Maintenance of viral suppression in HIV-1–infected HLA-B*57$^+$ elite suppressors despite CTL escape mutations

Justin R. Bailey,[1] Thomas M. Williams,[3] Robert F. Siliciano,[1,2] and Joel N. Blankson[1]

[1]Department of Medicine and [2]Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21287
[3]Department of Pathology, University of New Mexico School of Medicine and TriCore Reference Laboratories, Albuquerque, NM 87131

Rare human immunodeficiency virus 1–infected individuals, termed elite suppressors (ES), maintain plasma virus levels of <50 copies/ml and normal CD4 counts without therapy. The major histocompatibility complex class I allele group human histocompatibility leukocyte antigen (HLA)-B*57 is overrepresented in this population. Mutations in HLA-B*57–restricted epitopes have been observed in ES, but their significance has remained unclear. Here we investigate the extent and impact of cytotoxic T lymphocyte (CTL) escape mutations in HLA-B*57$^+$ ES. We provide the first direct evidence that most ES experience chronic low level viremia. Sequencing revealed a striking discordance between the genotypes of plasma virus and archived provirus in resting CD4$^+$ T cells. Mutations in HLA-B*57–restricted Gag epitopes were present in all viruses from plasma but were rare in proviruses, suggesting powerful selective pressure acting at these epitopes. Surprisingly, strong CD8$^+$ T cell interferon-γ responses were detected against some mutant epitopes found in plasma virus, suggesting the development of de novo responses to viral variants. In some individuals, relative CD8$^+$ T cell interleukin-2 responses showed better correlation with the selection observed in vivo. Thus, analysis of low level viremia reveals an unexpectedly high level of CTL escape mutations reflecting selective pressure acting at HLA-B*57–restricted epitopes in ES. Continued viral suppression probably reflects CTL responses against unmutated epitopes and residual or de novo responses against epitopes with escape mutations.

CORRESPONDENCE
Joel N. Blankson:
jblanks@jhmi.edu

Abbreviations used: ES, elite suppressors; LTNP, long-term nonprogressor; SIV, simian immunodeficiency virus; SFC, spot-forming cell.

Numerous studies of HIV-1 infection in humans and simian immunodeficiency virus (SIV) infection in macaques have demonstrated a correlation between CD8$^+$ T cell responses and the restriction of viral replication. There is a temporal relationship between the development of CD8$^+$ T cell responses and the decline in viremia after acute HIV-1 infection (1, 2). In addition, CD8$^+$ T cell depletion in SIV-infected macaques leads to higher viral loads and faster disease progression (3–5). Despite immune pressure from CTLs, most HIV-infected individuals experience high levels of ongoing viral replication. CTL escape mutations commonly develop during infection with HIV-1 (6–12) and SIV (13–17). In some cases, these escape mutations have correlated directly with loss of viral suppression (18–20). There is also a correlation at a population level between mutations at specific HIV-1 epitopes and the expression of MHC class I alleles capable of presenting those epitopes (21, 22). Collectively, these studies suggest that CTL escape is an important mechanism of immune evasion, allowing ongoing viral replication and progressive loss of CD4$^+$ T cells in most HIV-1–infected individuals.

Some HIV-1–infected individuals, termed long-term nonprogressors (LTNPs), maintain high CD4$^+$ T cell counts for many years without antiretroviral treatment (23–25). A subset of LTNP, termed elite suppressors (ES), maintains viral loads of <50 copies of HIV RNA/ml of plasma and normal CD4$^+$ T cell counts without therapy. A large proportion of ES express the MHC class I allele HLA-B*57 (26).

The online version of this article contains supplemental material.

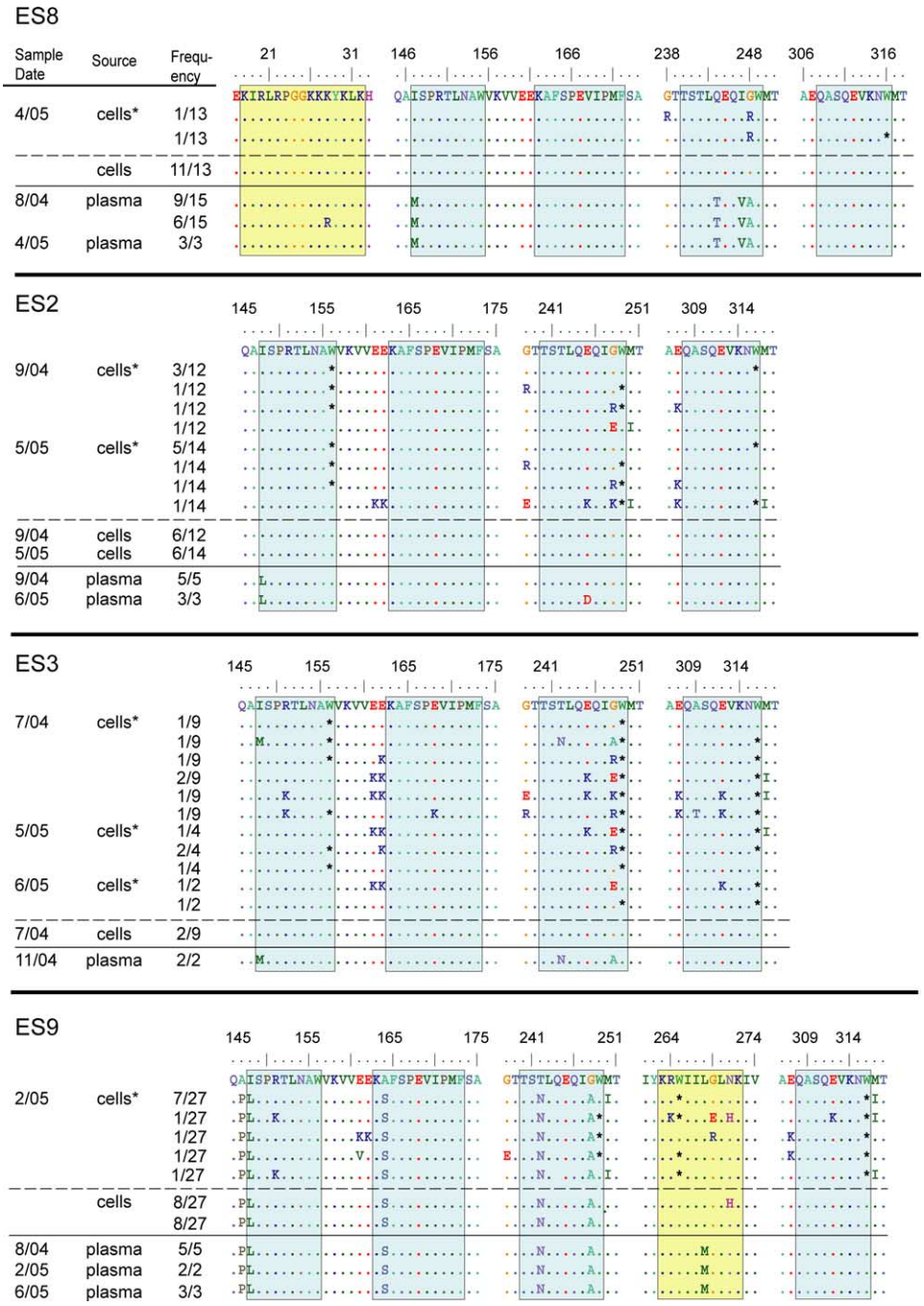

Figure 1. Sequences of HLA-B*57–restricted Gag epitopes and other CTL epitopes in *gag* genes amplified from proviral DNA and plasma of ES. Areas shaded in blue are optimal HLA-B*57–restricted epitopes. Areas shaded in yellow are non–HLA-B*57–restricted epitopes that stimulate $CD8^+$ T cell IFN-γ responses in that individual. Intervening *gag* sequence between these epitopes is not shown. The first sequence of each group is the *gag* clade B consensus sequence. Homology to this sequence is indicated by a dot. Stop codons are indicated by an asterisk (*) in place of an amino acid. Asterisks in the "Source" column indicate sequences from resting $CD4^+$ T cells with characteristic APOBEC3G-mediated G→A hypermutation. Sequences are listed in the following order: hypermutated proviral sequences, nonhypermutated proviral sequences, and sequence from plasma virus.

