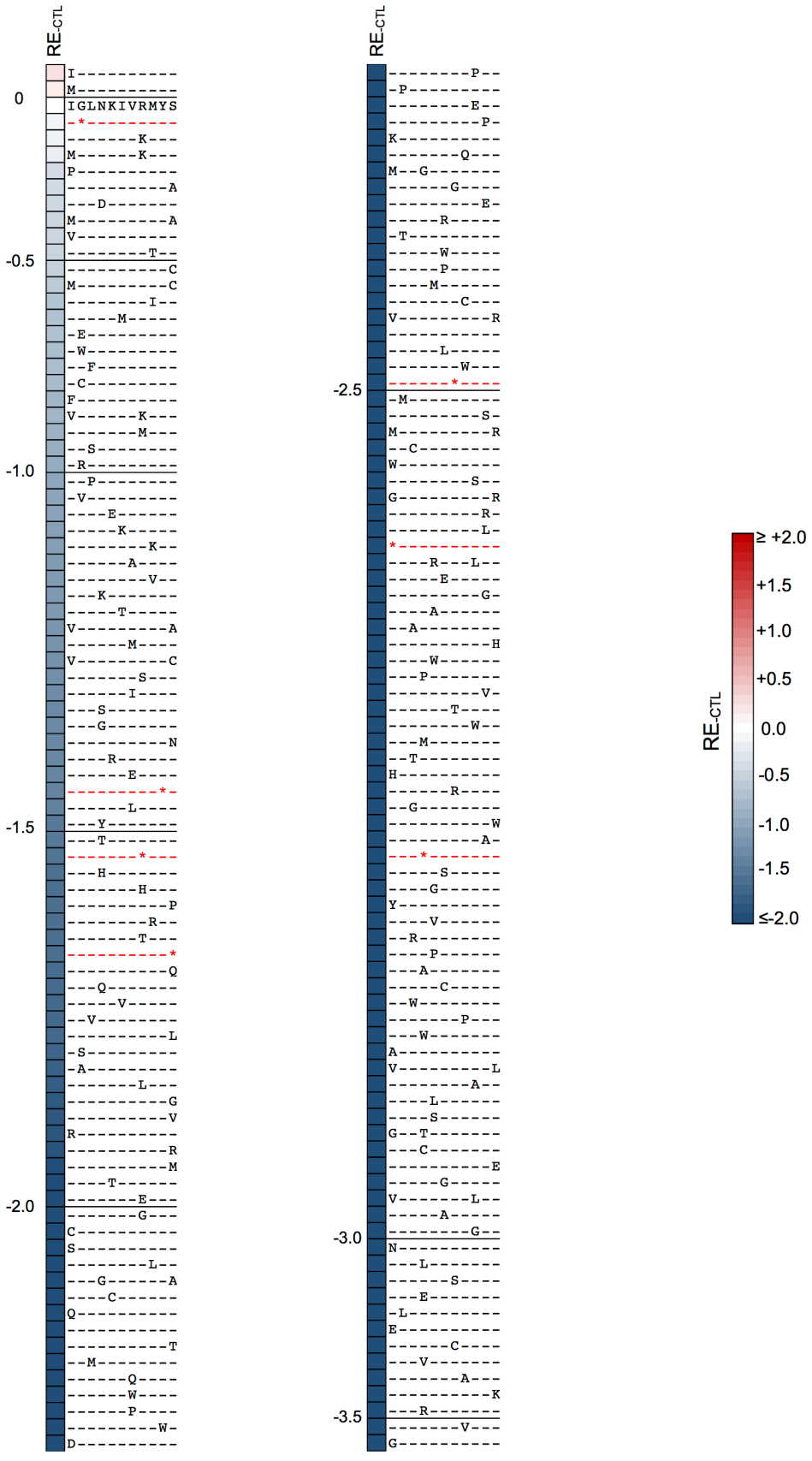
RE-CTL
0
-0.5
-1.0
-1.5
-2.0
RE-CTL
-2.5
-3.0
-3.5
RE-CTL
≥ +2.0
+1.5
+1.0
+0.5
0.0
-0.5
-1.0
-1.5
≤-2.0

Figure A-3: Effect of all variants in the GY9 library on relative enrichment. All variants within the GY9 epitope and flanking amino acids are listed in order of highest to lowed RE values. The box next to the variant sequence indicates the RE value of that variants, with red representing higher values and blue representing lower values.

