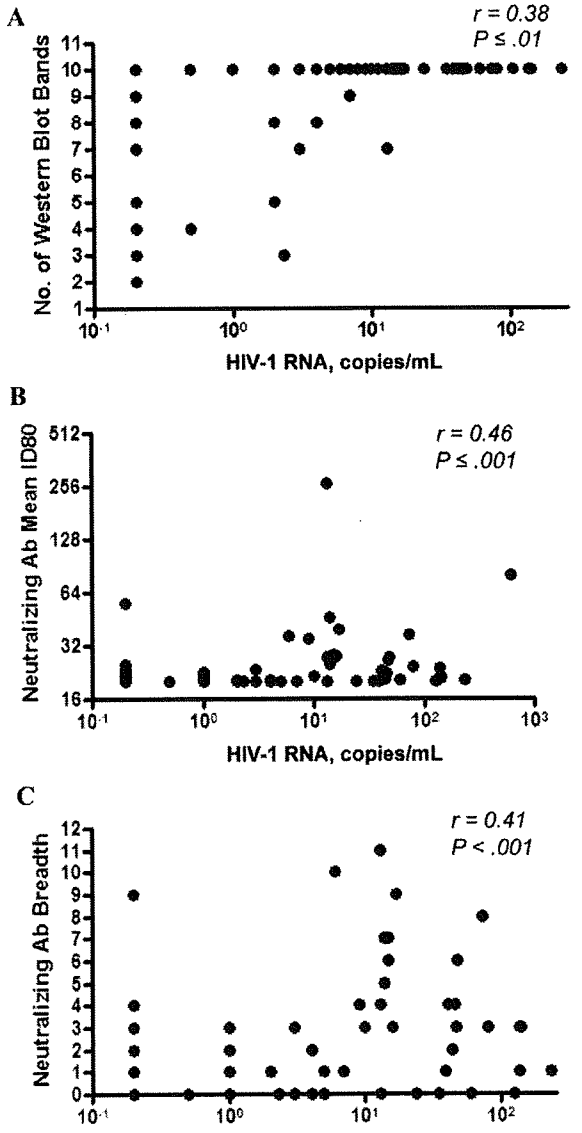

Figure 2. Antibody responses to human immunodeficiency virus type 1 (HIV-1) in elite controllers. Mean plasma virus load is plotted against *(A)* the number of virion proteins recognized by HIV-1 antibodies (Ab), as detected by Western blot; *(B)* the mean neutralizing antibody 80% inhibitory dose (ID80) titer; and *(C)* the breadth of neutralizing antibody response, as measured by number of heterologous HIV-1 Env-pseudoviruses neutralized by patient plasma. Statistical analysis was performed using the nonparametric Spearman test.

for each individual ranged from <20 to 264, with individual neutralizing antibody ID80 titers ranging as high as 1497. The breadth of neutralizing antibody responses ranged from 0 to 11 individual antibody-specific responses, with an average of 2. The potency and breadth of neutralization of heterologous virus directly correlated with the plasma HIV-1 RNA level ($r = 0.46$ and $P \leq .01$ for potency; $r = 0.41$ and $P \leq .01$ for breadth) (figure 2*B* and 2*C*). These data indicate that even the very low level of viremia found in elite controllers is directly correlated with the breadth and potency of the neutralizing antibodies response.

Absence of correlation between HIV-1–specific CD8⁺ T cell responses and virus load among elite controllers. We previously demonstrated that the overall breadth and magnitude of HIV-1–specific CD8⁺ T cell responses in elite controllers is lower than that in individuals with higher virus loads, albeit with a large amount of heterogeneity in responses, with both the highest and lowest responses detected in elite controllers [8]. To determine whether varying degrees of low level plasma viremia correlate with the responses, we used a single time point to compare HIV-1 RNA levels with the magnitude and breadth of CD8⁺ T cell responses (figure 3*A* and 3*B*). For the 53 individuals examined, neither the magnitude ($r = 0.22$; $P = .10$) nor breadth ($r = 0.19$; $P = .15$) of response correlated with plasma virus load. Responses were heterogeneous even among those with HIV-1 RNA levels of <0.2 copies/mL, with total magnitudes ranging from 200 to 13,660 SFCs and breadth ranging from 2 to 50 individual peptide-specific responses.

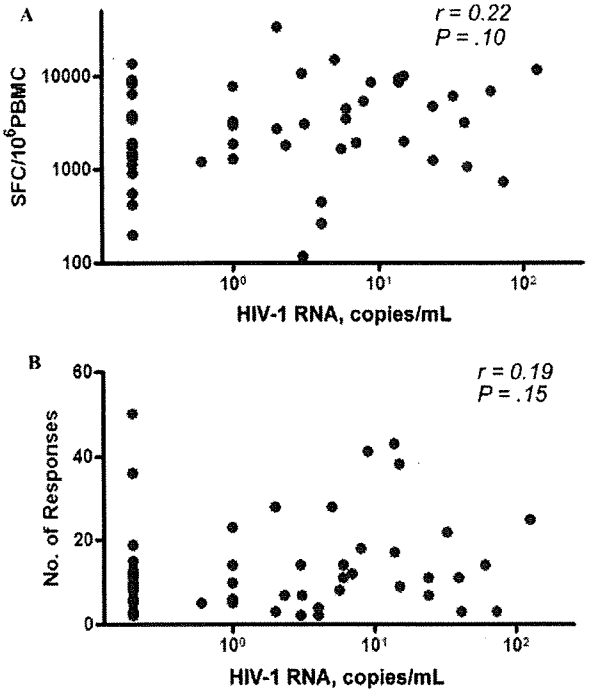

Figure 3. CD8⁺ T cell responses in elite controllers. *A*, Correlation between plasma virus load and total magnitude of the CD8⁺ T cell response, measured by enzyme-linked immunospot assay and presented in spot forming cells (SFC) per 1×10^6 peripheral blood mononuclear cells (PBMCs). *B*, Total breadth of the CD8⁺ T cell response, presented as the number of peptides targeted throughout the entire proteome. Statistical analysis was performed using the nonparametric Spearman test.

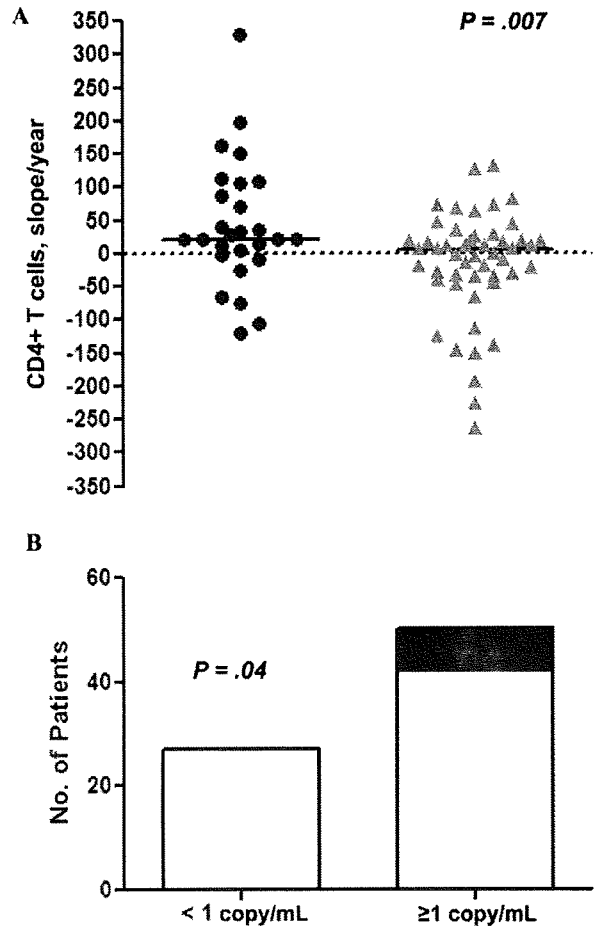

Figure 4. The change in absolute CD4⁺ T cell count per year was calculated by linear regression. *A*, The value of the slope is represented on the *Y* axis for subjects with human immunodeficiency virus type 1 (HIV-1) RNA levels <1 copy/mL (black circles) and for subjects with HIV-1 RNA levels ≥1 copy/mL (gray triangles). The median change in CD4⁺ T cell count per year is indicated with horizontal black bars. The *P* value for the difference between the groups was calculated using a 2-tailed Mann Whitney *U* test. *B*, The proportion of individuals with stable CD4⁺ T cell counts per year (white bars) and with significant CD4⁺ T cell count decreases per year (black bars) is compared between subjects with HIV-1 RNA levels <1 copy/mL and with HIV-1 RNA levels ≥1 copy/mL.