One interesting study showed that some HLA-B*57^+ ES have evidence of mutations in HLA-B*57–restricted epitopes in PBMC provirus, whereas others do not (27). The significance of these mutations has remained unclear. Because *gag* genes could not be amplified from plasma virus of ES with viral loads <50 copies/ml of plasma, it was not possible to determine whether the observed mutations gave the mutant virus a biologically relevant selective advantage. As efforts

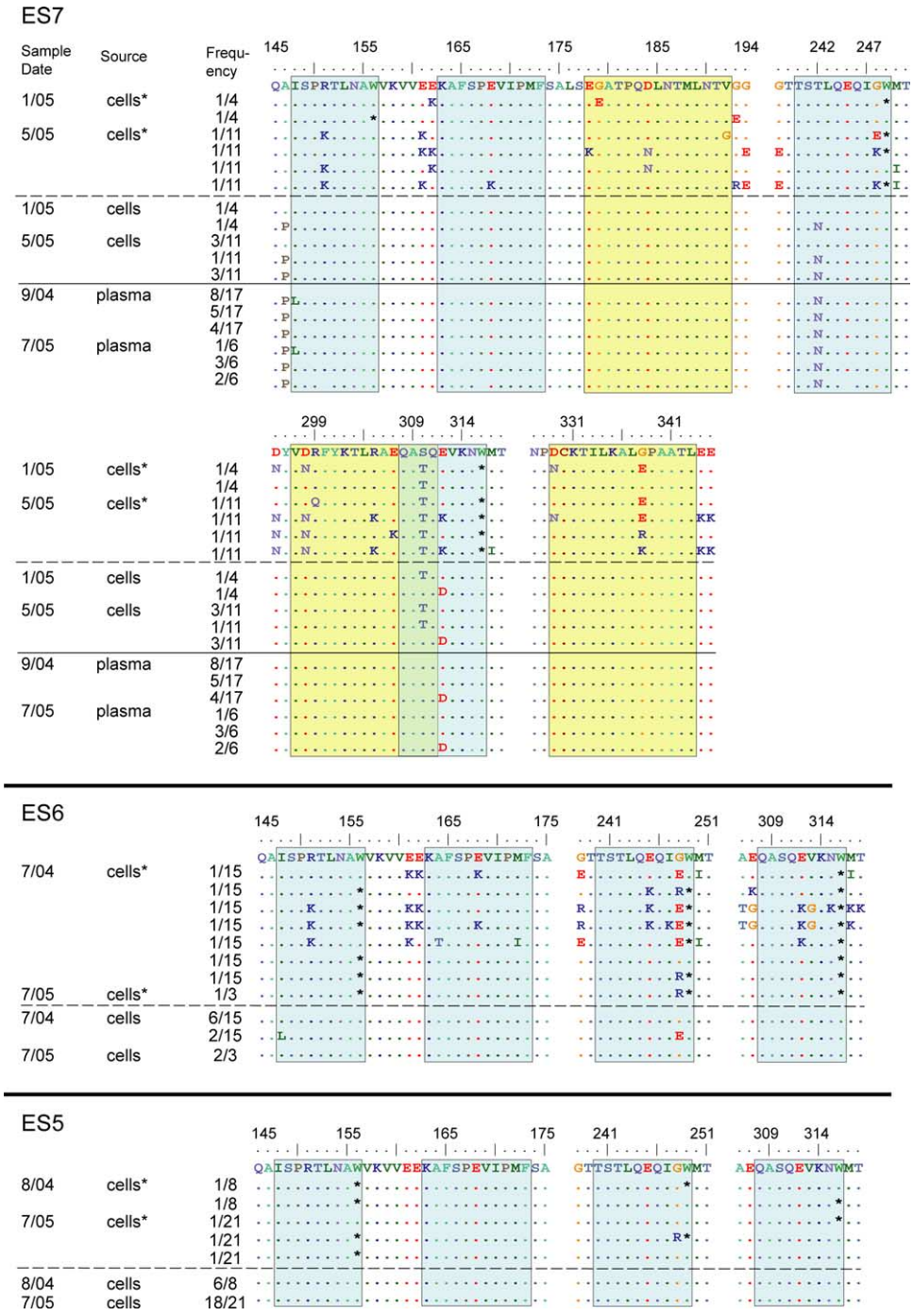

Figure 1 (continued)

to develop an effective vaccine against HIV-1 continue, it is extremely important to understand the extent and nature of $CD8^+$ T cell escape that is tolerated in ES without loss of viral suppression.

We have studied CTL escape in seven ES who have had documented HIV-1 infection for an average of 11 yr. All have maintained viral loads of <50 copies/ml of plasma upon repeated measurements, and none have received antiretroviral therapy. We used a novel approach to determine whether ongoing selective pressure by CTLs is important for the control of viremia in ES. Although ES maintain clinically undetectable viral loads, we show here that free virus can be detected in the plasma of most of these individuals using sensitive RT-PCR assays. We amplified near full-length *gag* genes from this plasma virus and from proviral DNA in resting $CD4^+$ T cells, allowing sequence comparisons between the two viral populations. We then tested IFN-γ responses in each subject against all well-defined HLA-B*57–restricted

epitopes in the HIV-1 genome as well as overlapping peptides spanning the entire *gag* gene. Finally, we measured relative IFN-γ production and relative IL-2 responses to wild-type Gag epitopes and potential plasma virus escape variants to determine how these responses related to the strong selective pressure observed in vivo.

RESULTS

All plasma viruses have mutations in HLA*B57-restricted Gag epitopes

Proviral *gag* and *nef* sequences were successfully PCR amplified from resting $CD4^+$ T cells of seven out of seven HLA-$B*57^+$ ES who had viral loads of <50 copies/ml (Fig. 1). In five out of seven of these subjects, *gag* sequences were also successfully amplified from free virions in the plasma by an extremely sensitive RT-PCR method, even though the viral load was consistently below the limit of detection of ultrasensitive clinical assays (50 copies/ml). In each of these five subjects, plasma virus sequences were successfully amplified on at least two different time points 8–10 mo apart. These results provide the first direct evidence that many ES with viral loads of <50 copies/ml experience a chronic state of extremely low level viremia. The number of independent *gag* sequences that could be amplified, particularly in ES3, was limited by the extremely low viral loads in these subjects. To eliminate the problem of PCR resampling (28), only genes amplified in independent PCR reactions were considered to be independent. Although defective *nef* genes have been demonstrated in some LTNPs (29–31), none of these subjects had any evidence of truncations or deletions of proviral *nef* genes (not depicted).

In all cases where plasma virus could be successfully amplified, there was a striking discordance between *gag* sequences obtained from this plasma virus and sequences obtained from archived provirus in resting T cells. This difference, which was observed at all time points, suggests strong selective pressure acting on the plasma virus (Fig. 1). Many of the differences between cellular proviral *gag* and plasma virus *gag* sequences occurred in well-defined HLA-B*57–restricted Gag epitopes, including ISPRTLNAW (IW9), KAFSPEVIPMF (KF11), and TSTLQEQIGW (TW10) (32). In the five individuals from whom both cellular provirus and plasma virus *gag* sequences could be amplified, the mean number of amino acid differences between provirus and plasma virus Gag ranged from 6.7 to 8.7 (Table I). A median of

Table I. Frequency of mutations in HLA-B*57–restricted epitopes

Study subject	Mean no. of A.A. differences[a] between provirus and plasma virus Gag	Mean no. of provirus→ plasma virus A.A. changes away from consensus[b]	Mean no. of provirus→ plasma virus A.A. changes toward consensus[b]	Predicted no. of A.A. differences at A146, I147, T242, and G248 of Gag[c,d]	Observed no. of A.A. differences at A146, I147, T242, and G248[d]	
ES8	7.5	7.5	0	0.07	2	
ES2	8.7	6.4	2.3	0.08	1	
ES3	8	7	1	0.07	3	
ES7	5.4	2.4	3	0.05	1.3	
ES9[e]	6.7	4.9	1.8	0.06	0	
median	7.5	6.4	1.8	0.07	1.3	

Study subject	Fold increase	p-value	Predicted no. of differences in HLA-B*57–restricted epitopes[c]	Observed no. of A.A. differences in HLA-B*57–restricted epitopes	Fold increase	p-value
ES8	28.6	<0.001	0.7	4	5.7	<0.001
ES2	12.5	<0.001	0.8	1.4	1.8	NS
ES3	42.9	<0.001	0.7	3	4.3	<0.01
ES7	26	<0.001	0.5	2	4	<0.025
ES9[e]	0	NS	0.6	0	0	NS
median	26	–	0.7	2	4	–

[a]Mean number of amino acid differences between Gag from provirus and Gag from plasma virus was calculated to include mutations that were present in <100% of the proviral Gag quasispecies or <100% of the plasma virus Gag quasispecies. Mutations were only counted in this analysis if they were detected in at least two independent *gag* genes, and hypermutated Gag sequences were not included in the analysis.

[b]Provirus→plasma virus Gag changes were separated into those where plasma virus diverged more than provirus from the subtype B consensus Gag sequence ("away from consensus") and those where changes in plasma virus Gag were reversions back to the consensus sequence ("toward consensus").

[c]Predicted number of amino acid differences at A146, I147, T242, and G248 of Gag or in HLA-B*57–restricted epitopes in Gag was calculated based upon the overall number of differences between provirus and plasma virus Gag and assuming a random distribution of mutations across the gene.

[d]Mutations at A146, I147, T242, and G248 were specifically examined because these residues fall within or immediately adjacent to HLA-B*57–restricted epitopes, and mutations at these specific amino acids are more frequent in viremic $HLA\text{-}B*57^+$ individuals than in viremic $HLA\text{-}B*57^-$ individuals. Mutations at A146 and T242 have been shown to act as CTL escape mutations.

[e]All Gag from both provirus and plasma virus in this individual had A146P, I147L, T242N, and G248A mutations. Thus, despite the presence of mutations, there was no change in plasma virus relative to provirus at these residues. However, there were an average of 1.5 changes at the HLA-B*27–restricted KK10 epitope in plasma virus Gag relative to provirus Gag, which is ninefold greater than the predicted number of changes at this epitope based upon the overall frequency of changes in Gag ($P < 0.001$).

