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Immune Escape Mutations Detected within HIV-1 Epitopes Associated with Viral Control During Treatment Interruption

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Abstract

We analyzed immune responses in chronically HIV-infected individuals who took part in a treatment interruption (TI) trial designed for patients who initiated anti-retroviral therapy within 6 months of seroconversion. In the two subjects that exhibited the best viral control, we detected CD8⁺ T cell responses against 1–2 Gag epitopes during the early weeks of TI and a subsequent increase in the number of epitopes recognized by the later time points. Each of these subjects developed mutations within the epitopes targeted by the highest magnitude responses. In the subject with the worst viral control, we detected responses against two Gag epitopes throughout the entire TI and no Gag mutations. The magnitude of these responses increased dramatically with time, greatly exceeding those detected in the virologic controllers. The highest levels of contemporaneous autologous neutralizing antibody activity were detected in the virologic controllers, and a subsequent escape mutation developed within the envelope gene of one controller that abrogated the response. These data suggest that immune escape mutations are a sign of viral control during TI, and that the absence of immune escape mutations in the presence of high-levels of viral replication indicates the lack of an effective host immune response.

INTRODUCTION

HIV-specific immune responses are typically diminished by the onset of anti-retroviral treatment, presumably as a result of decreased antigenic stimulation ^{1–3}. Treatment interruption (TI) trials have been tested in an effort to boost immune responses to autologous virus in treated patients and achieve better viral control in the long-term. Small-scale trials reported anecdotal cases where reduced viral replication was associated with increased immune responses in chronically HIV-infected subjects ^{4–8}. However, longer-term studies with larger patient cohorts demonstrated that the immune responses were not increased in all cases and that durable viral control was not achieved. In fact, viral loads returned to pre-treatment levels over time, CD4⁺ T cell counts declined substantially, and the incidence of opportunistic infections and mortality often increased ^{9–12}. More recent TI studies have focused on subjects that were initially treated within months of exposure. Initiation of treatment during this early phase of infection has been proposed as a way to preserve the early immune responses that are typically decimated during the widespread CD4⁺ T cell depletion that occurs during this stage of

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infection. These trials have resulted in augmented HIV immune responses in many cases, but long-term viral control has not been achieved¹³⁻¹⁵.

The TI study described in this report focused on HIV-infected subjects who were treated within 6 months of seroconversion, received treatment for at least 24 weeks, and maintained undetectable viral loads for at least 8 weeks prior to starting the protocol¹⁶. We performed detailed longitudinal analyses comparing the two subjects that exhibited the best viral control during TI and the subject that had the least amount of viral control off treatment. Our goal was to assess the interplay between immune responses, viremic control, and viral evolution in the corresponding viral genes. We detected increased levels of anti-Gag CD8⁺ T cell responses and neutralizing antibody (NAb) activity in each of these subjects during TI, regardless of virologic control. However, only the virologic controllers developed mutations within the genes targeted by the measured immune responses. Our data suggest that there is a strong pressure for mutations to occur within regions of the virus targeted by immune responses that are able to reduce viral replication during TI, and that the development of these mutations is likely responsible for the lack of durable viral control. Conversely, our data suggests that escape mutations are less likely to occur within epitopes targeted by immune responses that are ineffective at reducing viral replication, and that the emergence of immune escape mutations during TI is an indicator of an effective immune response.

METHODS

Study Participants¹⁶

To qualify for this study, participants must have initiated antiretroviral therapy within 6 months of HIV seroconversion, received treatment for at least 24 weeks, and maintained viral loads below 75 copies/ml for at least 8 weeks prior to entering the protocol. Participants were enrolled in a treatment interruption (TI) protocol that was approved by the UCSF IRB, and designed for patients who initiated antiretroviral therapy in early HIV infection. Under this protocol, treatment would be re-initiated if viral load exceeded certain thresholds (>200,000 copies/ml at any time or >50,000 copies/ml between weeks 4 and 7 of TI).

Reagents

Consensus Subtype B Gag (p17-p24) peptides were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Human Leukocyte Antigen class I typing

Molecular typing of *HLA-A*, *-B* loci was performed using a non-radioactive PCR based sequence specific oligonucleotide probe reverse line-blot assay (PCR-SSOP) (Dynal, Norway).

Determination of HIV-1 RNA and CD4⁺ T cell count

Plasma HIV-1 RNA was measured using the branched-chain DNA (bDNA) test version 3.0 (Seimens Diagnostics, Emeryville, CA; lower limit of detection 75 copies/ml). CD4⁺ T-cell counts were calculated using standard flow-cytometry methods.