These data suggest that mechanisms independent of the level of antigen in plasma drive the breadth and magnitude of measurable HIV-1–specific CD8⁺ T cell responses in elite controllers.

Absolute CD4⁺ T cell count decrease over time is more common among individuals with low-level plasma viremia. Elite controllers are characterized by stable high absolute CD4⁺ T cell counts [2], but CD4⁺ T cell decrease and progression to AIDS has been reported in isolated cases [16]. To determine the impact of low-level viremia on the absolute CD4⁺ T cell counts, we calculated the slope of change over time. For this analysis, 6 of 83 individuals, for whom only 2 CD4⁺ T cell counts were available, were excluded. Available CD4⁺ T cell counts spanned a median of 3.6 years (range, 1.0–17.3 years) of follow-up, with a median of 7 measurements per patient (range, 3–28 measurements); 5 individuals had 3 cell count measurements, and 9 had <24 months of follow-up.

The median absolute CD4⁺ T cell count was 828 cells/mm³; 6 individuals (8%) had a median cell count of <500 cells/mm³, and 2 (3%) had a median cell count of <300 cells/mm³. The median value of the slope per year was +11 cells/mm³ per year (interquartile range, −30 to +37 cells/mm³). Exclusion of individuals with <24 months of follow-up or less than 4 cell count measurements did not alter the results. We then compared the absolute CD4⁺ T cell counts and the value of the slope between individuals with median HIV-1 RNA levels <1 copy/mL and those with levels ≥1 copy/mL; we found that the absolute CD4⁺ T cell counts did not differ between the groups (median, 873 and 783 cells/mm³, respectively; $P = .15$; data not shown), but there was a significant difference in the slopes (median, +22 and +4 cells/mm³ per year, respectively; $P = .007$) (figure 4*A*).

The short duration of follow-up, the limited number of time points sampled in some persons, and the distribution of slope values suggested that some values might not be significantly different from zero and, thus, might not represent a significant change in absolute CD4⁺ T cell counts. We therefore determined the number of individuals that had a significant change in CD4⁺ T cell counts, as defined by slopes with values that were significantly different from zero ($P < .05$). This *P* value for a nonzero slope is part of the regression analysis described in the Methods. Five (19%) of 27 individuals with HIV-1 RNA levels <1 copy/mL and 3 (6%) of 50 individuals with HIV-1 RNA levels ≥1 copy/mL had significant CD4⁺ T cell increases; this difference between groups was not statistically significantly different ($P = .12$).

We also identified 8 individuals (10%) who had significant CD4⁺ T cell count decreases (all with $P \leq .05$) and found that all of them had HIV-1 RNA levels ≥1 copy/mL and none had HIV-1 RNA levels <1 copy/mL ($P = .04$) (figure 4*B*). Moreover, when we examined the correlation between HIV-1 RNA level and the slope of CD4⁺ T cell change, we found a weak but significant correlation, with higher levels of viremia associated with CD4 T cell count decrease ($r = -0.23$; $P = .04$).

These data suggest that, among elite controllers, there is a group of individuals that have significant progressive CD4⁺ T cell loss and that this is seen more commonly among individuals with viral loads ≥1 copy/mL. Conversely, a small fraction of individuals appear to have significant CD4⁺ T cell increases, suggesting some degree of immune reconstitution over time.

DISCUSSION

Although many studies have defined elite controllers as individuals without measurable viremia by commercially available

assays [2, 17, 18], we and others have shown, using ultrasensitive assays, that low-level plasma levels of virus can be detected in a subset of these individuals [3–6]. Here we show that 70% of elite controllers with a single measurement and 81% of elite controllers with multiple measurements had detectable plasma virus levels, with a median value of 2 copies/mL, demonstrating that most HIV-1 elite controllers have detectable low-level viral replication that fluctuates over time. Moreover, we show that very low level plasma HIV-1 RNA levels correlate with HIV-1–specific antibodies detected by Western blot and with the breadth and magnitude of heterologous neutralizing antibody responses. We also demonstrate that $CD4^+$ T cell count decrease is more frequent among individuals with low-level viremia than among those with plasma viral loads of <1 copy/mL.

These results are in disagreement with a recent report involving 14 elite controllers showing that only 36% of elite controllers had plasma viral loads >1 copy/mL, as measured by a similar single-copy assay [3]. The difference may be explained by the larger size of our cohort and our inclusion of multiple time points. It may also be affected by primer and/or probe mismatches, which were not examined in the other study. In the current study, 7 of 62 individuals in whom viral *gag* sequences could be obtained were excluded from the analysis on the basis of documented primer and/or probe mismatches.

Given the possibility of primer/probe mismatches, our data allow estimation of the frequency of viral loads <0.2 copy/mL. Of 28 individuals in whom we failed to obtain *gag* sequences, 17 had HIV-1 RNA levels <0.2 copies/mL. On the basis of the results from individuals for whom sequencing was successful, among whom we found PCR mismatches in 11%, we estimate that in 3 of these 28 individuals, HIV-1 RNA might be excluded because of primer/probe mismatch. These data thus suggest that as few as 22 (27%) of 83 elite controllers have undetectable viral loads in a single measurement. Of the 31 subjects assessed longitudinally, adjusting for possible primer/probe mismatch, we estimate that only 4 (13%) had viremia <1 copy/mL in multiple measurements.

Those with persistent plasma viremia <1 copy/mL at all time points were infected for a mean duration of 17 years (range, 13 to 21 years), only 2 carried the protective human leukocyte antigen allele B*57, all had minimal or absent neutralizing antibody activities, and in 2, only 2 and 4 HIV-1 specific bands, respectively, were detected on Western blot.

Recent studies have suggested that mechanisms independent of virus load play a role in $CD4^+$ T cell decrease in elite controllers [19]; specifically, it has been suggested that $CD4^+$ T cell decrease may be the result of immune activation, and this has been suggested to play a central role in chronic AIDS in general [20, 21] . Furthermore, in chronically HIV-1–infected individuals, plasma viremia has been reported to have minimal predictive power for $CD4^+$ T cell decrease [22]. Our data suggest that individuals with HIV-1 RNA levels ≥1 copy/mL are more likely to experience $CD4^+$ T cell decrease over time than are individuals with HIV-1 RNA levels <1 copy/mL, but a direct correlation between the level of viremia and $CD4^+$ T cells decrease cannot be confirmed without additional longitudinal virus load data. The extent to which low-level viremia might affect T cell activation, T cell phenotype, T cell responses, and $CD4^+$ T cell decrease will require additional studies. Treatment of chronic HIV-1 infection with highly active antiretroviral therapy to virus loads <50 copies/mL, as well as transient "blips" of viremia in these persons have not been associated with $CD8^+$ T cell immune activation [23, 24], but the relationship between immune activation and viral load may be very different in elite controllers, compared with that in persons who have experienced prolonged exposure to high levels of viremia. Moreover, although some significant $CD4^+$ T cell increases appear to be taking place, particularly in aviremic individuals, these findings need to be confirmed with larger and more-extended longitudinal datasets. It is possible that over time, in the absence of measurable viremia, some immune reconstitution takes place.

In addition to an effect on $CD4^+$ T cells, uncontrolled viral replication has been associated with virus-specific activation of $CD8^+$ T cells and progressive loss of effector function [25]. We have previously demonstrated that elite controllers have fewer $CD8^+$ T cell responses than do individuals with HIV-1 RNA levels of 50–2000 copies/mL [8], but the degree to which plasma viremia below that cut off affects $CD8^+$ T cell responses is unknown. In this study, a correlation between low-level viremia and the breadth and magnitude of HIV-1–specific $CD8^+$ T cell responses was not found, but we cannot rule out a potential effect on $CD8^+$ T cell function. We have shown that elite controllers can exhibit strong antiviral T cell function that would not be measured by the IFN-γ ELISPOT assay used in this study, are able to inhibit viral replication in vitro [26], and can select for human leukocyte antigen–associated mutations that impair viral fitness [9, 27]. Evaluation of T cell functional assays and effects on viral function in elite controllers will be important to pursue in future studies.

An interesting finding in this study is the marked heterogeneity in the breadth and magnitude of $CD8^+$ T cell responses among individuals with a plasma viral load <0.2 copies/mL. A possible explanation is that there is ongoing HIV-1 replication in sequestered sites that is driving $CD8^+$ T cell responses; another explanation might be related to differences in the quality or quantity of central memory $CD8^+$ T cells. In contrast to HIV-1–specific $CD8^+$ T cell responses, HIV-1–specific and heterologous neutralizing antibodies are directly correlated with plasma viremia. This discrepancy of findings between measurable antibodies and $CD8^+$ T cells suggests that the latter might be affected by mechanisms independent of plasma virus load

or that the assays used fail to quantify critical defects in effector mechanisms.