2 (range: 0–4) of these amino acid differences was within or immediately flanking HLA-B*57–restricted epitopes. A median of 1.3 (range: 0–3) of these differences was at A146, I147, T242, and G248 of Gag. These amino acids are within or immediately flanking HLA-B*57–restricted epitopes, and they have been shown to mutate more frequently in viremic HLA-B*57$^+$ individuals than in viremic individuals who are HLA-B*57$^-$ (33, 34). In ES8, ES2, ES3, and ES7, the number of changes at A146, I147, T242, and G248 was 12.5–42.9 times higher than would be expected if the amino acid differences between provirus and plasma virus Gag in these individuals were randomly distributed ($P < 0.001$). The absence of amino acid differences between provirus and plasma virus Gag in HLA-B*57–restricted epitopes in ES9 could be explained by the fact that this individual had A146P, I147L, T242N, and G248A mutations in all provirus and all plasma virus Gag sequences (Fig. 1). All viral clones obtained from the plasma showed at least one mutation in an HLA-B*57–restricted Gag epitope, and some of these changes were mutations previously described in HIV-1–infected HLA-B*57$^+$ patients with progressive disease, including I147L in the IW9 epitope as well as T242N and G248A in the TW10 epitope (33, 34). A146P, seen in plasma virus of both ES9 and ES7, is a processing mutation that blocks presentation of the IW9 epitope and causes escape from T cell responses to that epitope (33). Plasma viruses of ES9, ES3, and ES7 bear the T242N mutation in the TW10 epitope. This mutation has been extensively studied in HIV-1–infected patients with progressive disease and is known to cause escape from CD8$^+$ T cell IFN-γ responses (34, 35). A G248E mutation in the TW10 epitope appeared along with I147L in IW9 in 2 out of 10 nonhypermutated proviral clones of ES6, a subject from whom no plasma virus *gag* could be amplified. Some previously uncharacterized mutations were also observed in plasma viruses. These included I147M in the IW9 epitope in ES8 and ES3, the Q244T/I247V/G248A triple mutation in the TW10 epitope of ES8, and E245D in the TW10 epitope of some plasma clones of ES2.

Despite the presence of mutations in all plasma viral *gag* clones, most proviral cellular sequences were entirely wild-type at HLA-B*57–restricted epitopes, suggesting that these subjects were not initially infected with virus bearing HLA-B*57 escape mutations (Fig. 1). One subject, ES7, showed a mixture of wild-type and mutant clones in cellular provirus, but 23 out of 23 plasma clones bore HLA-B*57–restricted epitope mutations. Collectively, these data suggest that mutations in HLA-B*57–restricted epitopes in this population occurred sometime after infection and that these mutations likely continue to confer a selective advantage to plasma virus. Another subject, ES9, showed changes relative to subtype B consensus in multiple HLA-B*57–restricted epitopes in both the cellular provirus and plasma virus. It is therefore possible that this subject was initially infected with virus bearing escape mutations in HLA-B*57–restricted epitopes. This subject, who is HLA-B*27$^+$ as well as HLA-B*57$^+$, nonetheless showed discordance between cellular and plasma virus, with a L268M mutation at the HLA-B*27–restricted Gag epitope KRWIILGLNK (KK10) present in all (10 out of 10) plasma sequences and no (0 out of 27) cellular sequences. This mutation has been described previously as a precursor mutation required for the development of a R264K escape mutation in the same epitope (20, 36).

Substantial numbers of proviral clones from each subject showed characteristic APOBEC3G-mediated G to A hypermutation (Fig. 1; references 37–41). Hypermutation of proviral DNA has been observed previously in patients on suppressive highly active antiretroviral therapy and in chronically infected patients (42, 43). Consistent with previous observations in patients on highly active antiretroviral therapy, hypermutated sequences were not detected in the plasma (42). The fraction of hypermutated proviral clones varied between subjects, ranging from 2 out of 13 independent clones in ES8 to 13 out of 15 in ES3. The majority of the hypermutated proviral sequences did not carry the HLA-B*57–restricted epitope mutations found in plasma virus, which is consistent with the hypothesis that these are ancestral, archived viruses. The significance of this finding remains unclear, as similarly detailed studies involving limiting dilution PCR of proviral *gag* have not yet been performed in other HIV-infected populations. It is noteworthy that subjects with the highest proportion of hypermutated provirus nevertheless had detectable nonhypermutated plasma virus.

CD8$^+$ T cell IFN-γ responses focus on HLA-B*57–restricted Gag epitopes

Using ELISPOT assays for IFN-γ production, we assessed CD8$^+$ T cell IFN-γ responses to peptides representing the wild-type sequences of all well-characterized HLA-B*57–restricted epitopes in all HIV-1 genes as well as overlapping peptides spanning the entire *gag* gene (Fig. 2; reference 32). Responses were highly focused on HLA-B*57–restricted Gag epitopes, as described previously (26, 44), but responses to non–HLA-B*57–restricted Gag epitopes were also detected in three patients in at least two independent experiments with CD4$^+$ T cell–depleted PBMCs. ES9, the HLA-B*27$^+$ subject, responded to the HLA-B*27–restricted KRWIILGLNK (KK10) epitope. ES8 responded to a peptide spanning Gag amino acids 17–31 (KK15), which had a K26R mutation in a minority of plasma virus clones, and ES7 responded to three non-B57–restricted peptides spanning amino acids 178–192 (EV15), 297–311 (VQ15), and 329–343 (DL15), none of which showed mutations in plasma virus (Fig. 1). Responses to overlapping peptides containing HLA-B*57–restricted Gag epitopes were consistent with responses against optimized HLA-B*57–restricted Gag epitopes (not depicted). Although potential escape mutations were detected in this population, CD8$^+$ T cells from all subjects except ES9 showed IFN-γ response against at least one epitope that was not mutated in either cellular provirus or in plasma virus. ES3 showed plasma virus mutations in both targeted Gag epitopes, but an IFN-γ response to the IW9 epitope in the RT gene was also present. We therefore sequenced

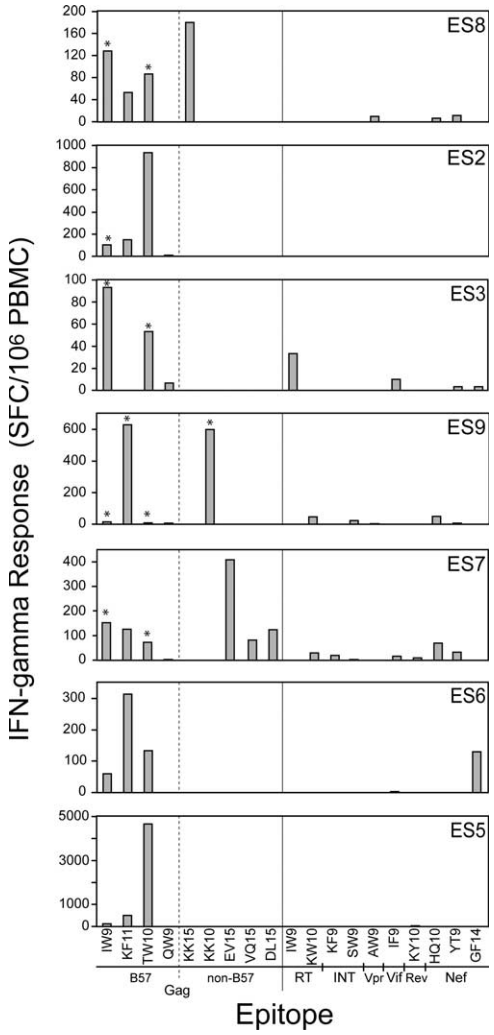

Figure 2. CD8+ T cell IFN-γ responses, expressed as SFCs per million PBMCs, to wild-type forms of well-defined HLA-B*57–restricted epitopes in all HIV-1 genes and responses to non-B57–restricted overlapping peptides spanning the entire *gag* gene. The first four peptides shown in Gag (IW9, KF11, TW10, and QW9) are HLA-B*57 restricted. Epitopes that are mutated in all plasma virus *gag* genes from that individual are indicated by an asterisk (*).

the RT gene from plasma virus in this subject and found no evidence of mutation in the targeted epitope in clones from seven out of seven independent RT-PCR reactions (not depicted). Thus, the presence of epitopes that remain free of escape mutations despite targeting by CD8+ T cells may be one key for maintenance of suppression in these ES.