IFN-gamma ELISPOT assay

HIV-1 specific cellular responses were quantified by IFN-gamma ELISPOT as previously described¹⁷. Cryopreserved PBMC were stimulated with peptide pools comprised of 15mers overlapping by 11 amino acids. Each peptide was represented in at least two pools to ensure that a positive response could be confirmed in each case. Peptides corresponded to subtype B consensus Gag (p17-p24) sequences. PBMC were plated at 50,000 – 100,000 cells per well and stimulated with 1 µg/ml of peptide. Efforts were focused on the Gag protein, because it has

been shown to be one of the most immunogenic viral proteins in early HIV infection¹⁸. All test wells were run in duplicate. Positive controls included cells stimulated with Staphylococcal enterotoxin B (SEB), and negative controls included cells cultured in medium alone without antigenic stimulation. The number of HIV-specific IFN-gamma secreting cells was counted by direct visualization. The total number of spot forming cells (SFC) detected in duplicate wells was averaged and twice the background control was subtracted. Average spot number was normalized to the number of SFC per 1×10^6 PBMC. The targeted epitopes for each subject were deduced by cross-referencing the list of peptides from the responding peptide pools with the current published list of defined HLA-restricted epitopes (<http://www.hiv.lanl.gov/content/immunology>).

Amplification and sequencing of HIV-1 *gag* genes

Viral RNA was extracted from plasma samples using the Dynabeads mRNA DIRECT kit (DynaL Biotech) according to manufacture's protocol. The *gag* gene was then reverse transcribed and amplified by PCR using the Titan One Tube RT-PCR kit (Roche). RT-PCR conditions were as follows: 94°C for 2 min, 35 cycles of 94°C for 10s, 56°C for 30s, 68°C for 1 min, and a final extension of 68°C for 1 min. The *gag* gene was further amplified by nested PCR using the High Fidelity PCR Master kit (Roche). PCR products were sequenced using a thermocycling method with fluorescent dye-labeled dideoxynucleotide chain terminator chemistry.

Amplification and sequencing of HIV-1 *gp160* genes

Virus was pelleted by centrifugation of patient plasma ($20,400 \times g$ for 60 min). Viral particles were disrupted by resuspension in lysis buffer (4 M guanidine thiocyanate, 0.1 M Tris HCl [pH 8.0], 0.5% sodium lauryl sarcosine, 1% dithiothreitol). RNA was extracted from viral lysates using oligo(dT) linked to magnetic beads (DynaL, Oslo, Norway). First strand cDNA was synthesized in a standard reverse transcription reaction using oligo(dT) primers and the Thermoscript reverse transcriptase enzyme (Invitrogen, Gaithersburg, MD). *Env* sequences were amplified with the BD AdvantageTM 2 PCR Enzyme System (Clontech, Mountain View, CA) with a forward primer containing a *PinAI* site and a reverse primer containing an *MluI* site. The 2.6kb amplification product contained the entire open reading frame for HIV-1 gp160 polyprotein. Amplification products were digested with *PinAI* and *MluI* (Gibco/BRL, Gaithersburg MD), purified by agarose gel electrophoresis, and then ligated into *PinAI*- and *MluI*-digested expression vectors. The expression vector contains a cytomegalovirus promoter, which drives expression of the envelope gene within transfected cells. Ligation reactions were used to transform competent *Escherichia coli* (Invitrogen, Carlsbad, CA). The resulting pool of expression plasmids contained the population of viral envelope genes. The expression plasmids were purified using silica column chromatography (Qiagen, Valencia, CA).

DNA sequencing was performed using a thermocycling method with fluorescent dye-labeled dideoxynucleotide chain terminator chemistry (ABI, Foster City, CA). Sixteen primers were used to generate sequence spanning the entire *gp160* open reading frame, approximately 2600 nucleotides. These primers anneal within conserved regions and are designed to provide double coverage (both negative and positive DNA strands are sequenced) across the entire envelope gene. Sequencing reactions were analyzed using an ABI 3700 sequencer (PE Biosystems, Foster City, CA). Envelope sequences were assembled and edited using SequencherTM software. Sequence alignments were obtained using ClustalW¹⁹ and manually edited in the regions of variable length in particular to suppress the gaps within N-glycosylation sites (identified using an NXYX motif where X is any amino acid except proline and Y is either serine or threonine).

Neutralization assay

A recombinant virus assay involving a single round of virus infection was used to measure neutralization^{20, 21}. Briefly, pseudotyped viral particles were produced by co-transfecting HEK293 cells with an expression vector carrying the patient-derived *gp160* gene (eETV) and an HIV-1 genomic vector carrying a luciferase reporter gene (pRTV1.F-lucP.CNDO- Δ U3). Forty-eight hours post-transfection, pseudovirus stocks were harvested and a small aliquot of each was screened for infectivity using U87 target cells expressing CD4, CCR5, and CXCR4. Pseudovirus stocks were then diluted based on infectivity and used within a range of relative light units (RLU) where neutralization titers are known to be unchanged by viral input. In the neutralization assay, diluted pseudovirus was incubated with serial dilutions of heat-inactivated (56°C for 1 hour) patient plasma for 1 hour at 37°C prior to the inoculation of the U87 target cells. The ability of antibody to neutralize viral infection was assessed by measuring luciferase activity seventy-two hours after viral inoculation in comparison to a control infection with aMLV pseudotyped virus. Neutralization titers are expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC₅₀).

