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 (LTNP), 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).

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.
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...
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 LTNP's (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 TSTLQEIQGW (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

| Study subject | Fold increase | p-value | Predicted no. of differences in HLA-B*57–restricted epitopes | Observed no. of 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           | 0             | NS      | 0.6                                                      | 0                                                         | 0             | NS      |
| median        | 26            |         | 0.7                                                      | 2                                                         | 4             |         |

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

| Study subject | Mean no. of A.A. differences between provirus and plasma virus Gag | Mean no. of provirus Gag A.A. changes away from consensus | Mean no. of plasma virus A.A. changes toward consensus | Predicted no. of A.A. differences at A146, I147, T242, and G248 of Gag | Observed no. of A.A. differences at A146, I147, T242, and G248 |
|---------------|---------------------------------------------------------------|----------------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| 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           | 6.7                                                           | 4.9                                                      | 1.8                                             | 0.06                                           | 0                                               |
| median        | 7.5                                                           | 6.4                                                      | 1.8                                             | 0.07                                           | 1.3                                             |

aMean 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.

bProvirus→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”).

cPredicted number of amino acid differences at A146, I147, T242, and G248 of Gag in HLA-B*57–restricted epitopes 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.

dMutations 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-B*57+ individuals than in viremic HLA-B*57− individuals. Mutations at A146 and T242 have been shown to act as CTL escape mutations.

eAll 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 K10 epitope in plasma virus Gag relative to provirus Gag, which is ninfold 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
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
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+.

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 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 IW9 and TW10. (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.
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 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, and ES6 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.

None of these individuals showed evidence of the I147M mutation, although virus from ES7, ES2, and ES6 did have a I147M 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.

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.
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. 3K).

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.

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 ES 9 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. ES 8 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 ES 6, 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 ES 8 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, ES 6 and ES 8, 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 similar to 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 anti-coagulated 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.

**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). Genomic DNA 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 ACAGGGGGAGAATTAGAT (HXB2 814–831) to include the Gag KK15 epitope in the amplicon. PCRs were performed at a concentration of DNA 10 ng/μl. 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 ACAGGGGGAGAATTAGAT (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 inner primer IW9, TSTTVKAACW (SW9), AVRHFPRIW (AW9), ISKKAKGWF (IF9), KAVRIKFLY (KY10), HTQGYFPDWQ (HQ10), YFPDWQ-NYT (YT9), and GPGKRYPLTFGWCF (GF14) (32). These sequences 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 (SFC)/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 | Plasma HIV-1 RNA | 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 |

*aAt study entry.  
*bAt all study time points.
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