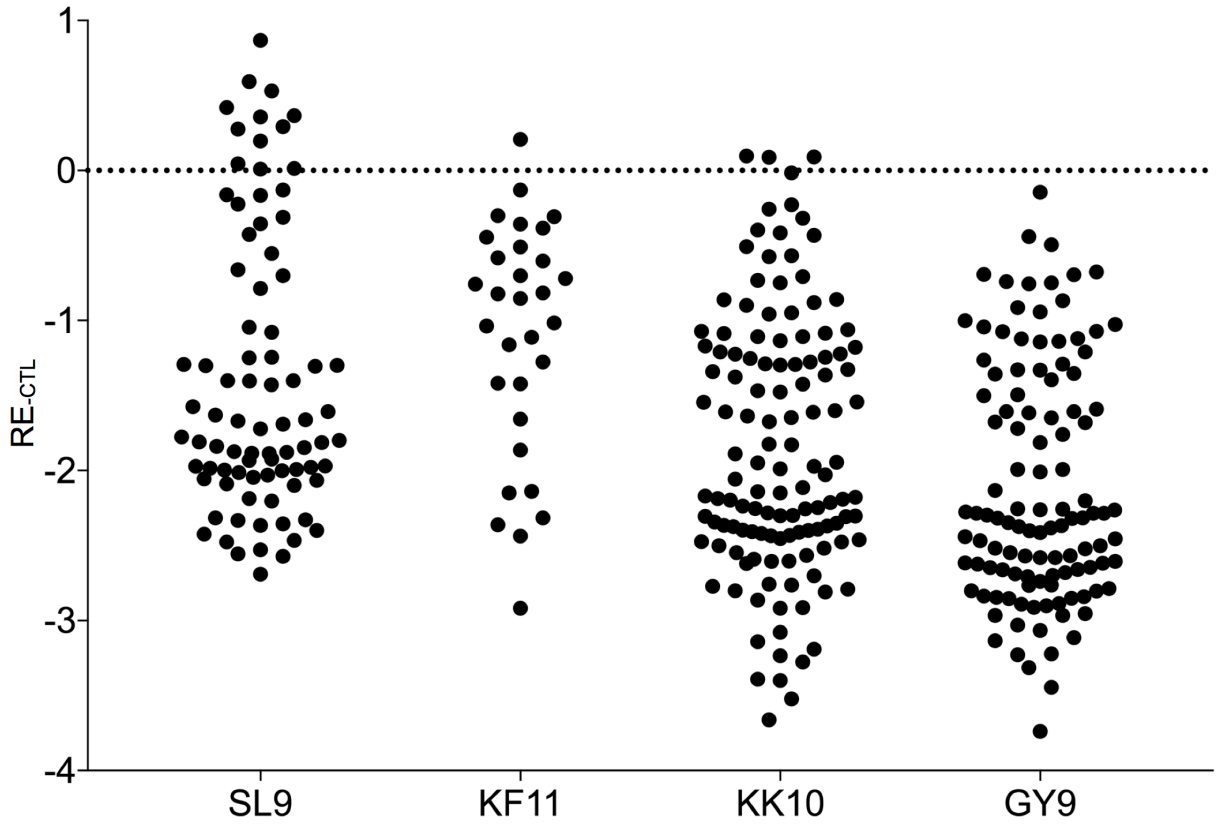

Figure A-4: GY9 is far less tolerant of mutations than all other examined epitopes. The RE values of variants within the epitope is shown for the SL9, KF11, KK10, and GY9 libraries grown in the absence of CTL pressure.