RESULTS

Viral Load and CD4⁺ T cell counts

Viral load and CD4⁺ T cell counts were closely studied in longitudinal samples from HIV-infected individuals before and during an extended treatment interruption (TI) lasting 24 weeks or longer (Figure 1). Each subject was treated with anti-retroviral therapy within six months of seroconversion (Table 1). Subjects were treated for at least 3.9 years before undergoing the planned TI. Viral loads remained undetectable (<75 RNA copies/ml) and CD4⁺ T cell counts increased or remained at a steady state in all subjects during anti-retroviral treatment. All subjects experienced an initial burst in viral replication within 4 weeks of interrupting treatment. This viral peak declined to a different degree in each subject before rising again to various levels. Two subjects had exceptionally low viral loads throughout the TI. Subject 313 achieved a viral load of < 75 copies/ml after an initial viremic peak above 5,000 copies/ml. Although this level of virologic control was only temporary, this subject was able to maintain viral loads under 10,000 copies/ml during the entire TI. Subject 311 maintained viral loads under 75 copies/ml for 10 weeks once the initial viremic peak was cleared, but viremia subsequently increased with time. Subject 410 never achieved a viral load below 10,000 copies/ml during the entire TI (Figure 1). In this report, the two subjects that maintained lower viral loads will be referred to as “virologic controllers”, and the subject that had continuously high levels of viral replication will be referred to as the “non-controller”.

Detection of Gag-Specific Cellular Immune Responses by IFN-gamma ELISPOT Assay

We compared the longitudinal Gag-specific CD8⁺ T cell responses of the two virologic controllers (311 & 313) to the responses detected in the non-controller (410) (Figure 2, Table 2). Very low magnitude Gag responses were detected in each of the subjects at the onset of TI (week 0), yet the magnitude and/or breadth of these responses changed substantially in each subject over time. Low magnitude responses to 2 overlapping epitopes were detected in subject 311 just prior to TI. The magnitude of these responses increased dramatically by week 8, coinciding with a decrease in viral load from a peak of 25,000 copies/ml to <75 copies/ml. The magnitude of these responses decreased by week 16, possibly due to the reduction in antigenic stimulus resulting from a prolonged period of undetectable viremia. The decrease in magnitude was immediately followed by an increase in viremia in this subject, which then declined to undetectable levels again within 4 weeks. Following 12 weeks of increasing viremia, the magnitude of these responses was augmented once again and a broadening of the response was detected, with several new epitopes being recognized. In subject 313, a low magnitude response against one Gag epitope was detected at 4 and 8 weeks of TI. The magnitude of this response

was augmented substantially at 24 weeks, following 18 weeks of steadily increasing plasma viremia. The breadth of the response also expanded by week 24, with two additional Gag epitopes recognized. A narrowly-directed Gag-specific response against two A2-restricted epitopes was detected in subject 410 throughout the duration of TI. Plasma viremia peaked by week 4 of TI, and then dropped slightly for several weeks before increasing once again by week 20. The magnitude of the original Gag responses greatly increased with time, but no additional epitopes were recognized.

Gag Sequence Analysis

Plasma samples were obtained from each subject at various time points throughout TI. The population of *gag* genes from each plasma sample was amplified by RT-PCR and population-based sequencing was performed. The amino acid sequence of the p17 and p24 regions of Gag have been aligned chronologically for each patient in Fig. 3. The CD8 responses that were detected in each patient during TI have been highlighted. Listed above each epitope is the week (s) at which the response was detected, and listed below each epitope is the HLA class I restriction of that specific response. An asterisk near the HLA restriction indicates that the response had the greatest relative magnitude of IFN-gamma release. The Gag sequence of subject 311 was analyzed at weeks 4 and 48 of TI (Fig. 3A). Several amino acid changes were detected within targeted epitopes by week 48. Of particular interest, two mutations were detected within the B8 epitope (ELKSLYNTV). The *gag* sequence of subject 313 was analyzed at 4, 8, and 24 weeks of TI (Fig. 3B). One amino acid change was detected by week 24, which was located at an MHC I anchor position within the A11 restricted epitope (VATLYCVHQRI). The *gag* sequence of subject 410 was analyzed at 4, 8, and 24 weeks of TI. No amino acid mutations were detected at any of the time points (Fig. 3C).