Taken together, our data demonstrate that, using an assay 250-fold more sensitive than current commercial assays, most elite controllers have persistent low-level plasma viremia and that humoral immune responses but not $CD8^+$ IFN-γ ELISPOT responses correlate with the level of viremia. Moreover, we show that $CD4^+$ T cell loss is more common among elite controllers with detectable virus levels, suggesting that future studies using this assay could help identify elite controllers who are at risk for this complication.

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Differential Neutralization of Human Immunodeficiency Virus (HIV) Replication in Autologous CD4 T Cells by HIV-Specific Cytotoxic T Lymphocytes[▽]

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Defining the antiviral efficacy of CD8 T cells is important for immunogen design, and yet most current assays do not measure the ability of responses to neutralize infectious virus. Here we show that human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte (CTL) clones and cell lines derived from infected persons and targeting diverse epitopes differ by over 1,000-fold in their ability to retard infectious virus replication in autologous CD4 T cells during a 7-day period in vitro, despite comparable activity as assessed by gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay. Cell lines derived from peripheral blood mononuclear cells stimulated in vitro with peptides representing targeted Gag epitopes consistently neutralized HIV better than Env-specific lines from the same person, although ineffective inhibition of virus replication is not a universal characteristic of Env-specific responses at the clonal level. Gag-specific cell lines were of higher avidity than Env-specific lines, although avidity did not correlate with the ability of Gag- or Env-specific lines to contain HIV replication. The greatest inhibition was observed with cell lines restricted by the protective HLA alleles B*27 and B*57, but stimulation with targeted Gag epitopes resulted in greater inhibition than did stimulation with targeted Env epitopes even in non-B*27/B*57 subjects. These results assessing functional virus neutralization by HIV-specific CD8 T cells indicate that there are marked epitope- and allele-specific differences in virus neutralization by in vitro-expanded CD8 T cells, a finding not revealed by standard IFN-γ ELISPOT assay currently in use in vaccine trials, which may be of critical importance in immunogen design and testing of candidate AIDS vaccines.

Many current human immunodeficiency virus (HIV) vaccine strategies are focused not on preventing infection but on preventing disease progression by induction of virus-specific CD8 T-cell responses (16). As such, there is a great urgency to define the relative contribution of cytotoxic T lymphocytes (CTLs) to viral control during chronic infection. However, numerous studies have failed to show a relationship between breadth or magnitude of CD8 T-cell responses, as measured by gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay, and viral load (1, 5, 12), a major predictor of disease progression. Moreover, in a recent phase IIB trial of a recombinant HIV-adenovirus vaccine, CD8 T-cell responses to HIV as measured by IFN-γ were induced in vaccinees but failed to lower set point viral loads in immunized persons who subsequently became infected, raising concerns that this entire approach may be untenable (16). The ability of vaccine-induced responses to inhibit primary virus replication in autologous CD4 T cells, the ultimate function likely required of these cells, was not measured and has rarely been measured.

IFN-γ ELISPOT approaches to defining CD8 T-cell efficacy may be of limited value in defining effector function, as IFN-γ is the main cytokine that continues to be expressed throughout the pathway to T-cell exhaustion (3, 4, 33). The ability of CD8 T cells to secrete multiple cytokines has been associated with long-term nonprogressive infection but also has not been directly linked to viral control (6, 24, 39). Most of these studies measure the ability of uninfected cells pulsed with supraphysiologic concentrations of synthetic viral peptide to trigger cytokine production by T cells and thus fail to account for differences that may be associated with antigen processing and presentation (22, 31) and kinetics of T-cell lysis in relation to new progeny virion production (36). Indeed, recent studies indicate that preformed viral Gag protein processed in the cytoplasm upon viral entry can sensitize target cells for lysis by Gag-specific CTLs within 4 h of infection, whereas endogenous envelope synthesis over a 24-h period is required for cells to be targeted by envelope-specific CTLs (29). Detailed IFN-γ ELISPOT studies of breadth and specificity of HIV-specific CD8 T cells from HIV controllers who maintain levels of viremia below the limit of detection by current assays indicate that these cells are actually of lower response magnitude and more narrowly directed than those in persons with progressive infection (27), although the ability of cells from these persons to inhibit virus replication in autologous CD4 T cells appears to be enhanced (30). Previous limited studies in humans using CTL clones and HIV infection of HLA-matched cell lines infected with laboratory virus strains indicated superior inhibition of viral replication by Gag- and Nef-specific CTL clones,

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compared to reverse transcriptase (RT)-specific clones (37, 38). Recent studies of viral suppression by CTL clones derived from simian immunodeficiency virus-infected monkeys have shown marked differences in activity among multiple clones specific for the same epitopes and that in vitro suppression of virus replication does not correlate with the ability to produce IFN-γ, tumor necrosis factor alpha, or interleukin-2 (IL-2) (9). Similar studies in humans have shown that the detection of cross-clade CD8 T-cell reactivity by IFN-γ ELISPOT assay does not predict the ability to neutralize viruses containing the same variant sequences (3). Together, these studies suggest differences in antiviral efficacy that depend on the epitope targeted and other as-yet-undefined properties of the CTLs. To date, no studies have examined the ability of CD8 T cells of differing specificities derived from the same person to inhibit virus in infected autologous CD4 T cells.

Here we address the relative antiviral efficacy of CD8 T-cell responses in a functional assay that measures the ability to neutralize virus in autologous CD4 T cells. HIV-specific CD8 T-cell lines and clones from infected persons were tested for the ability to inhibit replication of primary HIV isolates over 7 to 10 days in vitro, using an adaptation of a previously reported assay (37). By expanding peripheral blood mononuclear cells (PBMCs) through stimulation with peptides representing optimal epitopes targeted in vivo, we were able to assess the relative antiviral efficacy of different responses within a single individual. This approach incorporates numerous steps that influence virus replication in vivo, including viral entry, antigen processing, epitope presentation, epitope recognition by CD8 T cells, infected cell lysis, and subsequent spread of infection to uninfected cells. Given recent population-based IFN-γ ELISPOT studies indicating that Gag-specific responses are associated with lower viral loads and Env-specific responses with higher viral load (20), we concentrated our studies on epitopes targeted within these two proteins. In order to examine the polyclonal populations that exist in vivo and also to compare the relative efficacies of multiple responses present in vivo in individual infected persons, we established effector cells of differing specificities by short-term cultures by stimulation with epitopic peptides shown by IFN-γ ELISPOT assay to be targeted in each person. Our results indicate marked differences in antiviral efficacy of CTL responses induced in HIV infection and significant superiority of in vitro-expanded Gag-specific cell lines over Env-specific cell lines.

MATERIALS AND METHODS

Study subjects. HIV-infected individuals were recruited from outpatient clinics at local Boston hospitals following institutional review board approval and written informed consent. PBMCs were obtained and cryopreserved as previously described (27). CD4 counts and viral loads were determined as described previously (27). All subjects were not on anti-HIV therapy at the time of testing. HLA types of the subjects are shown in Table 1.

Viruses. In addition to the CXCR4-utilizing HIV-1 strain NL4-3, the primary isolate X4 92HT599 and the primary CCR5-utilizing HIV-1 strain R5 91US056, were obtained from the AIDS Research and Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, MD). CCR5-utilizing HIV-1 isolates SE and JGC were isolated from HIV-infected patients as described previously (14). HIV-1 laboratory strain NL4-3 was also modified to express one or more mutations in $p24^{gag}$ as previously described (8).

Virus sequencing. Nested PCR for viral DNA or RNA was performed as previously described (2). PCR fragments were population sequenced to identify regions of sequence variation. All fragments were sequenced bidirectionally on an ABI 3100 PRISM automated sequencer (Applied Biosystems, Foster City, CA).

TABLE 1. Gag- and Env-specific CTL lines generated from HIV-1-infected individuals

Patient	CTL line generated	
	Gag specific (n = 14)	Env specific (n = 12)
013646A (HLA-A3/26 and HLA-B15/27)	A3-RY10 A3-KK9 A3-RK9 B27-KK10	A3-TK10 A3-RR11 B27-GY10
013196G (HLA-A2/30 and HLA-B44/57)	A2-SL9 B57-IW9 B57-KF11 B57-QW9	A2-SAV10 A2-RA9 A30-KQY9 A30-IY9
A01 (HLA-A2/3 and HLA-B35/55)	A3-RY10 A3-KK9 A3-RK9	A3-TK10 A3-RR11
CR0023W (HLA-A2/11 and HLA-B35/40)	A11-AK11 B35-PY9 B35-WF9	A11-SK9 B35-VL11 B35-DL9

ELISPOT assay. IFN-γ ELISPOT assays were performed as described, using overlapping peptides spanning the designated viral proteins, or optimally defined epitopes (1). Input cells ranged from 10,000 to 100,000. To calculate the number of specific spot-forming cells (SFC), the number of spots in the negative control wells was subtracted from the counted number of spots in each well. The magnitude of epitope-specific response was calculated as SFC per million cells.