Relative CD8+ T cell IFN-γ responses to wild-type and mutant epitopes correlate poorly with the persistence of plasma virus–bearing mutations in the targeted epitopes

We sought to determine whether the presence of plasma virus variants with mutations in targeted epitopes correlated with relative CD8+ T cell IFN-γ responses to particular epitopes. IFN-γ responses by CD8+ T cells from each subject in response to plasma virus variants of targeted epitopes were analyzed and compared with the response to the corresponding wild-type peptide (Fig. 3). Surprisingly, mutant plasma virus epitopes generally stimulated IFN-γ responses that were as good as or better than responses to the corresponding wild-type epitopes. ES8 showed nearly equivalent responses to the I147M plasma virus variant and the wild-type proviral form of IW9 and a slightly diminished response to the K28R variant of the non–HLA-B*57–restricted KK15 peptide (Fig. 3, A and C). This subject unexpectedly showed a significantly greater IFN-γ response to the Q244T/I247V/G248A plasma virus variant of TW10 than to the wild-type proviral epitope (Fig. 3 B). In the case of ES2, although I147L was the only HLA-B*57–restricted epitope mutation present in all plasma viruses, CD8+ T cells from this subject showed equivalent IFN-γ responses to the I147L and wild-type form of the IW9 epitope (Fig. 3 D). This subject had a diminished IFN-γ response to the E245D variant of TW10 that was found in some plasma viruses (Fig. 3 E). Like ES8, ES3 showed equivalent responses to the I147M and wild-type variants of IW9 (Fig. 3 F). In this subject, there was a strong response to the wild-type form of the TW10 epitope but no detectable response to the T242N/G248A double mutant detected in the plasma (Fig. 3 G). This double mutation has been previously shown to stimulate a decreased IFN-γ response by CD8+ T cells relative to the wild-type TW10 peptide (34). ES9 had nearly equivalent responses to the wild-type and mutant versions of the KF11 epitope (Fig. 3 H). In addition, response to the L268M plasma virus variant of the HLA-B*27–restricted KK10 epitope was equivalent to or greater than responses to the wild-type and N271H forms found in resting CD4+ T cells (Fig. 3 I). ES7 also showed equivalent IFN-γ responses to wild-type and I147L plasma virus variants of IW9 (Fig. 3 J). As observed in ES3, ES7 responded poorly to the T242N variant of TW10 (Fig. 3 K). Although no plasma virus *gag* sequences could be amplified in ES6, this subject had a minority population of cellular virus with the I147L mutation in IW9 and G248E mutation in TW10. Like the other subjects tested, ES6 had equivalent responses to wild-type and I147L forms of IW9 (Fig. 3 L). Like ES8, this subject had a significantly greater response to a mutant variant of TW10 (G248E) than to the wild-type peptide (Fig. 3 M). Collectively, these results suggest that although selective pressure apparently leads to the appearance of plasma virus variants with mutations in CTL epitopes, some of these mutant epitopes stimulate CD8+ T cell IFN-γ responses that equal or surpass the responses stimulated by wild-type epitopes. The responses of ES8 and ES6 to their variant TW10 epitopes are particularly suggestive of de novo responses. Overall, relative CD8+ T cell IFN-γ responses correlated poorly with persistence of plasma virus variants with mutations in the targeted epitopes.

Mutations found in plasma virus *gag* can potentially mediate CTL escape

We asked whether peptides with the mutations found in plasma viruses were recognized as well as the corresponding

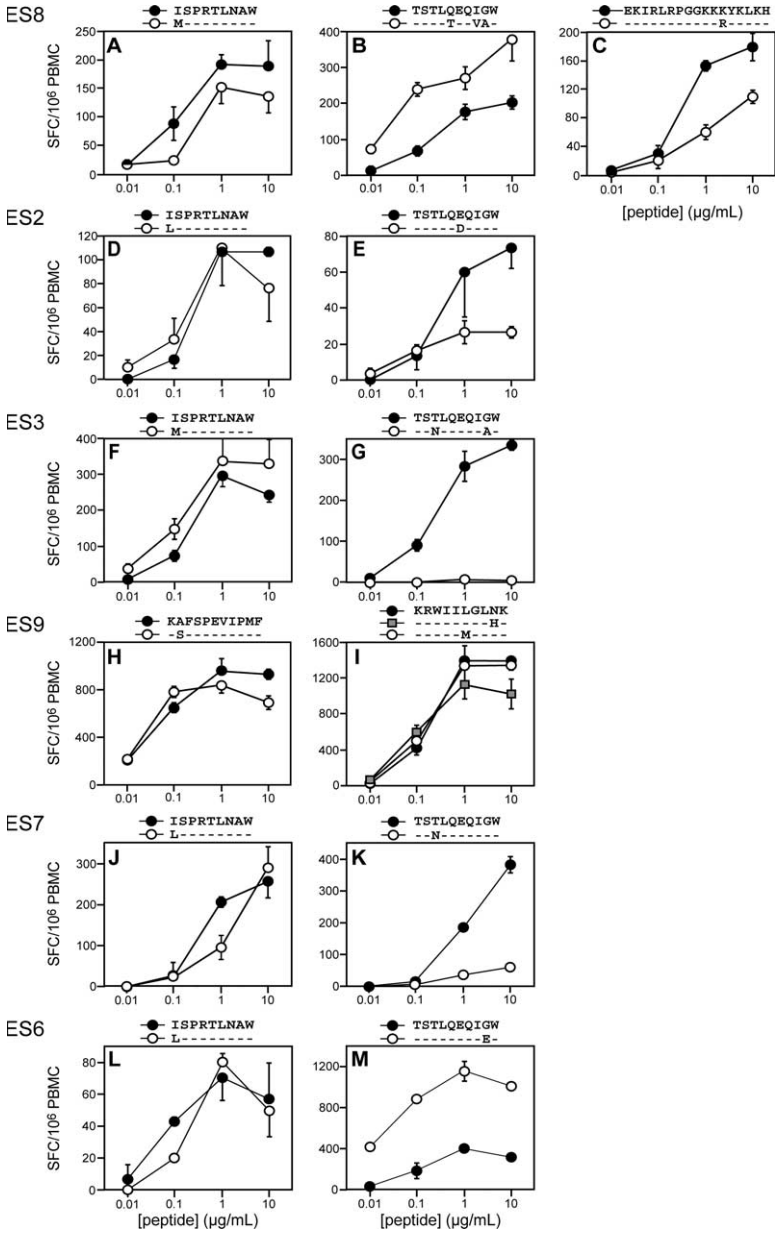

Figure 3. IFN-γ responses to autologous mutant epitopes. IFN-γ responses by $CD8^+$ T cells from each subject against wild-type targeted epitopes and mutant epitopes found in plasma virus or minor proviral populations in that individual. ●, responses against wild-type epitopes; ○, responses to plasma virus mutant epitopes. (A–C) Responses by ES8 to wild-type and mutant IW9, TW10, and EH15. (D and E) Responses by ES2 to wild-type and mutant IW9 and TW10. (F and G) Responses by ES3 to wild-type and mutant IW9 and TW10. (H and I) Responses by ES9 to wild-type and mutant KF11 and KK10. (J and K) Responses by ES7 to wild-type and mutant IW9 and TW10. (L and M) Responses by ES6 to wild-type and mutant IW9 and TW10.

wild-type epitopes by CTLs from other HLA-B*57$^+$ subjects. To examine this possibility, we assessed recognition of each mutant plasma virus epitope by epitope-specific T cells from other study subjects whose virus did not possess that particular mutation (Fig. 4 and Fig. S1, which is available at http://www.jem.org/cgi/content/full/jem.20052319/DC1). The L268M mutation in KK10 could not be tested in this manner because no other subjects were HLA-B*27$^+$. All mutations aside from I147L and I147M in the IW9 epitope were found to result in considerable loss of recognition by T cells of multiple subjects. For example, the Q244T/I247V/G248A triple mutant TW10 epitope that arose in ES8 was not recognized by T cells from ES2, ES3, or ES7, although T cells from each of these individuals recognized the wild-type TW10 epitope (Fig. 4 A and Fig. S1). None of these individuals had the Q244T/I247V/G248A triple mutation.

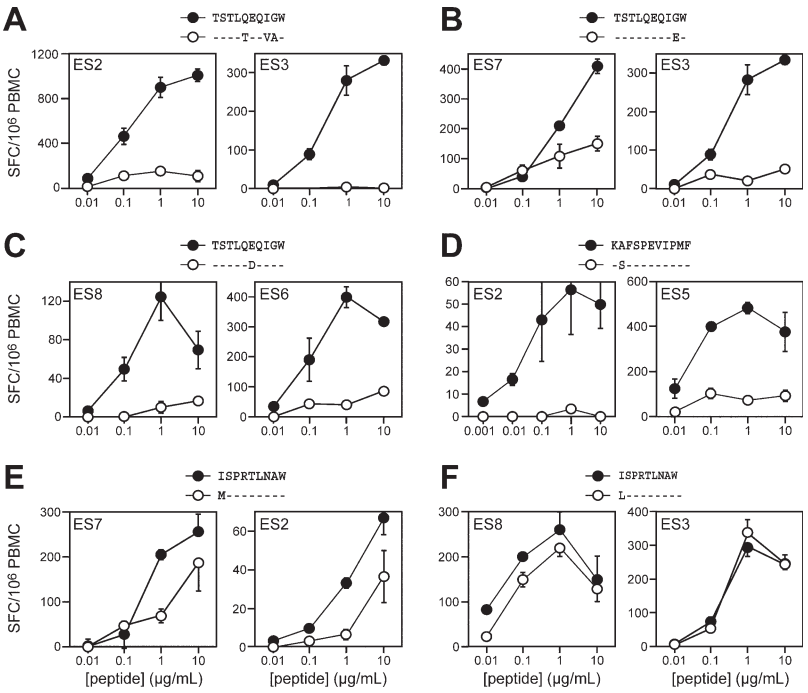

Figure 4. IFN-γ responses to nonautologous mutant epitopes. IFN-γ responses against wild-type and mutant epitopes by CD8$^+$ T cells from subjects whose virus does not have the mutation being tested. The subject providing PBMCs is indicated above each graph. ●, responses against wild-type epitopes; ○, responses to mutant epitopes. (A) Responses to wild-type TW10 and Q244T/I247V/G248A plasma virus mutant of TW10. (B) Responses to TW10 and G248E variant. (C) Responses to TW10 and E245A variant. (D) Responses to KF11 and A163S variant. (E) Responses to IW9 and I147M variant. (F) Responses to IW9 and I147L variant. Similar responses to these epitopes by additional subjects are shown in Fig. S1.