Autologous and Heterologous Neutralizing Antibody Activity

Neutralizing antibody (NAb) activity was assessed in longitudinal plasma samples in each study subject using a cell-based infectivity assay (Fig. 4) 20, 21. Plasma was tested for autologous NAb activity against virus isolated from the last time point prior to the start of anti-retroviral treatment and from various time points during TI. Patient plasma was also tested for heterologous NAb activity using HIV-1 reference strains; JRCSF, NL4-3, and SF162. As a positive control for HIV-specific neutralization, plasma from a drug-naïve, chronically infected patient with known broadly NAb activity was tested against all patient-derived viruses and controls. As a control for non-specific inhibition of viral entry, plasma was tested against virus pseudotyped with amphotropic murine leukemia virus (aMLV) envelope. For neutralization titers to be scored as positive, inhibition levels needed to be at least three fold greater than any background inhibition detected in the aMLV infection. Green shading on IC₅₀ data tables in Figure 4 denotes IC₅₀ values that scored positive for HIV-specific neutralization. Black boxes indicate NAb titers against contemporaneous autologous virus. The data are also represented graphically in Figure 4, with each point on the graph representing the IC₅₀ of one plasma sample against one autologous viral isolate. Each line on the graph represents the autologous neutralization of one viral sample over time.

Each subject exhibited unique patterns of autologous and heterologous NAb activity during TI (Fig. 4). Autologous NAb titers were not detected in subject 311 until week 6 of TI. At this time point, low-level titers were detected against the early pre-treatment virus and viruses from up to 16 weeks of TI. The titers against these viruses increased with time, with the highest titers being detected at 24 and 36 weeks of TI. Interestingly, no autologous neutralization was detected against virus from time points after 16 weeks, suggesting that a viral sequence change occurred some time after 16 weeks that eliminated the ability of the plasma to neutralize autologous virus. Heterologous neutralization titers against the reference strain, SF162, increased over time in this subject, with the highest titers being detected at 36 weeks. Positive

heterologous titers were also detected against NL43 in this subject by week 36. The broadly neutralizing control plasma was able to neutralize viruses from this subject at each time point with similar low-level inhibitory activity, suggesting that the switch in autologous neutralization sensitivity that occurred at week 16 did not cause a dramatic overall change in heterologous neutralization sensitivity.

Positive autologous NAb titers were detected in subject 313 at each time point during TI. The magnitude of these titers increased over time, with the highest levels being detected at 24 weeks. High-levels of contemporaneous autologous NAb activity were detected in this subject and continued to increase in magnitude over time. Heterologous NAb activity against NL4-3 and SF162 also increased with time, reaching the highest levels by 24 weeks. Similar to subject 311, the control plasma inhibited infection of virus from each time point of subject 313 with similar activity.

The pattern of the NAb response detected in subject 410 was quite different than those detected in subjects 311 and 313. Subject 410 had high-titer autologous neutralizing responses against the early pre-treatment virus throughout TI, but did not develop a robust autologous response against contemporaneous or later viruses. The magnitude of the neutralization titers against the early pre-treatment virus peaked at week 8 and then decreased slightly by week 24. Heterologous neutralization against NL4-3 and SF162 followed a similar pattern, with titers increasing until week 8 and then decreasing by week 24. Similar to subjects 311 and 313, the control plasma was able to neutralize virus from each time point with similar activity levels.

Envelope Sequence Analysis

Population-based sequencing was performed on the *gp160* envelope genes isolated from longitudinal time points from each subject. The amino acid sequence alignments of the *gp160* genes of each subject are shown in Figure 5. In subject 311, several nucleotide mixtures were detected at different time points within various regions of *gp120*. However, only two mixtures progressed to amino acid changes, and both were in the C3 region (positions 351 and 359). Interestingly, the nucleotide mixture at position 359 was first detected at 16 weeks and its appearance coincided with an escape from the autologous NAb response. In subject 313, multiple nucleotide mixtures were detected within *gp120* and *gp41* at various time points of TI. Many of these mixtures were detected within the variable regions of *gp120*, with two leading to amino acid changes. However, none of these sequence changes were associated with escape from the autologous NAb response. Multiple nucleotide mixtures and amino acid mutations were also detected within the *gp120* and *gp41* regions of subject 410 at various time points during TI. Sequence changes within the V1 region of *gp120* at positions 143 and 145 occurred by the earliest time point of TI, which selected for amino acid changes that likely differed from the pre-treatment virus. These sequence changes may explain the difference in neutralization sensitivity of the pre-treatment virus compared to the virus isolated from the later time points of TI. Multiple mixtures were detected within the V1 variable region, as well as in other areas of *gp120* and *gp41* at the later time points of TI. Most noticeably, a selection toward double leucine (L) residues was detected at positions 452 and 453 in a region of *gp120* just upstream of the V5 variable region.

DISCUSSIONS

The purpose of the current study was to examine the differences in immune responses and viral escape mutations within HIV-infected individuals that control viral replication well during TI compared to those that maintain high-levels of viral replication. The strategy of TI following early initiation of antiretroviral therapy resulted in robust immune control of HIV replication in a subset of subjects. These subjects achieved levels of viral control typically observed in patients treated with antiretroviral therapy or in the small proportion of untreated individuals

referred to as elite controllers. Although immune control was impressive in this subset of subjects, escape mutations rapidly emerged and viral replication levels subsequently rose. Interestingly, immune escape mutations within the gag gene were not detected in the non-controller, despite high-levels of viral replication and high-titer CD8⁺T cell responses. These results suggest that the TI led to the selective loss of the immune responses best able to reduce viremia in the absence of anti-viral therapy.