Effector cell preparation. PBMCs were isolated from whole blood of HIV-infected individuals by Ficoll-Hypaque density gradient centrifugation. To generate HIV-specific CD8 T-cell lines, one aliquot (2 to 4 M PBMCs) was stimulated with 10 μg/ml of specific peptides for 90 min and irradiated. After washing, the cells were incubated with a second aliquot of unstimulated autologous PBMCs and 10 to 20 M irradiated allogeneic PBMCs in RPMI 1640 medium containing 50 U/ml of IL-2 in addition to 0.5 μg/ml of a CD3 CD4-bispecific monoclonal antibody, which results in the selective expansion of CD8 cells (17). These cultures were maintained at 37°C and 5% CO_2 for 10 to 12 days. Epitope-specific cell lines were progressively enriched by stimulation with specific epitope peptides presented by irradiated autologous PBMCs. Bulk CD8 T cells were generated from freshly isolated PBMCs by the addition of CD3 CD4-bispecific monoclonal antibody or by positive selection with anti-CD8 antibody-coated beads (30). The generated CD8 T-cell lines/clones were stimulated in vitro in the presence of peptides representing epitopes that were identified in IFN-γ ELISPOT assays, as described previously (1). The degree of enrichment in specificity of cell lines is reported as the "percent of response," defined as the magnitude of response of the expanded cell line to the stimulating peptide, divided by the sum of all epitope specificities (stimulating peptide and all other peptides targeted at baseline) detectable in the resultant cell line. T-cell clones were prepared by limiting dilution cloning and screening with recombinant HIV-vaccinia viruses as described previously (32), and clonality was determined by T-cell receptor sequencing, as described previously (23).

Target cell preparation. Primary CD4 T cells were generated from freshly isolated PBMCs by the addition of CD3 CD8-bispecific monoclonal antibody (34, 35) or by positive selection with anti-CD4 antibody-coated beads (30). Greater than 95% of these primary cells coexpressed CD3 and CD4 by flow cytometric analysis. These CD4 T cells were stimulated with PHA for 3 days before infection with HIV.

Viral inhibition assay. Inhibition of viral replication was assessed in a previously established assay system (36, 37). Autologous CD4 lymphocytes were stimulated with PHA at 1 μg/ml and infected at day 3 with the designated HIV-1 isolates at a multiplicity of infection of 0.1 or as otherwise specified for 4 h at 37°C, washed twice, resuspended in medium, and plated at 5×10^5 cells per well in a 24-well plate. To assess inhibition, effector cells then were added at a ratio of 1:1 or as otherwise specified in a total of 2 ml of medium in the presence of IL-2 at 50 U/ml. At 2- to 4-day intervals, the cocultures were fed by removing and replacing one-half of the culture supernatant with fresh medium. The removed

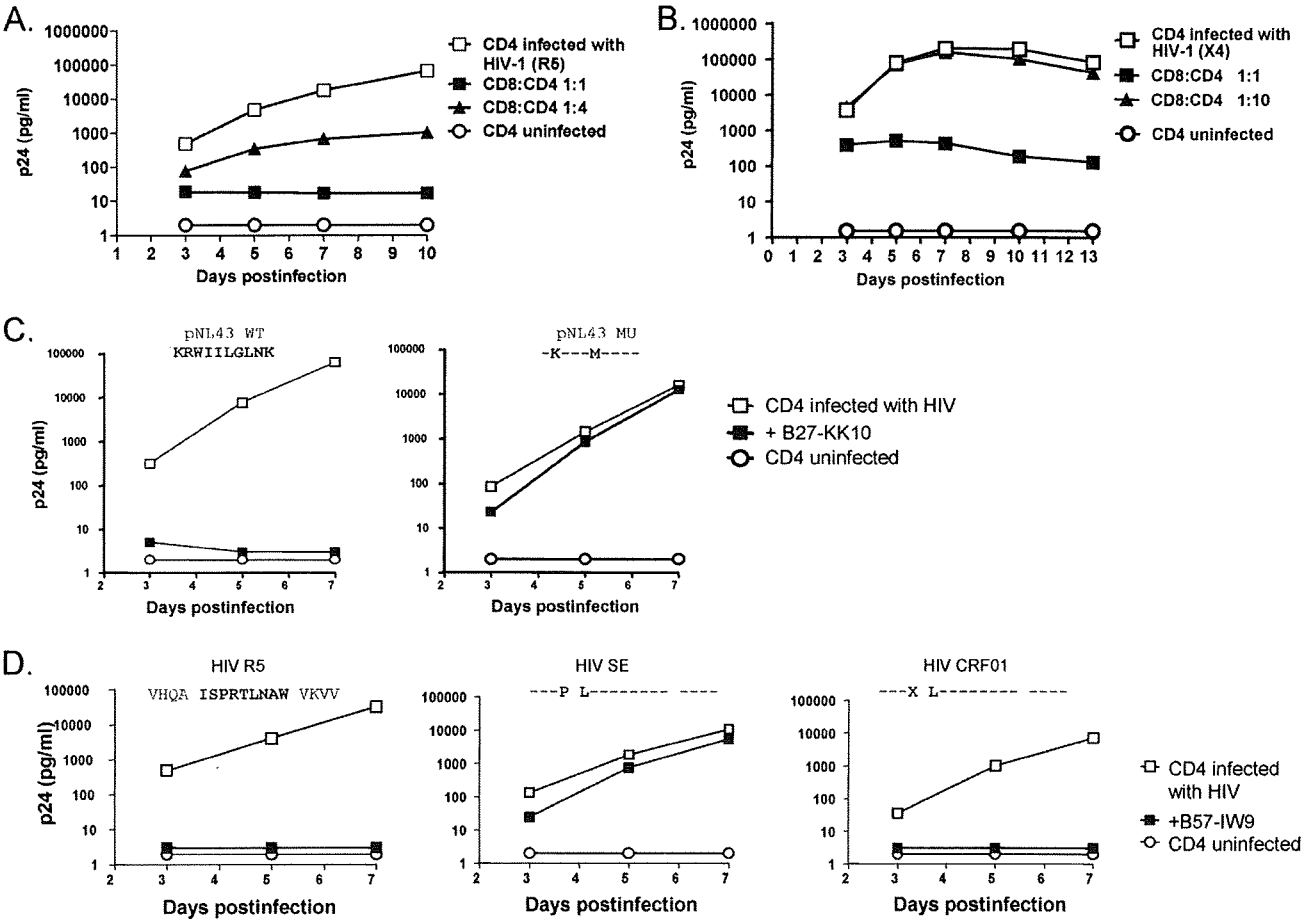

FIG. 1. HIV-specific CD8 T cells can potently suppress replication of primary HIV isolates in autologous CD4 cells. (A) Bulk CD8 T cells expanded in vitro from an elite controller (subject 013196g) by stimulation of PBMCs with CD3:CD4-bispecific monoclonal antibody inhibit HIV R5 replication in autologous CD4 T cells at the indicated effector/target cell ratios. The control of uninfected CD4 T cells showed that there were no autologous viruses grown out from the tested subject during the period of the assay. (B) Bulk CD8 T cells directly isolated from peripheral blood of the same subject by positive selection with anti-CD8 antibody-coated magnetic beads suppressed HIV X4 replication in autologous CD4 T cells at a 1:1 ratio of CD8 to CD4 T cells. (C) The Gag epitope KK10-specific, HLA-B*27-restricted CD8 T-cell clone recognized pNL4-3 wild-type virus but did not recognize an engineered escape variant which contains R-to-K and L-to-M mutations within the KK10 epitope and thus abrogates HLA class I binding with the peptide. (D) The B*57-restricted cell line specific for the epitope IW9 in Gag inhibited the R5 and CRF_01 viruses over time, whereas it had no demonstrable effect on HIV SE virus, which contains a single-amino-acid substitution, A-to-P, known to alter the peptide processing.

supernatant was cryopreserved for later p24 antigen quantitation by a standard quantitative enzyme-linked immunosorbent assay (commercial kit; Dupont, Boston, MA). Log inhibition units were calculated as $-\log_{10}$ (p24 with CTL/p24 without CTL) at day 7 in culture.