As shown in Fig. 4 B, the G248E mutant TW10 epitope detected in ES6 was poorly recognized by T cells from ES7 and ES3, although T cells from each individual recognized wild-type TW10. Interestingly, as shown in Fig. 3, these TW10 mutants were strongly recognized by CTLs from ES8 and ES6, the subject in which the mutation arose. Thus, it is appears likely that both patients developed de novo responses to these variant peptides. The E245D mutant TW10 epitope detected in ES2 was not recognized by T cells from ES8 or ES6, although T cells from each of these individuals recognized the wild-type TW10 epitope (Fig. 4 C). T cells from ES2, ES5, ES8, ES6, and ES7 recognized the A163S variant of the KF11 epitope poorly, although T cells from each recognized wild-type KF11 peptide (Fig. 4 D and Fig. S1). Collectively, these data suggest that the Q244T/I247V/G248A triple mutation in the TW10 epitope of ES8, the E245D mutation in the TW10 epitope of ES2, the G248E mutation in the TW10 epitope of ES6, and the A163S mutation in the KF11 epitope of ES9 can potentially act as CTL escape mutations because epitopes with these mutations are not recognized by some CTLs specific for other forms of the same epitope.

The I147M and I147L mutant variants of the IW9 epitope were recognized as well or nearly as well as the wild-type form of IW9 by T cells from several subjects. T cells from ES7, ES2, ES5, and ES6 showed similar IFN-γ responses to wild-type IW9 and the I147M variant (Fig. 4 E and Fig. S1). None of these individuals showed evidence of the I147M mutation, although virus from ES7, ES2, and ES6 did have an I147L mutation in the IW9 epitope. As shown in Fig. 4 F, T cells from ES8 and ES3 showed equivalent IFN-γ responses to the wild-type and I147L forms of IW9, although virus in both individuals had an I147M rather than an I147L mutation. Collectively, these results suggest that unlike the other mutations studied, the I147L and I147M mutations in IW9 do not significantly decrease IFN-γ responses by T cells specific for wild-type or other mutant variants of the IW9 epitope. These mutations may therefore be relatively poor mediators of CTL escape or they might affect the processing of Gag, preventing the presentation of the optimal epitope. This phenomenon has been reported with the I46P mutation that immediately precedes this IW9 epitope (33).

Relative CD8$^+$ T cell IL-2 responses to wild-type and mutant epitopes correlate with the viral variants found in plasma

The relative magnitude of CD8$^+$ T cell IFN-γ responses to wild-type proviral epitopes and mutant plasma virus epitopes could potentially explain the apparent selective advantage of the mutant plasma virus in two subjects, ES3 and ES7. All plasma clones in ES3 carried the T242N/G248A mutation in the TW10 epitope, which stimulated a significantly lower IFN-γ response than the wild-type epitope in this subject (Fig. 3 G). All plasma clones in ES7 carried both the I146P

mutation in the IW9 epitope and T242N in the TW10 epitope. I146P has been clearly shown to block presentation of the IW9 epitope, which would limit any T cell response to that epitope (33). In addition, the T242N variant of TW10 stimulated a lower IFN-γ response than wild-type TW10 in this individual (Fig. 3 K).

The relative magnitude of $CD8^+$ T cell IFN-γ responses to wild-type proviral epitopes and mutant plasma virus epitopes could not explain the apparent selective advantage of the mutant plasma virus relative to proviral wild-type virus in the remaining ES. We therefore examined another aspect of $CD8^+$ T cell function: the capacity of $CD8^+$ T cells from ES to secrete IL-2 in response to wild-type HLA-B*57–restricted epitopes found in cellular provirus and to mutant variants found in plasma virus (Fig. 5). $CD8^+$ IL-2 secretion has been shown to be an important correlate of protection in mouse models of chronic lymphocytic choriomeningitis virus infection (45, 46). In addition, studies have shown that the ability of $CD8^+$ T cells to secrete IL-2 in response to HIV antigen is lost in HIV-infected patients with progressive disease but maintained in LTNPs (47–49). As shown in Fig. 5, ES8 showed diminished IL-2 secretion in response to the Q244T/I247V/G248A plasma virus mutant of TW10 relative to wild-type TW10, even though IFN-γ response in this individual was greater to the mutant epitope than to the wild-type epitope. Although plasma virus could not be amplified in ES6, the IL-2 response against wild-type TW10 and a G248E variant found as a minor proviral population was tested. The IL-2 response was greater against wild-type TW10 than against the G248E mutant, even though the IFN-γ response was greater to the G248E mutant. As with the IFN-γ responses, the IL-2 responses were considerably greater to the wild-type TW10 epitope than to the mutant plasma virus epitopes in ES2, ES3, and ES7. The IL-2 responses to the plasma variants and wild-type IW9 were essentially equivalent in all of the ES. With the exception of some *gag* clones in ES2, the TW10 and IW9 mutants were present on the same viral clones and thus the substantial difference in the IL-2 response to the TW10 epitopes could explain the selective advantage of the plasma variants. Overall, $CD8^+$ T cell IL-2 responses to mutant plasma virus epitopes relative to wild-type epitopes correlated with the selective advantage of mutant plasma virus observed in vivo for ES8, ES3, ES2, and ES7. Although in vivo selection could not be proven in ES6, T cells from this subject also showed greater IL-2 secretion against wild-type TW10 than against a mutant variant.

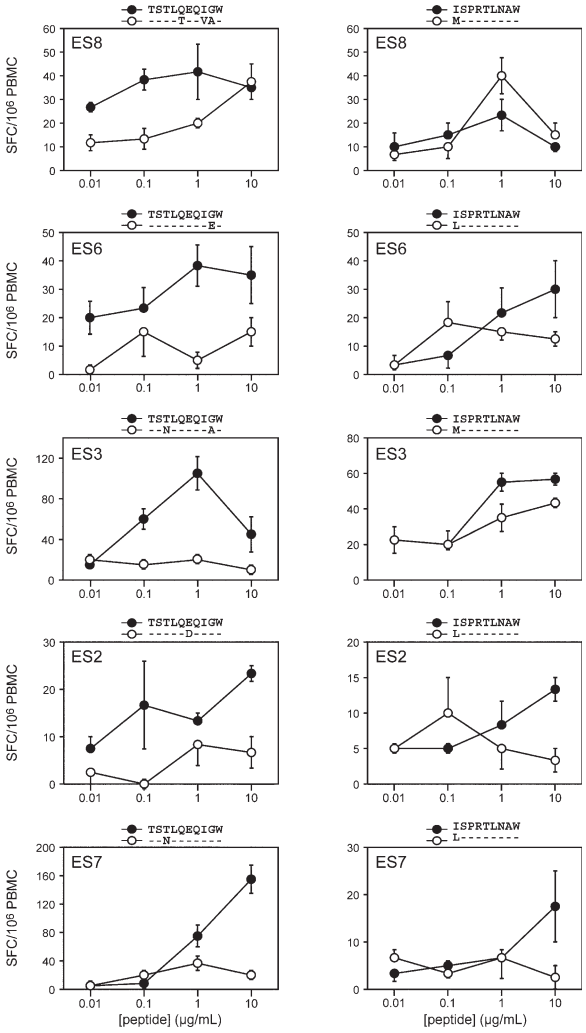

Figure 5. IL-2 responses to autologous mutant epitopes. IL-2 responses by $CD8^+$ T cells from each subject against wild-type targeted epitopes and mutant epitopes found in plasma virus or minor proviral populations in that individual. ●, responses against wild type epitopes; ○, responses to plasma virus mutant epitopes.

DISCUSSION

We have shown that extremely low but detectable levels of plasma virus persist chronically in ES with viral loads of <50 copies/ml of plasma. Comparison of *gag* gene sequences from this plasma virus and sequences from proviruses in resting $CD4^+$ T cells provides evidence of strong selective pressure favoring viruses with changes in HLA-B*57–restricted epitopes in Gag. Despite the low frequency of mutations in proviral cellular *gag*, mutations in HLA-B*57–restricted epitopes were seen in every plasma virus amplified, suggesting powerful ongoing selective pressure targeting these epitopes. This study demonstrates that CTL escape mutations arise even in HIV-1–infected patients who have the best possible suppression of viremia. Thus, rather than the absolute presence or absence of mutations, the number and nature of mutations relative to the breadth of the immune response may be critical to understanding immune control in ES.

Different subjects showed varying fractions of proviral clones bearing mutations in HLA-B*57–restricted epitopes. Archiving of viral quasispecies in resting $CD4^+$ cells is a topic under active investigation, especially with regard to drug resistance mutations (50–52). The extremely low frequency of proviruses with CTL escape mutations in *gag* in most ES may suggest that the mutant plasma viruses have never replicated

at high enough levels in these subjects to allow archiving. These results also suggest that viruses with escape mutations are not necessarily more likely to evade the immune response and revert to a latent phase of infection in resting $CD4^+$ T cells.