Important lessons can be learned by studying the interplay between the cellular and humoral immune responses within the virologic controllers. For instance, subject 311 elicited robust CD8⁺ T cell responses and high-titer autologous NAb activity early during TI. A combination of robust immune responses is likely what led to the extraordinary viral control within this subject. Breakthrough viremia coincided with the appearance of escape mutations within both the *envelope* and *gag* genes, which likely caused reduced effectiveness of these immune responses. Subject 313 also controlled viremia extremely well during TI, and subsequently developed an amino acid mutation within the highest magnitude Gag epitope. The development of this mutation coincided with increased viremia levels, suggesting reduced effectiveness of the immune response. Interestingly, this subject was able to maintain high-levels of autologous NAb activity against contemporaneous viral isolates throughout the entire TI, despite the appearance of multiple amino acid mutations within the variable regions of the envelope gene. The lack of escape from the NAb response in this subject may be due the positioning of the targeted epitopes within the envelope gene. The NAb epitopes may be located within conserved regions of the envelope protein that would greatly reduce viral fitness if an escape mutation were to occur. The combination of protective CTL responses and high-titer autologous NAb activity likely contributed to this subject's viral control.

An interesting paradox arose from the analysis of subject 410. This subject did not control viral replication well during TI, despite having a high-magnitude CD8⁺ T cell response and no escape mutations. This suggests that the targeted immune responses were ineffective at reducing viremia, and therefore did not elicit a selective pressure on the virus to escape. In addition, CTL escape mutations within these targeted epitopes may have elicited a negative effect on viral replicative fitness that greatly outweighed the positive effect of evading the immune response²²⁻²⁶. Since, we measured CD8⁺ T cell responses using the IFN-gamma ELISPOT assay, we were unable to determine other factors that might have influenced the effectiveness of the responses, such as skewed maturation-state of immune cells, diminished proliferative capacity, reduced perforin expression, or inability to generate adequate quantities of other essential anti-viral cytokines, such as TNF- α and IL-2²⁷⁻³⁶. Future studies on these subjects measuring these parameters would likely yield valuable information regarding the essential components of protective immune responses. Furthermore, there may have been other CD8⁺ T cell responses directed against viral epitopes outside of the Gag region that played a role in the differential viral control detected among the study subjects. Overall, the immune response of subject 410 lacked an overall adaptability that was exhibited by the virologic controllers. For example, we detected a high-titer NAb response against archived virus from an early pre-treatment time-point in subject 410 during TI, but did not detect NAb activity against any current viral isolates. This suggests that the immune system continued to recognize the initial viral antigens that were encountered during early infection, but did not adapt to recognize new viral isolates.

Taken together, these data provide evidence that early initiation of antiretroviral therapy followed by TI can induce immune responses that are temporarily effective at reducing viral replication. However, viral escape selection rapidly occurs within regions of the virus targeted by the immune responses most effective at reducing viremia. The appearance of such escape mutations are likely responsible for the lack of long-term viral control that has been reported in subjects undergoing this treatment strategy.

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Figure 1. Viral loads and CD4⁺ T cell counts

HIV-1 RNA (copies/ml, pink line in each panel) was measured using the Bayer (Emeryville, CA) branched-chain DNA (bDNA) test version 3.0 (lower limit of detection 75 copies/ml). CD4⁺ T cell counts (cells/mm³, blue line in each panel) were calculated using standard flow cytometry methods.

Figure 2. Gag-Specific CD8⁺ T cell responses Measured by IFN- γ ELISPOT Assay

PBMC were stimulated with pools of 15-mer peptides that overlapped each other by 11 amino acids each. Peptide sequences corresponded to the HIV-1 subtype B consensus Gag sequence for p17-p24. Each peptide was represented in at least two pools to ensure that a positive result could be confirmed in each case. Peptide pools are listed along the X-axis (G1-G19). Reactions were set up in duplicate wells, and the total number of IFN- γ secreting spot-forming cells (SFC) was averaged and twice the background control (media alone) was subtracted. The resulting average spot number was normalized to the number of SFC per 1×10^6 PBMC. Error bars represent the standard deviation between replicate samples. Inset: viral load following TI. The time points when ELISPOT analysis was performed are indicated with arrows.

wk 48
 311 wk 4 MGARASVLISG GELDRWEKIR LRPGGKKKKYK LKHIVWASRE LERFAVNPLG LETSEGCQRI
 311 wk 48 -----K-----
 B8 A23
 wk 8, 16, 48 wk 48
 311 wk 4 LGQLQPALQT GSEELKSLYN TVATLYCVHQ KLEIKDTKEA LKIEIEEQNK SKKKAQQAQA
 311 wk 48 -----S-----R-----I-V-----R-DV-----D-----
 A1 *B8 B8