Statistical analyses. Spearman rank-correlation, Mann-Whitney, and Wilcoxon matched-pairs tests were performed using GraphPad Prism version 4.0a. All tests were two-tailed, and P values of $P < 0.05$ were considered significant.

RESULTS

HIV-specific CD8 T cells can potently suppress HIV replication in vitro. In initial experiments, we examined the ability of bulk CD8 T cells from HIV-infected persons to inhibit virus replication. In contrast to previous studies examining the ability of CTLs to inhibit laboratory strains of HIV replication in HLA-matched cell lines (36–38), here we tested multiple primary viruses, including both X4 and R5 viruses, and used autologous CD4 T cells that were expanded and infected in vitro. Initial experiments were performed using cells from elite controllers, persons who spontaneously control HIV without the need for medication (27). Since outgrowth of autologous virus in CD4 T cells is markedly delayed in these individuals (data not shown), we were able to use controlled inoculums of primary HIV isolates to infect these CD4 T cells.

Using PBMCs obtained from an elite controller (subject 013196g) that were expanded nonspecifically in vitro by stimulation with a CD3:CD4-bispecific monoclonal antibody, which results in selective expansion of CD8 T cells, marked inhibition of replication was observed at CD8/CD4 T-cell ratios of 1:1 and 1:4 (Fig. 1A). Similar experiments were performed with CD8 T cells directly isolated from fresh peripheral blood in the absence of any IL-2 or initial exogenous stimulation, again revealing marked inhibition of virus replication in autologous CD4 T cells at cell ratios present in vivo and

marked diminution of inhibition when the added CD8 T cells were diluted 10-fold (Fig. 1B), providing clear evidence of active virus neutralization of the reference viruses by circulating CD8 T cells.

The above studies examined bulk CD8 T cells. To further define potential contribution of HIV-specific CD8 T cells to the observed inhibition, we next established HIV-specific CD8 T-cell lines and clones by repeated in vitro stimulation with synthetic peptides representing immunodominant HIV epitopes. Given the reported superiority of Gag-specific CTLs in disease outcome and the enrichment for HLA-B*27 and -B*57 in persons who spontaneously control HIV replication, we first focused on responses to known B*27 and B*57 epitopes. There was essentially complete suppression of wild-type NL4-3 virus replication by a Gag-specific, HLA-B*27-restricted CD8 T-cell clone specific for the KK10 wild-type epitope (KRWIILG LNK, residues 263 to 272). In contrast, infection with a virus engineered to contain two mutations (R264K and L268M) within the KK10 epitope that arise in vivo and abrogate HLA class I binding (15) led to complete loss of antiviral effect by the CD8 T-cell clone specific for the wild-type epitope (Fig. 1C). Likewise, using a cell line from a second donor (subject 013196g) that recognize the Gag IW9 epitope (ISPRTLNAW, residues 147 to 155) in the context of HLA-B*57, complete suppression of wild-type virus was observed, whereas infection with a virus containing a single-amino-acid substitution (A146P) known to arise just proximal to the epitope in vivo and alter the epitope processing (11) in the setting of HLA-B*57 resulted in 1,000-fold-higher p24 antigen in the supernatant at 7 days. In contrast, a common conservative mutation (I147L) within the epitope did not compromise the antiviral efficacy of these cells (Fig. 1D). The assay thus accurately replicates specific steps known to influence recognition of infected cells by HIV-specific CTLs that are not assessed in standard IFN-γ ELISPOT assays. Together, these data indicate that circulating HIV-specific CD8 T cells can markedly inhibit HIV replication in autologous CD4 T cells, in an assay that is sensitive to both the sequence of the infecting virus and critical steps in antigen processing.

HIV-specific CD8 T-cell clones differ in their antiviral efficacy, depending on antigen specificity. We next examined the ability of HIV-specific CD8 T-cell clones specific for both structural and accessory proteins and restricted by different HLA class I alleles to inhibit virus replication. Clones were established by limiting dilution using a CD3-specific monoclonal antibody as a stimulus for T-cell proliferation and had comparable potency by IFN-γ ELISPOT and comparable killing in cytotoxicity assays with exogenous peptide-pulsed autologous or HLA-matched B-lymphoblastoid cell lines (B-LCL) (Fig. 2A and B). Next, we evaluated the production of p24 antigen in exogenously infected autologous CD4 T cells in the presence or absence of added CD8 T cells, and expressed the data as log inhibition units (38). CD8 T-cell clones differed markedly in their ability to inhibit virus production, with results strikingly similar for three different primary isolates (Fig. 2C), all of which had been sequenced and were known to present the relevant cognate epitopes (data not shown). Of note, the least antiviral activity was observed for an Env-specific CD8 T-cell clone and an RT-specific clone despite exhibiting robust IFN-γ production in ELISPOT assay and potent killing in cytotoxic assay triggered by exogenously added peptides. These data indicate that at a clonal level, using primary viruses and infected autologous CD4 cell lines, there are marked differences in the ability of CD8 T cells to inhibit virus replication, despite comparable activity by the IFN-γ ELISPOT assay.

Simulation of PBMCs with Gag epitopes results in more effective virus neutralization than stimulation with Env epitopes. Previous IFN-γ ELISPOT studies have shown that broader Gag-specific CD8 T-cell responses are associated with lower viral load in vivo, whereas broader Env-specific responses are associated with higher viral loads (20), and that cells are sensitized for lysis by Gag-specific CTLs much earlier after infection than Env-specific CTLs (29). We therefore compared the ability of Gag-specific and Env-specific cell lines established from four HIV-infected individuals (Table 1) to inhibit replication of a primary X4 virus and a primary R5 virus. For each subject, PBMCs were stimulated in vitro in the presence of peptides representing epitopes shown to be targeted by that individual in an IFN-γ ELISPOT assay (data not shown). For each subject, 5 to 8 individual epitope-specific cell lines were established, for a total of 26 different cell lines tested.

For each cell line generated, the magnitude and specificity of these epitope-specific CD8 T-cell lines were first evaluated by IFN-γ ELISPOT assay (Fig. 3A), the standard assay used to assess anti-HIV responses in vaccine trials. Although there were differences in the number of rounds of restimulation that each of the lines had received in establishing the epitope-specific lines, there were no differences among the lines in terms of the magnitude of Gag-specific responses versus Env-specific responses (11,684 ± 3,868 versus 11,793 ± 2,388 SFC per million cells; $P = 0.5203$) or in terms of the percentage of the enriched response that was specific for the stimulating Gag versus Env epitopes (78.43% ± 14.99% versus 87.42% ± 7.452%; $P = 0.1985$) when they were assessed in an IFN-γ ELISPOT assay. These data indicate comparable in vitro expansion of PBMCs with Gag and Env epitopes, as assessed by IFN-γ production.

We next evaluated the ability of each of these 26 cell lines to inhibit the outgrowth of primary HIV isolates in autologous CD4 T cells infected with different strains of HIV. For each subject, we attempted to grow HIV-specific lines to epitopes known to be restricted by the subject's HLA alleles and shown to be targeted by the bulk expanded CD8 T-cell lines. An example of neutralization of an X4 and R5 virus using multiple cell lines derived from one HIV-infected individual (subject 013646a) is shown in Fig. 3B, demonstrating over 1,000-fold differences in p24 antigen production using the enriched cell lines by day 7 in culture, depending on the antigenic specificity of the cell lines tested. Furthermore, Gag-specific CD8 T-cell lines in this individual were more effective than Env-specific cell lines in suppression of both R5 and X4 virus replication.