Despite CTL escape mutations in plasma virus, the ES studied maintained suppression of viremia. This may be at least partly due to the fact that all ES aside from ES9 showed $CD8^+$ T cell IFN-γ responses against one or more epitopes that showed no evidence of mutations in either cellular provirus or plasma virus. This has been observed in chronically HIV-infected patients with progressive disease as well, and it is not sufficient for suppression in those individuals (27, 53). It has also been shown, however, that $CD8^+$ T cell responses against HIV-1 are generally more functionally impaired in patients with progressive disease than in ES (54). A small number of fully functional $CD8^+$ T cell responses targeting unmutated epitopes in ES may mediate more effective control of viremia than a broad array of poorly functional responses in progressors.

In addition to IFN-γ responses against unmutated epitopes, $CD8^+$ T cells in some ES showed IFN-γ responses to mutant plasma virus epitopes that equaled or surpassed their responses to the corresponding wild-type proviral epitopes. Although this was not conclusively proven here, the recognition of escape mutant variants of the TW10 epitope in addition to the corresponding wild-type epitope by $CD8^+$ T cells could be the result of de novo $CD8^+$ T cell responses to escape mutants. Development of de novo responses has recently been described at the TW10 epitope in HIV-1–infected infants and at the HLA-A*11–restricted Gag 349–359 epitope in an HIV-1–infected adult (35, 55). It is therefore possible that the development of de novo responses to escape mutants may be an important mechanism for maintaining viral suppression in ES.

$CD8^+$ T cell IFN-γ responses to escape mutant did not correlate in all subjects with the apparent selective advantage of mutant plasma viruses. This suggests that some IFN-γ responses against wild-type and mutant plasma virus epitopes may result from $CD8^+$ T cell responses that are not functionally equivalent. We therefore examined another aspect of $CD8^+$ T cell function: the capacity of $CD8^+$ T cells from ES to secrete IL-2 in response to wild-type HLA-B*57–restricted epitopes found in cellular provirus and to mutant variants found in plasma virus. The relative IL-2 secretion responses against the epitopes tested correlated with the selective advantage of the mutant viruses found in plasma. ES8 showed very good evidence of selective pressure, with 18 out of 18 plasma *gag* clones bearing HLA-B*57–restricted epitope mutations and 0 out of 13 proviral clones with mutations. Although a better IFN-γ response was seen against the mutant TW10 epitope, a stronger IL-2 secretion response was seen against the wild-type TW10 epitope found in cellular provirus. Although plasma virus could not be amplified from ES6, $CD8^+$ T cells from this subject showed a more vigorous IL-2 secretion response against the wild-type TW10 epitope than against the mutant variant found in a minor proviral population. Collectively, these results suggest that in some cases, relative $CD8^+$ T cell IL-2 secretion responses stimulated by wild-type and corresponding mutant epitopes correlate better with selection of the variants found in plasma than the relative magnitude of IFN-γ responses.

Subject ES8 is illustrative of the complex interaction between viral evolution and evolution of $CD8^+$ T cell response. Without plasma virus sequencing, we might have concluded from IFN-γ results that no CTL escape had occurred in this subject. In fact, it is likely that escape did occur, and $CD8^+$ T cell responses to escape mutant TW10 epitopes were present but functionally inferior to the $CD8^+$ T cell responses to the wild-type epitopes. Although the mutations observed here have not led to an increase in viremia to clinically detectable levels, they may be of critical importance in allowing further viral evolution and CTL escape (20, 36). It will be important to follow these ES to observe any further changes in viral sequence or $CD8^+$ T cell response, which could then be correlated to changes in viral replication and disease progression.

In summary, we have shown that most HLA-B*57^+ ES with viral loads of <50 copies/ml of plasma maintain a chronic state of extremely low level viremia. Mutations in HLA-B*57–restricted Gag epitopes were seen in all plasma viruses but a minority of cellular proviruses. These escape mutants were present in all plasma virus clones, suggesting that selection pressure at these epitopes is very strong. The ES studied maintained $CD8^+$ T cell IFN-γ responses to both wild-type epitopes and many mutant plasma virus epitopes, but these responses on their own were generally a poor predictor of the observed selection pressure. We have not ruled out the possibility that the variations seen in the IW9 epitope represent processing mutations that affect presentation of the optimal epitope. Two patients, ES6 and ES8, appeared to develop strong de novo IFN-γ responses to the escape variants of the TW10 epitope. In these two subjects, $CD8^+$ IL-2 secretion responses showed better correlation with the plasma viral variants that were selected in vivo. It thus appears that $CD8^+$ T cell responses to some mutant plasma viral epitopes were functionally different from those to wild-type epitopes. CTL escape mutations apparently arise as a result of selective pressure acting at HLA-B*57–restricted epitopes in ES, but suppression of viremia is maintained. This could be due to partially functional and de novo responses against mutant epitopes and a highly functional $CD8^+$ T cell response to at least one epitope that remains free of escape mutations. The relationship between $CD8^+$ T cell response and selection of escape mutations in ES may provide key insights into the mechanisms of effective suppression of HIV-1.

MATERIALS AND METHODS

Subjects. Inclusion criteria were as follows. Subjects had positive HIV-1 Western blots and consistently had viral loads of <50 copies/ml without antiretroviral therapy. High resolution HLA typing was performed as described previously (56), and all patients were determined to be positive for the HLA-B*57 allele group. The salient clinical features are shown in Table II. This protocol was approved by the Institutional Review Board of Johns Hopkins

University. Informed consent was obtained before phlebotomy. Blood anticoagulated in citrate dextrose was centrifuged on Ficoll-Hypaque gradients to separate PBMCs and plasma. Plasma was centrifuged a second time to remove any remaining PBMCs.

Plasma HIV-1 RNA quantification. Viral RNA was quantified using the ultrasensitive Roche Amplicor Monitor System Version 1.5 assay (Roche Molecular Systems, Inc.), which has a lower limit of quantification of 50 copies/ml.

Genomic DNA and RNA isolation. Magnetic bead depletion was performed on PBMCs to enrich for resting $CD4^+$ T cells as described previously (57). Genomic DNA was purified from resting $CD4^+$ T cells using the Puregene kit (Gentra). 10–20 ml of plasma from each patient was ultracentrifuged at 25,200 *g* for 2 h at 4° to concentrate plasma virions. Viral RNA was isolated from pelleted virions using the QIAGEN Viral RNA isolation kit (QIAGEN). In brief, supernatant was removed after centrifugation, and pelleted virions were resuspended in the supplied lysis buffer. RNA was isolated according to the recommended protocol.

PCR and RT-PCR amplification of gag and nef. To prevent resampling, *gag* and *nef* genes were amplified from provirus in genomic DNA by limiting dilution "digital" nested PCR (28). *Gag* was amplified using primers described previously (27). *Nef* was also amplified using primers described previously (58). Nested PCR for ES8 was performed with 5′ inner primer AGCGGGGGAGAATTAGAT (HXB2 814–831) to include the Gag KK15 epitope in the amplicon. PCRs were performed at a concentration of DNA that would produce less than one out of three positive PCR reactions to ensure clonality. PCR products were gel purified using the QIAquick Gel Extraction kit (QIAGEN) and directly sequenced using an ABI PRISM 3700 DNA analyzer (Applied Biosystems). Chromatograms were manually examined for the presence of double peaks indicative of two templates per sequencing reaction. Such sequences were discarded. The RT gene was amplified from viral RNA as described previously (52). *Gag* was amplified from viral RNA by a one-step RT-PCR protocol with SSII (Invitrogen) with the primers described (27). Nested PCR was then performed using the same primers used for genomic DNA. Negative control reactions without RT run with each RNA sample were invariably negative. RT-PCR products were TOPO TA cloned (Invitrogen) before sequencing. To avoid resampling, only products of independent PCR reactions were considered to be independent viral clones in subsequent analysis. Sequences were assembled using CodonCode Aligner, version 1.3.1, aligned using ClustalX, and the alignments were manually adjusted in Bioedit. Sequences were translated in Bioedit, and the mean number of amino acid differences between all provirus Gag and all plasma virus Gag sequences from each patient was calculated. The predicted number of amino acid differences at HLA-B*57–restricted epitopes in Gag as well as at A146, I147, T242, and G248 was calculated using the total number of differences between provirus and plasma virus Gag for each patient and assuming a random distribution of mutations. These values were then compared with the actual number of mutations observed at these amino acids. The significance of differences between the predicted and observed number of mutations at these sites was calculated using the chi-square test. Sequences have been submitted to GenBank, accession numbers DQ315156–DQ315359.