 311 wk 4 ADTGNRSQVS QNYPIVQNLO GQMVHQAIASP RTLNAWVKV EEAFAFSPEVI PMFSALSEGA
 311 wk 48 XX--N-----

 311 wk 4 TPQDLNTMLN TVGGHQAAMQ MLKETINEEA AEWDRLHPVH AGPIAPGQMR EPRGSDIAGT
 311 wk 48 -----

 wk 48
 311 wk 4 TSTLQEQIGW MTNPPPIVPG EYKRWIILG LNKIVRMYSPT SILDIRQGP KEPRFDYVDR
 311 wk 48 -----H-----
 B8

 wk 48
 311 wk 4 FYKTLRAEQA SQEVKNWMTETLLVQNANPD CKTILKALGPAATLEEMMTA CQGVGGPGHK
 311 wk 48 -----G-----
 B8 B8/B51 overlapping

 311 wk 4 ARVL
 311 wk 48 ----

 wk 4 GCRQI
 wk 8
 wk 24

 wk 4, 8, 24
 wk 4 IGQLQPSLQT GSEELRSLYN TVATLYCVHQ RREVKDTKEA LDKIEEQNK IKKKAQQAQQ
 wk 8 X-----V-----
 wk 24
 *overlapping A11

 wk 4 AAADAGTNKQ VSQNYPIVQN IQGQMVHQAI SPRTLNAWVK VVEEKAFSPE VIPMFSALSE
 wk 8 -----NS-----
 wk 24 -----

 wk 4 GATPQDLNLM LNTVGGHQAAMQLKETINEEAEADWDLRHP VQAGVPAPGQ IREPRGSDIA
 wk 8 -----
 wk 24 -----

 wk 24
 wk 4 GTTSTLQEQI AWMTNPPPIP VGEIYKRWII LGLNKIVRMY SPTSILDIRG GPKEPRFDYV
 wk 8 -----
 wk 24 -----
 B44...

 wk 24 wk 24
 wk 4 DRFYKTLRAE QASQEVKNWMTETLLVQNAN PDCKTILKAL GPAATLEEM TACQGVGGPGK wk 4, 8, 24
 wk 8 -----
 wk 24 -----
 B44 cont. overlapping B44 *A11...

 wk 4 HKARVL
 wk 8 -----
 wk 24 -----
 A11 cont.

410 wk 4	MGARASVLSG	GQLDRWEKIR	LRPGGKKQYK	LKHIVWASRE	LERFAVNPGL	LETAEGCRQI
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
		wk 4, 8, 24				
410 wk 4	LEQLQPALQT	GSEELRSLYN	TVATLYCVHQ	RIDVKDTKEA	LEKIEEEQNK	SKKRAQLVAA
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
		*A2				
410 wk 4	GAGNSNQGAS	QNFPIVQNLO	GQMVHQPISP	RTLNAWVKVI	EEKAFSPEVI	PMFSALSEGA
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
		wk 4, 8, 24				
410 wk 4	TPQDLNTMLN	TVGGHQAAMQ	MLKHTINEEA	ADWDRHLHPPQ	AGPVAPGQIR	DPRGSDIAGT
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
		*A2				
410 wk 4	TSTLQEQITW	MTSNPPPIVG	EIYKRWIILG	LNKIVRMYS	VSILDIKQGP	KEPFRDYVDR
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
410 wk 4	FYKVLRAEQA	SQDVKNWMT	TLLVQNANPD	CKTILKALGP	AATLEEMMTA	CQGVGGPGHK
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
410 wk 4	ARVL					
410 wk 8	----					
410 wk 24	----					
410 wk 4	MGARASVLSG	GQLDRWEKIR	LRPGGKKQYK	LKHIVWASRE	LERFAVNPGL	LETAEGCRQI
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
		wk 4, 8, 24				
410 wk 4	LEQLQPALQT	GSEELRSLYN	TVATLYCVHQ	RIDVKDTKEA	LEKIEEEQNK	SKKRAQLVAA
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
		*A2				
410 wk 4	GAGNSNQGAS	QNFPIVQNLO	GQMVHQPISP	RTLNAWVKVI	EEKAFSPEVI	PMFSALSEGA
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
		wk 4, 8, 24				
410 wk 4	TPQDLNTMLN	TVGGHQAAMQ	MLKHTINEEA	ADWDRHLHPPQ	AGPVAPGQIR	DPRGSDIAGT
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
		*A2				
410 wk 4	TSTLQEQITW	MTSNPPPIVG	EIYKRWIILG	LNKIVRMYS	VSILDIKQGP	KEPFRDYVDR
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
410 wk 4	FYKVLRAEQA	SQDVKNWMT	TLLVQNANPD	CKTILKALGP	AATLEEMMTA	CQGVGGPGHK
410 wk 8	-----	-----	-----	-----	-----	-----
410 wk 24	-----	-----	-----	-----	-----	-----
410 wk 4	ARVL					
410 wk 8	----					
410 wk 24	----					