After expansion of these detailed studies to all Gag and Env epitopes targeted by four persons (two elite controllers, 013646a and 013196g; one person with treated acute infection, A01; and one person with chronic untreated infection, CR0023w, all of whom had no autologous viruses grown out during the period of the assay), consistent superiority in the antiviral efficacy of Gag- versus Env-specific responses was observed, with similar results obtained when infecting with R5

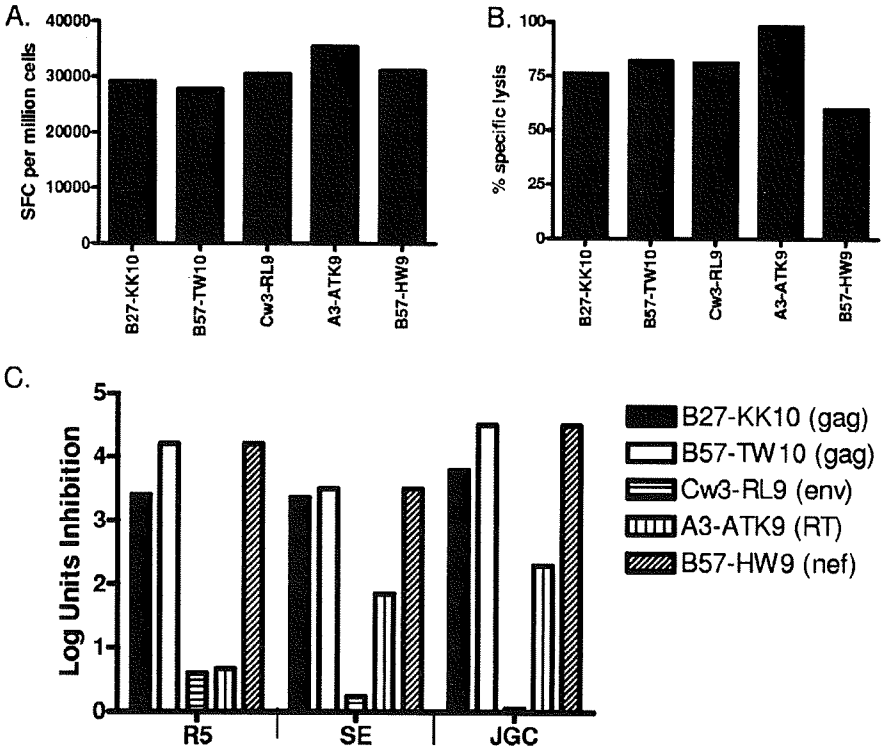

FIG. 2. CD8 T-cell clones differ in antiviral efficacy depending on antigen specificity. (A and B) HIV-specific CD8 T-cell clones were isolated from HIV-infected individuals' peripheral blood by limiting dilution. The B27-KK10-specific clone was isolated from an elite controller with 1,060 SFC per million PBMCs assessed directly ex vivo in an IFN-γ ELISPOT assay, a B57-TW10-specific clone from a chronic progressor who did not recognize TW10 peptide in the IFN-γ ELISPOT assay at that time, a Cw3-RL9-specific clone from an elite controller with 1,360 SFC per million PBMCs, an A3-ATK-specific clone from an HIV-infected individual during a time the IFN-γ ELISPOT assay was technically not available, and a B57-HW9-specific clone from an elite controller with 1,640 SFC per million PBMCs. These clones were tested in a standard IFN-γ ELISPOT assay (A) and a 4-h chromium release assay with peptide-pulsed autologous or HLA-matched B-LCL targets (B). (C) The same clones were tested for antiviral function using autologous CD4 T cells infected with different primary HIV isolates. Peptide-specific CD8 T-cell clones differed in their antiviral efficacy with similar potency against the R5, SE, and JGC HIV isolates. Data are expressed as log inhibition units, calculated as $-\log_{10}$ (p24 with CTL/p24 without CTL) at day 7 in culture.

and X4 viruses (Fig. 3C). Of note, even in cases where the same HLA allele presented both Gag and Env epitopes, in each case the Gag-specific cell lines derived by repeated peptide-specific stimulation resulted in greater inhibition than the Env-specific cell lines. Moreover, serial assessment of cell lines that were repeatedly stimulated revealed that progressive increase in magnitude and specificity for Gag-specific responses was associated with greater antiviral efficacy (Fig. 4A), whereas progressive enrichment for Env-specific responses always resulted in diminished antiviral efficacy (Fig. 4B). Although there were differences in the number of rounds of stimulations that each of the lines underwent in establishing the epitope-specific lines, overall there was no difference in the number of rounds of stimulations when comparing Gag- and Env-specific cell lines (data not shown), suggesting that the enrichment process itself did not account for the difference in antiviral efficacy of Gag-specific cell lines compared to Env-specific cell lines.

One possible explanation for the observed differences in antiviral efficacy between Gag and Env specificities is that mutations might be more likely to arise in envelope epitopes, due to the known greater plasticity of HIV Env over other expressed proteins (21). This could result in stimulation with a peptide that did not match the autologous peptide or in vitro selection for virus escape mutants that were not present at baseline. To address these possibilities, autologous virus was sequenced from each person, revealing that baseline mutations (compared to the X4 and R5 virus strains used in these assays) were present within the targeted epitope for a minority of the cell lines (one Gag epitope mutant and five Env epitope mutants in R5 virus and two Gag epitope mutants and five Env epitope mutants in X4 virus [data not shown]). Whether the antiviral efficacy of all cell lines was examined (Fig. 5A; $P < 0.0001$), or just those for which the autologous virus sequence was homologous to the experimental X4 and R5 viruses (Fig. 5B; $P = 0.0004$ and $P = 0.0005$, respectively), there were highly significant differences observed in the ability of Gag-specific versus Env-specific CD8 T-cell lines to inhibit virus replication. Sequencing of culture supernatants revealed there was no sequence change in the viruses that grew out despite CTL selection pressure during the 7-day coculture period (data not shown). Together these data indicate that viral escape mutations in Env protein did not account for the consistently lower antiviral efficacy of Env-specific CD8 T-cell lines compared to Gag-specific lines from the same person.

The strong correlation between HIV viral control and certain HLA class I alleles, particularly HLA-B*57 and -B*27 (19,