ELISPOT analysis. The IFN-γ ELISPOT assay was performed as described previously (59). In brief, 96-well plates that had been precoated with monoclonal antibody to IFN-γ were purchased from Mabtech. 10^5 cells/well were stimulated with different concentrations of peptides for 16–18 h. The plates were washed with PBS/ 0.1% Tween 20 (Sigma-Aldrich) before incubation with a biotinylated anti–IFN-γ monoclonal antibody (7-B6-1; Mabtech) for 3 h at room temperature. After washes with PBS/ 0.1% Tween 20, a 1-h incubation with avidin–peroxidase complex was performed (APC; Vector Laboratories). The NovaRed peroxidase substrate (Vector Laboratories) was then used to detect IFN-γ–producing cells. The IL-2 ELISPOT was performed with a kit (Mabtech) as per the manufacturer's instruction. 2×10^5 cells/well were used. All plates were evaluated with an automated ELISPOT reader system (Carl Zeiss MicroImaging, Inc.) with KS4.8 software by an independent scientist in a blinded fashion (Zellnet Consulting). Screening with the IFN-γ ELISPOT assay was performed with pools of five overlapping 15-mer Gag peptides (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), and confirmatory assays with $CD4^+$ T cell–depleted PBMCs were performed. Responses were also tested against the following HLA B*57–restricted optimal epitopes: ISPRTLNAW (IW9), KAFSPEVIPMF (KF11), TSTLQEQIGW (TW10), QASQEVKNW (QW9), IVLPEKDSW (IW9), KITTESIVIW (KW10), KTAVQMAVF (KF9), STTVKAACW (SW9), AVRHFPRIW (AW9), ISKKAKGWF (IF9), KAVRLIKFLY (KY10), HTQGYFPDWQ (HQ10), YFPDWQNYT (YT9), and GPGIRYPLTFGWCF (GF14) (32). These peptides were synthesized by the peptide facility at the Johns Hopkins School of Medicine Oncology Center. Samples were run in triplicate. Negative controls routinely had 0–1 spot-forming cells (SFCs)/10^5 PBMCs for the IFN-γ ELISPOT assay and 0–3 SFCs/2×10^5 PBMCs for the IL-2 ELISPOT assay. A positive response was defined as a mean value that was >3 SFC/10^5 PBMCs. One patient (ES7) had consistently high levels of SFCs in his negative control well in four separate experiments. For this patient, a positive response was defined as a mean value that was five times greater than the negative control.

Online supplemental material. IFN-γ responses to nonautologous mutant epitopes by subjects not shown in Fig. 4 is shown in Fig. S1, which is available at http://www.jem.org/cgi/content/full/jem.20052319/DC1.

Table II. Patient characteristics

Subject	Race/Gender/Age	First positive HIV test	CD4 count[a] (cells/μl)	Plasma HIV-1 RNA[b] (copies/mL)	HLA-A	HLA-B
ES2	AA/F/50	1986	383	<50	02, 31	570301, 510101
ES3	AA/F/53	1991	677	<50	25, 68	5702, 510101
ES5	AA/F/54	1990	704	<50	23, 68	570301, 5802
ES6	AA/F/49	1992	773	<50	23	570301, 1503
ES7	AA/M/51	1994	1125	<50	30, 32	570301, 8101/8102
ES8	AA/M/53	2003	458	<50	02, 03	570301, 440201/4419N
ES9	AA/F/55	1999	800	<50	02, 30	570301, 2703

[a]At study entry.
[b]At all study time points.

We would like the thank Jun Lai for excellent technical support.

This work was funded by grants from the National Institutes of Health (AI43222, AI51178, and K08 AI51191-04) and the Howard Hughes Medical Institute.

The authors have no conflicting financial interests.

Submitted: 18 November 2005
Accepted: 12 April 2006

REFERENCES

1. Koup, R.A., J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D.D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* 68:4650–4655.
2. Borrow, P., H. Lewicki, B.H. Hahn, G.M. Shaw, and M.B. Oldstone. 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol.* 68:6103–6110.
3. Matano, T., R. Shibata, C. Siemon, M. Connors, H.C. Lane, and M.A. Martin. 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. *J. Virol.* 72:164–169.
4. Schmitz, J.E., M.J. Kuroda, S. Santra, V.G. Sasseville, M.A. Simon, M.A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B.J. Scallon, et al. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science.* 283:857–860.
5. Jin, X., D.E. Bauer, S.E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C.E. Irwin, J.T. Safrit, J. Mittler, L. Weinberger, et al. 1999. Dramatic rise in plasma viremia after $CD8^+$ T cell depletion in simian immunodeficiency virus–infected macaques. *J. Exp. Med.* 189:991–998.
6. Phillips, R.E., S. Rowland-Jones, D.F. Nixon, F.M. Gotch, J.P. Edwards, A.O. Ogunlesi, J.G. Elvin, J.A. Rothbard, C.R. Bangham, and C.R. Rizza. 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature.* 354:453–459.
7. Couillin, I., B. Culmann-Penciolelli, E. Gomard, J. Choppin, J.P. Levy, J.G. Guillet, and S. Saragosti. 1994. Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the human immunodeficiency virus 1 NEF protein. *J. Exp. Med.* 180:1129–1134.
8. Koenig, S., A.J. Conley, Y.A. Brewah, G.M. Jones, S. Leath, L.J. Boots, V. Davey, G. Pantaleo, J.F. Demarest, and C. Carter. 1995. Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. *Nat. Med.* 1:330–336.
9. Borrow, P., H. Lewicki, X. Wei, M.S. Horwitz, N. Peffer, H. Meyers, J.A. Nelson, J.E. Gairin, B.H. Hahn, M.B. Oldstone, and G.M. Shaw. 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat. Med.* 3:205–211.
10. Price, D.A., P.J. Goulder, P. Klenerman, A.K. Sewell, P.J. Easterbrook, M. Troop, C.R. Bangham, and R.E. Phillips. 1997. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc. Natl. Acad. Sci. USA.* 94:1890–1895.
11. Goulder, P.J., C. Brander, Y. Tang, C. Tremblay, R.A. Colbert, M.M. Addo, E.S. Rosenberg, T. Nguyen, R. Allen, A. Trocha, et al. 2001. Evolution and transmission of stable CTL escape mutations in HIV infection. *Nature.* 412:334–338.
12. Jones, N.A., X. Wei, D.R. Flower, M. Wong, F. Michor, M.S. Saag, B.H. Hahn, M.A. Nowak, G.M. Shaw, and P. Borrow. 2004. Determinants of human immunodeficiency virus type 1 escape from the primary $CD8^+$ cytotoxic T lymphocyte response. *J. Exp. Med.* 200:1243–1256.
13. Evans, D.T., D.H. O'Connor, P. Jing, J.L. Dzuris, J. Sidney, J. da Silva, T.M. Allen, H. Horton, J.E. Venham, R.A. Rudersdorf, et al. 1999. Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. *Nat. Med.* 5:1270–1276.
14. Chen, Z.W., A. Craiu, L. Shen, M.J. Kuroda, U.C. Iroku, D.I. Watkins, G. Voss, and N.L. Letvin. 2000. Simian immunodeficiency virus evades a dominant epitope-specific cytotoxic T lymphocyte response through a mutation resulting in the accelerated dissociation of viral peptide and MHC class I. *J. Immunol.* 164:6474–6479.
15. Allen, T.M., D.H. O'Connor, P. Jing, J.L. Dzuris, B.R. Mothe, T.U. Vogel, E. Dunphy, M.E. Liebl, C. Emerson, N. Wilson, et al. 2000. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature.* 407:386–390.
16. O'Connor, D.H., T.M. Allen, T.U. Vogel, P. Jing, I.P. DeSouza, E. Dodds, E.J. Dunphy, C. Melsaether, B. Mothe, H. Yamamoto, et al. 2002. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. *Nat. Med.* 8:493–499.
17. Peyerl, F.W., D.H. Barouch, W.W. Yeh, H.S. Bazick, J. Kunstman, K.J. Kunstman, S.M. Wolinsky, and N.L. Letvin. 2003. Simian-human immunodeficiency virus escape from cytotoxic T-lymphocyte recognition at a structurally constrained epitope. *J. Virol.* 77:12572–12578.
18. Barouch, D.H., J. Kunstman, M.J. Kuroda, J.E. Schmitz, S. Santra, F.W. Peyerl, G.R. Krivulka, K. Beaudry, M.A. Lifton, D.A. Gorgone, et al. 2002. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. *Nature.* 415:335–339.
19. Goulder, P.J., R.E. Phillips, R.A. Colbert, S. McAdam, G. Ogg, M.A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, et al. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat. Med.* 3:212–217.
20. Feeney, M.E., Y. Tang, K.A. Roosevelt, A.J. Leslie, K. McIntosh, N. Karthas, B.D. Walker, and P.J. Goulder. 2004. Immune escape precedes breakthrough human immunodeficiency virus type 1 viremia and broadening of the cytotoxic T-lymphocyte response in an HLA-B27-positive long-term-nonprogressing child. *J. Virol.* 78: 8927–8930.
21. Moore, C.B., M. John, I.R. James, F.T. Christiansen, C.S. Witt, and S.A. Mallal. 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science.* 296:1439–1443.
22. Leslie, A., D. Kavanagh, I. Honeyborne, K. Pfafferott, C. Edwards, T. Pillay, L. Hilton, C. Thobakgale, D. Ramduth, R. Draenert, et al. 2005. Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA. *J. Exp. Med.* 201:891–902.
23. Cao, Y., L. Qin, L. Zhang, J. Safrit, and D.D. Ho. 1995. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* 332:201–208.
24. Harrer, T., E. Harrer, S.A. Kalams, T. Elbeik, S.I. Staprans, M.B. Feinberg, Y. Cao, D.D. Ho, T. Yilma, A.M. Caliendo, et al. 1996. Strong cytotoxic T cell and weak neutralizing antibody responses in a subset of persons with stable nonprogressing HIV type 1 infection. *AIDS Res. Hum. Retroviruses.* 12:585–592.
25. Pilgrim, A.K., G. Pantaleo, O.J. Cohen, L.M. Fink, J.Y. Zhou, J.T. Zhou, D.P. Bolognesi, A.S. Fauci, and D.C. Montefiori. 1997. Neutralizing antibody responses to human immunodeficiency virus type 1 in primary infection and long-term-nonprogressive infection. *J. Infect. Dis.* 176:924–932.
26. Migueles, S.A., M.S. Sabbaghian, W.L. Shupert, M.P. Bettinotti, F.M. Marincola, L. Martino, C.W. Hallahan, S.M. Selig, D. Schwartz, J. Sullivan, and M. Connors. 2000. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc. Natl. Acad. Sci. USA.* 97:2709–2714.
27. Migueles, S.A., A.C. Laborico, H. Imamichi, W.L. Shupert, C. Royce, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, C.W. Hallahan, and M. Connors. 2003. The differential ability of HLA B*5701+ long-term nonprogressors and progressors to restrict human immunodeficiency virus replication is not caused by loss of recognition of autologous viral gag sequences. *J. Virol.* 77:6889–6898.
28. Liu, S.L., A.G. Rodrigo, R. Shankarappa, G.H. Learn, L. Hsu, O. Davidov, L.P. Zhao, and J.I. Mullins. 1996. HIV quasispecies and resampling. *Science.* 273:415–416.
29. Alexander, L., E. Weiskopf, T.C. Greenough, N.C. Gaddis, M.R. Auerbach, M.H. Malim, S.J. O'Brien, B.D. Walker, J.L. Sullivan, and R.C. Desrosiers. 2000. Unusual polymorphisms in human immunodeficiency virus type 1 associated with nonprogressive infection. *J. Virol.* 74:4361–4376.