Figure 3. Longitudinal Gag Sequence Alignments

Viral RNA was extracted from longitudinal plasma samples from each subject at various time points during TI. The population of *gag* genes from each plasma sample was amplified by RT-PCR and population-based sequencing was performed. The amino acid sequence of the p17 and p24 regions of Gag have been aligned chronologically for each subject. Nucleotide mixtures that prevented the determination of a specific amino acid are displayed as Xs. The CD8 responses detected in each patient during TI are highlighted in orange. Listed above each epitope is the week(s) at which the response was detected, and listed below each epitope is the HLA class I restriction of that specific response. An asterisk indicates the response had the highest relative magnitude of IFN- γ release.

410

Viral Env	Plasma Samples					Broadly Neut Control
	Pre-Tx	0 wk	4 wk	8 wk	24 wk	
aMLV	28	<20	23	<20	20	<20
Pre-Tx	50	119	683	1166	724	47
4 wk	40	51	59	95	171	47
8 wk	33	24	32	58	176	105
24 wk	68	<20	41	24	46	59
JRCSF	30	<20	25	32	41	165
NL4-3	48	45	308	1657	1640	702
SF162	131	58	548	6202	5336	10566

Figure 4. Neutralizing Antibody Activity

Neutralizing antibody activity was measured in longitudinal plasma samples from each study subject using a cell-based infectivity assay. Plasma was tested for autologous neutralizing activity against virus isolated from the last time point prior to the start of treatment (Pre-Tx) and from various time-points during TI. Patient plasma was also tested for heterologous neutralizing activity using HIV-1 reference strains; JRCSF, NL4-3, and SF162. As a positive control, plasma from a drug-naïve, chronically infected patient with known broadly neutralizing antibody activity was tested against all patient-derived viruses and controls. Neutralization titers are expressed as the reciprocal of the plasma dilution that inhibited virus infection by 50% (IC_{50}). Green shading on the table indicates a neutralizing titer three times greater than the negative control infection using murine leukemia virus (aMLV) pseudotyped viral particles. Black boxes represent antibody titers measured against contemporaneous autologous virus samples. Each point on the graphs represents the IC_{50} of one plasma sample against one autologous viral isolate. Each line on the graphs represents the autologous neutralization of one viral sample over time.

Subject 311

wk4 MRVKEIRKNCQRL--WKNGIMLGLMIMCSATEKLWVIVYGVFVWKEANTTLFCASDAKAYDTEVHNWATHACVPTDPNPQEVVLENTENEMWKNOM
 wk16
 wk24
 wk36
 V1 V2
 wk4 VDQMHEDIISLWDQSLKPCVKLTPLCVTLNCTNVNTTESVNAINSVNAINTNSSSSWEMGEIKNCSEFNITTSIRNQMCKEYALPYKLDVVPIDGDN
 wk16
 wk24
 wk36
 V2
 wk4 SYTLINCNSTSVITQACPKVSEPIPIHYCAPAGFAILKCNKTFNGTGPCTNVSTVQCTHGIRPVVSTQILLNGSLAEZEVVIRSENFNTNNAKTIIVQLN
 wk16
 wk24
 wk36
 V3 V4
 wk4 KSIPIINCTRPNNNTSKSIHI--GPGRAFATGAIMGNIRQAHCNLSGAAMGEALKQIVTKIKKQPG--NKILIVFNQSSGGDPEIVMHSFNCGGEFFYCN
 wk16
 wk24
 wk36
 V4 V5
 wk4 QLEPISFWN---DTAGPNIDINGTIIILPCRIKQIINRQEVGKAMYAPPIRGQIKCSSNITGILLTRDGGNVGTNTEIRPVGGMDDKNRSELYKIKV
 wk16
 wk24
 wk36
 wk4 VKIEPLGVAPTAKRRVVRQEKRAVIGIGAVLGLGAGSTMGAASMTLVQARILLSGIVQQNNILRAIEAQOHLQLTVWGIKQLQARVLAVERLYK
 wk16
 wk24
 wk36
 wk4 DQQLLGIGWCSGKLICTTAVPNASWSNKLKDIWNMTWMEWEREIDNYTSLIYTLLEESQNQKEKNEQLLELDKWAISLWNPFDITNWLWYIKIFIMI
 wk16
 wk24
 wk36
 wk4 VGGVLGLRIITVISIVNRVRQGYSPLSFQTRLPTFRGPDREFGIEEGGERDRDRSLVDGFLALIWDDLRLSLCLFSYHRLRDLILVIRIVELLGR
 wk16
 wk24
 wk36
 wk4 GNEVLKYWNLLQYWSQELKNSAITLFNATAIAVAGGTRVIEVLQRAIRHPRIRGGIERALL
 wk16
 wk24
 wk36