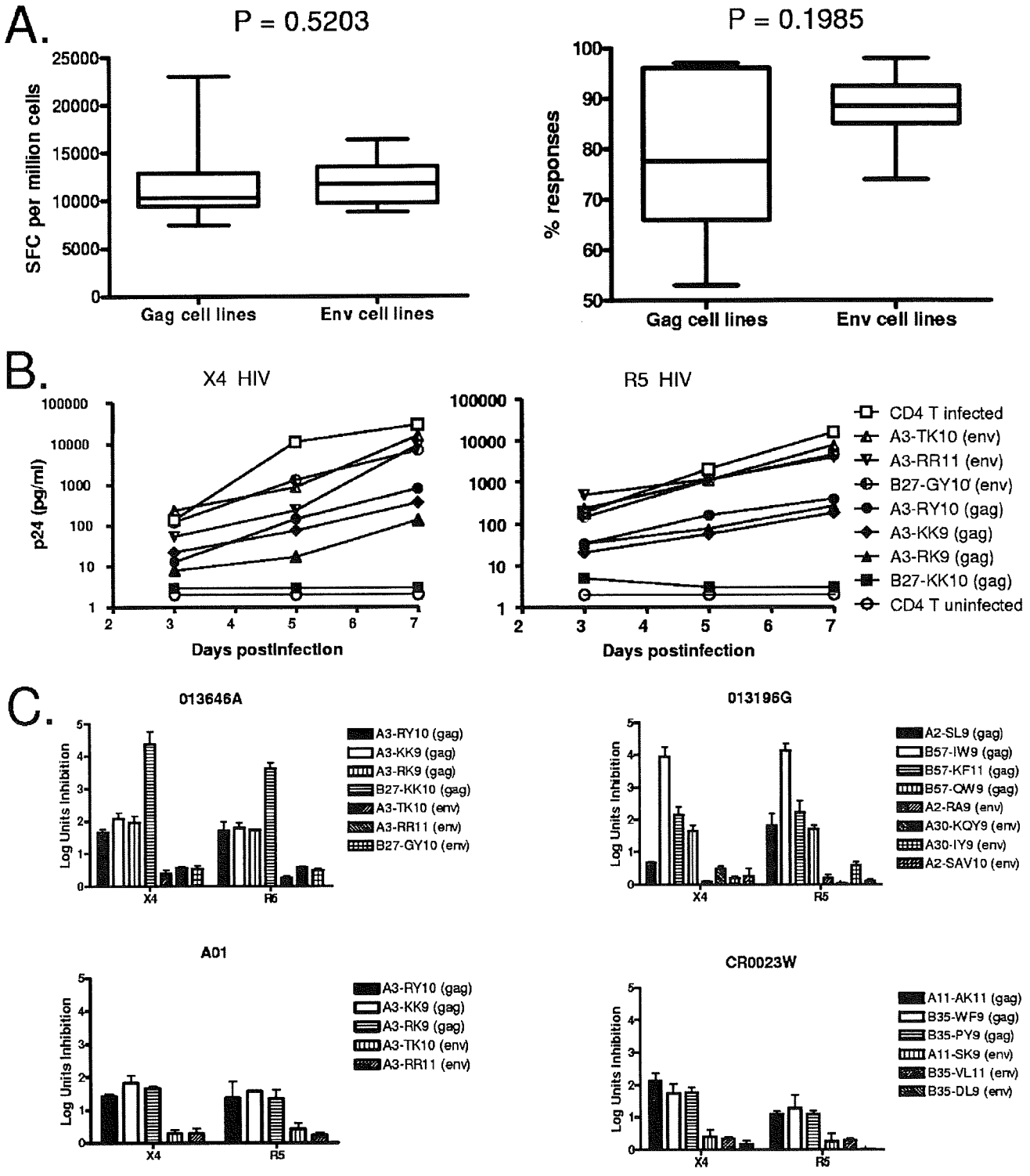

FIG. 3. Different antiviral efficacies of epitope-specific CD8 T-cell lines enriched in vitro from PBMCs. (A) The generated cell lines were stimulated in vitro in the presence of peptides representing epitopes in an IFN-γ ELISPOT assay. There was no difference among all lines in terms of the magnitude (SFC per million cells) or specificity (% response) of response specific for Gag (n = 14) versus Env epitopes (n = 12) and calculated as the percentage of the epitope-specific response of the overall HIV-specific CD8 T-cell response. Statistical comparisons were made using the Mann-Whitney test. (B) Example of differences in the antiviral efficacy of epitope-specific CD8 T-cell lines generated from a single subject (013646a) against X4 and R5 viruses during a 7-day period. The control uninfected CD4 T cells showed that there were no autologous viruses grown out from the tested subjects during the period of the assay. (C) Summary of data demonstrating different antiviral efficacies for Gag- and Env-specific cell lines against X4 and R5 in log units of inhibition after 7 days of culture. Inhibition was evaluated in multiple assays for each cell line at least twice (mean ± standard deviation).

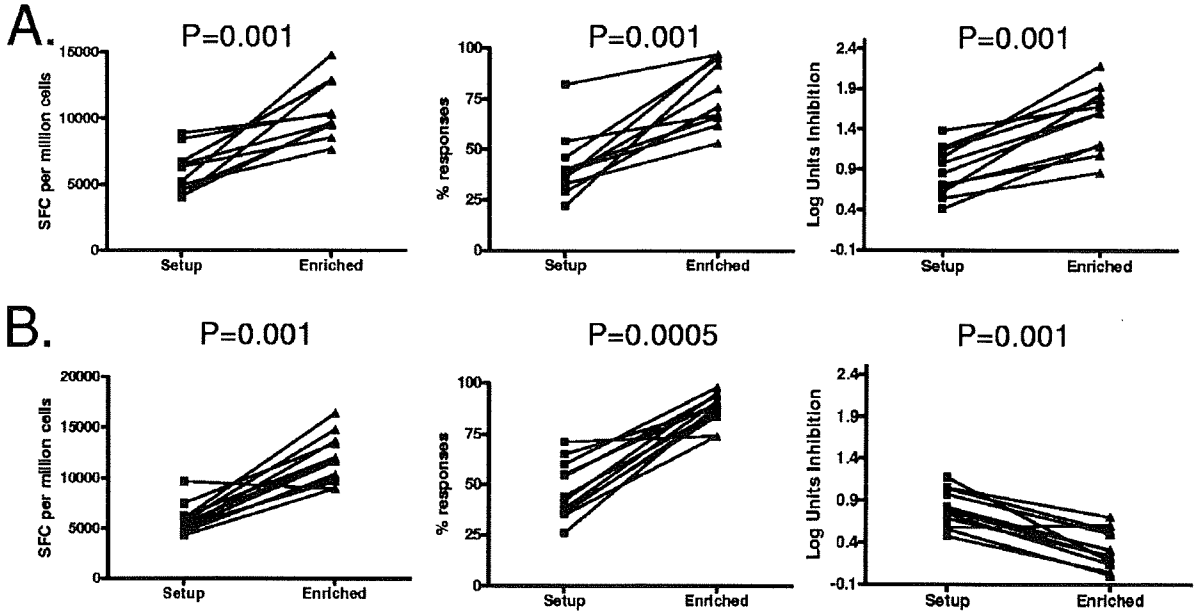

FIG. 4. Enrichment for Gag-specific responses enhances neutralization, whereas enrichment for Env-specific responses diminishes neutralization. PBMCs were stimulated in vitro with the cognate HIV Env or Gag epitope (Table 1). After a single round of stimulation starting with bulk PBMCs to establish epitope-specific cell lines (setup), the resultant cell lines were assessed for HIV-specific activity by IFN-γ ELISPOT and in the viral inhibition assay. After progressive enrichment of the setup lines for Gag or Env specificities using targeted epitopes as a stimulus (enriched), assays were repeated. (A) The progressive increase in magnitude (SFC per million cells) and specificity (% responses) for Gag-specific responses enhanced their ability to inhibit viral replication, as measured by log inhibition units (n = 11). (B) The progressive increase in magnitude and specificity for Env-specific responses diminished their ability to inhibit viral replication (n = 12). Statistical comparisons were made using the Wilcoxon matched-pairs test.

20, 26), supports the hypothesis that CTLs recognizing epitopes restricted by these HLA molecules provide an important antiviral function. Given more HLA-B*27 and -B*57-restricted cell lines specific for Gag epitopes than specific for Env epitopes, another possible explanation for the observed differences is enhanced antiviral potency of the HLA-B*27 and -B*57-restricted cell lines. To address this possibility, statistical analysis was performed between HLA-B*27/B*57-restricted lines and non-HLA-B*27/B*57-restricted lines, as well as among those non-HLA-B*27/B*57-restricted cell lines. HLA-B*27/B*57-restricted cell lines were more effective than non-HLA-B*27/B*57-restricted lines in inhibition of virus, with similar results obtained when infecting with R5 and X4 viruses (Fig. 5C; P = 0.0135 and P = 0.0437, respectively). Furthermore, among the non-HLA-B*27/B*57-restricted cell lines, there were highly significant differences observed in the ability of Gag-specific compared to Env-specific CD8 T cells to inhibit virus replication (Fig. 5D; P = 0.0001). These data provide a link between protective HLA alleles and virus neutralization but also indicate that the significantly different antiviral efficacy of Gag-specific versus Env-specific CD8 T-cell lines derived from repeated in vitro stimulation of PBMCs is not due to certain protective alleles but is antigen specific.

In vitro-derived Gag-specific CD8 T lines are of higher avidity than Env-specific lines. In order to address possible mechanisms of lack of control, we next determined the functional avidity of the Gag and Env-specific CTL lines. An example is shown in Fig. 6A, demonstrating 1,000-fold differences in the sensitizing dose of peptides required to yield 50% maximal CTL triggering of IFN-γ production (SD_{50}), depending on the antigenic specificity of the cell lines tested. Furthermore, Env-specific cell lines were of significantly lower avidity than Gag-specific cell lines (Fig. 6B; P = 0.0005), despite the fact that these cell lines had been established with equal amounts of stimulating peptide. Consistent with previous studies (38), there was no significant correlation between functional avidity and antiviral activity when Gag-specific cell lines were analyzed as a group, and this was also the case when the Env-specific cell lines were examined together (Fig. 6C; P = 0.5837 and P = 0.115, respectively). These results, extended here by the use of primary HIV isolates and autologous infected CD4 T cells, indicate that specificity rather than avidity is critical for the ability of HIV-specific CD8 T cells to inhibit virus replication in vitro, although there may be an avidity threshold for optimal antiviral activity (38).

DISCUSSION

These data, employing an assay that measures the ability of CD8 T cells to limit growth of primary HIV isolates in autologous infected CD4 T cells, indicate that there are marked differences in antiviral efficacy of CTLs induced by natural infection, based on the protein and epitope targeted. Moreover, they demonstrate for the epitopes tested here that in vitro expansion of Gag-specific cell lines from infected persons results in far greater virus neutralization than in vitro expansion of Env-specific cell lines. Importantly, these differences were not detectable using current approaches to assess im-