30. Deacon, N.J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D.J. Hooker, D.A. McPhee, A.L. Greenway, A. Ellett, C. Chatfield, et al. 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science*. 270:988–991.
31. Kirchhoff, F., T.C. Greenough, D.B. Brettler, J.L. Sullivan, and R.C. Desrosiers. 1995. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N. Engl. J. Med*. 332:228–232.
32. Brander, C., and P. Goulder. 2000. The Evolving field of HIV CTL epitope mapping: new approaches for the identification of novel epitopes. *In* HIV Molecular Database. B. Korber, C. Brander, B. Walker, R.A. Koup, J. Moore, and B. Haynes, editors. Los Alamos National Laboratory. I-1-19.
33. Draenert, R., S. Le Gall, K.J. Pfafferott, A.J. Leslie, P. Chetty, C. Brander, E.C. Holmes, S.C. Chang, M.E. Feeney, M.M. Addo, et al. 2004. Immune selection for altered antigen processing leads to cytotoxic T lymphocyte escape in chronic HIV-1 infection. *J. Exp. Med*. 199:905–915.
34. Leslie, A.J., K.J. Pfafferott, P. Chetty, R. Draenert, M.M. Addo, M. Feeney, Y. Tang, E.C. Holmes, T. Allen, J.G. Prado, et al. 2004. HIV evolution: CTL escape mutation and reversion after transmission. *Nat. Med*. 10:282–289.
35. Feeney, M.E., Y. Tang, K. Pfafferott, K.A. Roosevelt, R. Draenert, A. Trocha, X.G. Yu, C. Verrill, T. Allen, C. Moore, et al. 2005. HIV-1 viral escape in infancy followed by emergence of a variant-specific CTL response. *J. Immunol*. 174:7524–7530.
36. Kelleher, A.D., C. Long, E.C. Holmes, R.L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, et al. 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27–restricted cytotoxic T lymphocyte responses. *J. Exp. Med*. 193:375–386.
37. Sheehy, A.M., N.C. Gaddis, J.D. Choi, and M.H. Malim. 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature*. 418:646–650.
38. Zhang, H., B. Yang, R.J. Pomerantz, C. Zhang, S.C. Arunachalam, and L. Gao. 2003. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature*. 424:94–98.
39. Yu, Q., R. Konig, S. Pillai, K. Chiles, M. Kearney, S. Palmer, D. Richman, J.M. Coffin, and N.R. Landau. 2004. Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. *Nat. Struct. Mol. Biol*. 11:435–442.
40. Mangeat, B., P. Turelli, G. Caron, M. Friedli, L. Perrin, and D. Trono. 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature*. 424:99–103.
41. Stopak, K., C. de Noronha, W. Yonemoto, and W.C. Greene. 2003. HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. *Mol. Cell*. 12:591–601.
42. Kieffer, T.L., P. Kwon, R.E. Nettles, Y. Han, S.C. Ray, and R.F. Siliciano. 2005. G→A hypermutation in protease and reverse transcriptase regions of human immunodeficiency virus type 1 residing in resting CD4+ T cells in vivo. *J. Virol*. 79:1975–1980.
43. Janini, M., M. Rogers, D.R. Birx, and F.E. McCutchan. 2001. Human immunodeficiency virus type 1 DNA sequences genetically damaged by hypermutation are often abundant in patient peripheral blood mononuclear cells and may be generated during near-simultaneous infection and activation of CD4(+) T cells. *J. Virol*. 75:7973–7986.
44. Altfeld, M., M.M. Addo, E.S. Rosenberg, F.M. Hecht, P.K. Lee, M. Vogel, X.G. Yu, R. Draenert, M.N. Johnston, D. Strick, et al. 2003. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. *AIDS*. 17:2581–2591.
45. Wherry, E.J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. *J. Virol*. 78:5535–5545.
46. Wherry, E.J., V. Teichgraber, T.C. Becker, D. Masopust, S.M. Kaech, R. Antia, U.H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol*. 4:225–234.
47. Betts, M.R., M.C. Nason, S.M. West, S.C. De Rosa, S.A. Migueles, J. Abraham, M.M. Lederman, J.M. Benito, P.A. Goepfert, M. Connors, et al. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T-cells. *Blood*. In press.
48. Zimmerli, S.C., A. Harari, C. Cellerai, F. Vallelian, P.A. Bart, and G. Pantaleo. 2005. HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. *Proc. Natl. Acad. Sci. USA*. 102:7239–7244.
49. Arrode, G., J.S. Finke, H. Zebroski, F.P. Siegal, and R.M. Steinman. 2005. CD8+ T cells from most HIV-1-infected patients, even when challenged with mature dendritic cells, lack functional recall memory to HIV gag but not other viruses. *Eur. J. Immunol*. 35:159–170.
50. Hermankova, M., S.C. Ray, C. Ruff, M. Powell-Davis, R. Ingersoll, R.T. D'Aquila, T.C. Quinn, J.D. Siliciano, R.F. Siliciano, and D. Persaud. 2001. HIV-1 drug resistance profiles in children and adults with viral load of <50 copies/ml receiving combination therapy. *JAMA*. 286:196–207.
51. Zhang, H., Y. Zhou, C. Alcock, T. Kiefer, D. Monie, J. Siliciano, Q. Li, P. Pham, J. Cofrancesco, D. Persaud, and R.F. Siliciano. 2004. Novel single-cell-level phenotypic assay for residual drug susceptibility and reduced replication capacity of drug-resistant human immunodeficiency virus type 1. *J. Virol*. 78:1718–1729.
52. Ruff, C.T., S.C. Ray, P. Kwon, R. Zinn, A. Pendleton, N. Hutton, R. Ashworth, S. Gange, T.C. Quinn, R.F. Siliciano, and D. Persaud. 2002. Persistence of wild-type virus and lack of temporal structure in the latent reservoir for human immunodeficiency virus type 1 in pediatric patients with extensive antiretroviral exposure. *J. Virol*. 76:9481–9492.
53. Koibuchi, T., T.M. Allen, M. Lichterfeld, S.K. Mui, K.M. O'Sullivan, A. Trocha, S.A. Kalams, R.P. Johnson, and B.D. Walker. 2005. Limited sequence evolution within persistently targeted CD8 epitopes in chronic human immunodeficiency virus type 1 infection. *J. Virol*. 79: 8171–8181.
54. Migueles, S.A., A.C. Laborico, W.L. Shupert, M.S. Sabbaghian, R. Rabin, C.W. Hallahan, D. Van Baarle, S. Kostense, F. Miedema, M. McLaughlin, et al. 2002. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat. Immunol*. 3:1061–1068.
55. Allen, T.M., X.G. Yu, E.T. Kalife, L.L. Reyor, M. Lichterfeld, M. John, M. Cheng, R.L. Allgaier, S. Mui, N. Frahm, et al. 2005. De novo generation of escape variant-specific CD8+ T-cell responses following cytotoxic T-lymphocyte escape in chronic human immunodeficiency virus type 1 infection. *J. Virol*. 79:12952–12960.
56. Wu, J., S. Bassinger, B.B. Griffith, and T.M. Williams. 2000. Analysis of HLA class I alleles via direct sequencing of PCR products. *In* ASHI Laboratory Manual. Lenexa, Kansas. 7.1–7.9.
57. Chun, T.W., D. Finzi, J. Margolick, K. Chadwick, D. Schwartz, and R.F. Siliciano. 1995. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nat. Med*. 1:1284–1290.
58. Huang, Y., L. Zhang, and D.D. Ho. 1995. Characterization of nef sequences in long-term survivors of human immunodeficiency virus type 1 infection. *J. Virol*. 69:93–100.
59. Keating, S.M., R.C. Bollinger, T.C. Quinn, J.B. Jackson, and L.M. Carruth. 2002. Cross-clade T lymphocyte-mediated immunity to HIV type 1: implications for vaccine design and immunodetection assays. *AIDS Res. Hum. Retroviruses*. 18:1067–1079.