Subject 313

wk4 MRVKGIRKNCQRL--WRNGIMLGLMIMCSATD--LWVIVYGVFVWKEANTTLFCASDAKAYDTEVHNWATHACVPTDPNPQEVVLENTENEMWKNOM
 wk8
 wk16
 wk24
 V1 V2
 wk4 VEQMHEDIISLWDQSLKPCVKLTPLCVTLNCTNVNTINANFS--REIKKEGEIKNCSEFNITTSIRNQMCKEYALPYKLDVVPIDNSISNKSSTSNF
 wk8
 wk16
 wk24
 V2
 wk4 TSYRLISCNSTSVITQACPKVSEPIPIHYCAPAGFTILKCKDKKFKGKDKNVSTVQCTHGIRPVVSTQILLNGSLAEZEVVIRSDNFSENNAKTIIVQL
 wk8
 wk16
 wk24
 V3 V4
 wk4 NETVIINCTRPNNNTSK--GIRMGPGRAFYTTEGIIIGDIRQAHCNLSRTQWNRKTELELVARKLGEQY--NKEIVFNQASGGDPEIVAHSEFNCGGEFFYCN
 wk8
 wk16
 wk24
 V4 V5
 wk4 TSLFSSSTWSLNSITWNLNANSSVENDITITICRIKQPINMNGEVGKAMYAPPINGQLNCSSNITGILLTRDGGNVGTNTEIRPVGGMDDKNRSELYK
 wk8
 wk16
 wk24
 wk4 KVVRIEPLGIAPTEAKRRVVRQEKRAVIGIGAVLGLGAGSTMGAASMTLVQARILLSGIVQQNNILRAIEAQOHLQLTVWGIKQLQARVLAVERLY
 wk8
 wk16
 wk24
 wk4 LHDQQLLGIGWCSGKLICTTAVPNASWSNKLKDIWNMTWMEWEREISNYTGLIYNLLEESQNQKEKNEQLLELDKWAISLWNPFDITNWLWYIRLFI
 wk8
 wk16
 wk24
 wk4 MIVGGVLGLRIVCAVFSIVNRVRQGYSPLSFQTRLPAFRGPDREFGIEEGGERDRDRSLVLTGFLPLIWDDLRLSLCLFSYHRLRDLILVIRIVELLG
 wk8
 wk16
 wk24
 wk4 RRGNEVLKYWNLLQYWSQELKNSAITLFNATAIAVAGGTRVIEVLQRAIRHPRIRGGIERALL
 wk8
 wk16
 wk24

Figure 5. Longitudinal Envelope Sequence Alignments

Viral RNA was extracted from longitudinal plasma samples from each subject at several time points during TI. The population of *env* genes from each plasma sample was amplified by RT-PCR and population-based sequencing was performed. The amino acid sequences of the

gp160 genes from each subject at the various time-points were aligned according to HXB2 positioning. Amino mixtures are displayed as Xs.

Characteristics of the Study Participants

Table 1

Patient ^a	Age	ELISA at Diagnosis	Days from Onset of Symptoms	Baseline Values at Initiation of Therapy				Initial Therapy	Days on Therapy Before First Treatment Interruption
				ELISA	Western Blot Number of Bands	CD4+ Count (cells/mm ³)	HIV Viral Load (bDNA Copies/ml)		
311	40	Pos	60	Pos	Pos (3 bands)	704	63,540	AZT,3TC,NFV	1452
313	38	Pos	141	Pos	Pos (6 bands)	375	20,160	AZT,3TC,NFV	1547
410	43	Pos	82	Pos	Pos (6 bands)	270	79,470	AZT,3TC,NFV	1199

pos = positive

Table 2
Epitopes Recognized by the CD8⁺ T cell Response of Each Subject during TI

The table contains a list of the epitopes targeted by the CD8⁺ T cell response of each subject at several longitudinal time-points during TI. The targeted epitopes for each subject were deduced by cross-referencing the list of peptides from the responding peptide pools with the current published list of defined HLA-A-restricted epitopes (<http://www.hiv.lanl.gov/content/immunology>).

Subject	HLA Restriction	Targeted Epitope	0 wk	8 wk	16 wk	48 wk
311	B8	LRPGGKKKYKHKHIV	-	-	-	+
	A23	KYKHKHIVW	-	-	-	+
	B8	ELRSLYNTV	+	++	+	++
	A1	GSEELRSLY	+	++	+	++
	B8	EVKDTKEAL	-	-	-	+
	B8	NPPIPVGEIYKRWIILGLNKIV	-	-	-	+
	B8	VKNWMTETLL	-	-	-	+
	B8/B51	VQNANPDCKTILKAL	-	-	-	+
	A11	VATLYCVHQRI	+	+	+	++
	B44	RDYVDRFYKTL	-	-	-	+
313	B44	AEQASQEVKNWMT	-	-	-	+
	A11	ACQGVGGPGHK	-	+	+	+
410	A2	SLYNTVATL	+	+	++	+++
	A2	GHQAAMQMLKE	+	+	++	+++