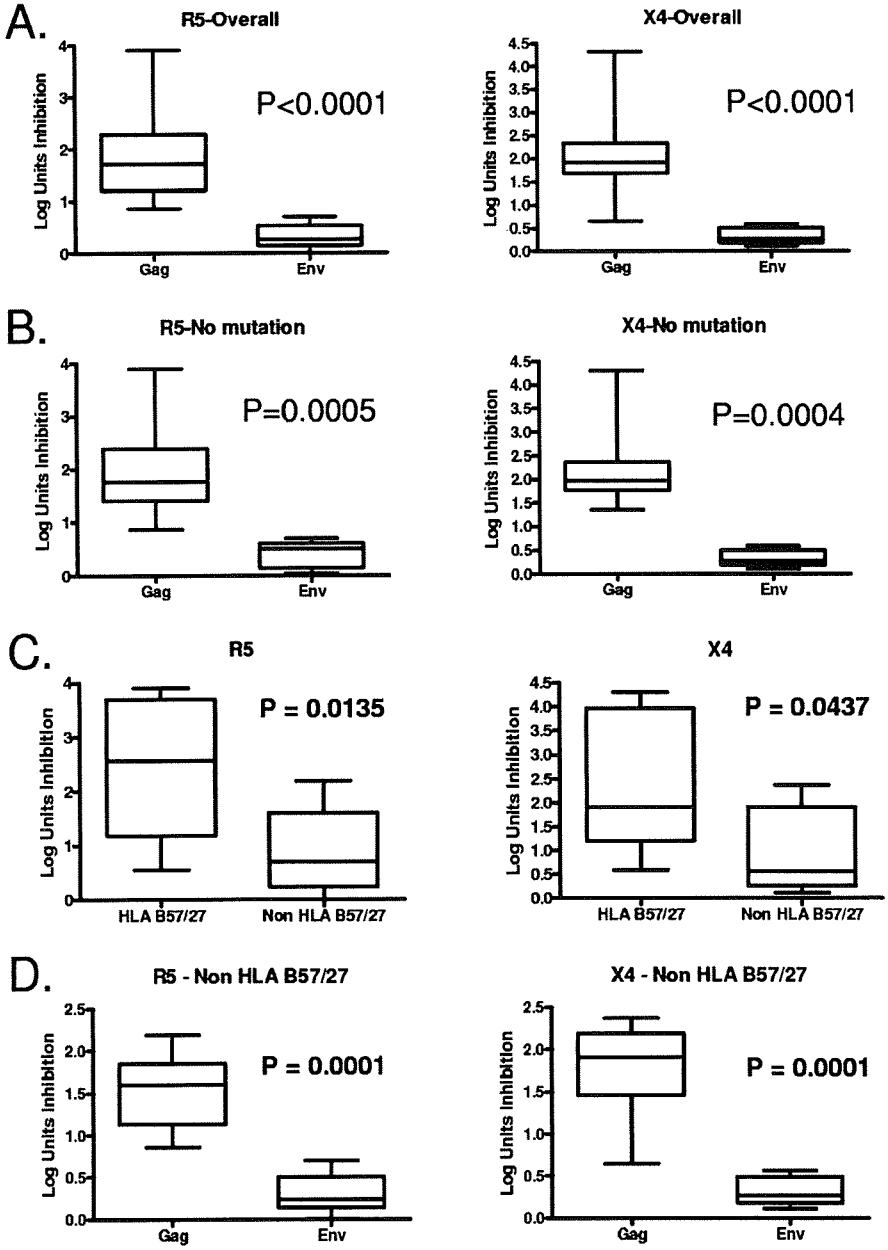

FIG. 5. Gag-specific CD8 T-cell lines are more effective than Env-specific lines in control of HIV replication. (A) Significant differences in antiviral efficacy against both R5 and X4 virus were observed between Gag-specific (n = 14) and Env-specific (n = 12) cell lines, using either an R5 virus (left panel) or X4 virus (right panel). (B) Similar significant differences in the ability to inhibit virus replication were detected when we compared Gag-specific (n = 13 for R5; n = 12 for X4) and Env-specific (n = 7 for either R5 or X4) cell lines for which the autologous viral sequences were homologous to the experimental R5 or X4 virus. (C) Antiviral efficacy against both R5 and X4 virus appeared greater for HLA B*27/B*57-restricted lines (n = 5) compared to non-HLA B*27/B*57-restricted lines (n = 21). (D) Among non-HLA B*27/B*57-restricted cell lines, we observed that Gag-specific (n = 10) responses inhibited R5 or X4 virus replication significantly better than Env-specific (n = 11) responses. Statistical comparisons were made using the Mann-Whitney test.

mune function, including IFN-γ ELISPOT assays and cytotoxicity assays using recombinant vaccinia viruses to sensitize target cells for lysis. These data also provide a link between protective HLA alleles and their functional ability to neutralize HIV in vitro, showing greater neutralization by HLA-B*57- and HLA-B*27-restricted cells. Results were consistent using bulk expanded CD8 T cells, CD8 T cells directly isolated from infected persons, CTL clones, and CD8 T-cell lines enriched for HIV-specific responses, as well as primary HIV isolates, showing that substantial inhibition of HIV can be observed at CD8/CD4 T-cell ratios present in vivo. Although the response magnitude of these cell lines/clones was relatively low as measured in IFN-γ ELISPOT assays with stimulation of peptides, the specificity in the enriched epitope-specific responses rep-

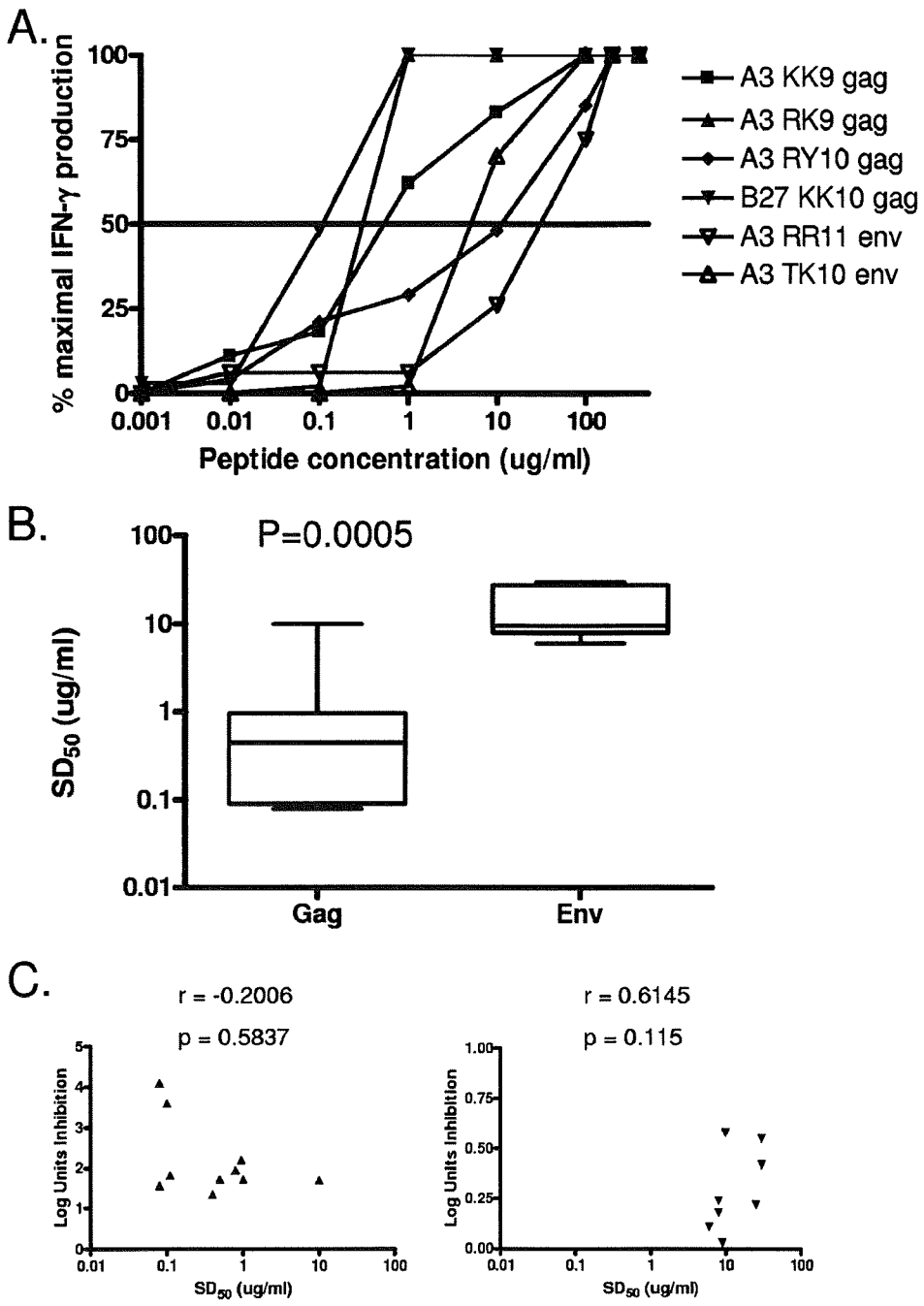

FIG. 6. Gag-specific cell lines are of higher avidity than Env-specific cell lines. (A) An example demonstrates that the indicated cell lines differed markedly in functional avidity by peptide titration in the IFN-γ ELISPOT assay. (B) Summary data demonstrate that Gag-specific responses (n = 10) were of higher avidity than Env-specific responses (n = 8). Statistical comparisons were made using the Mann-Whitney test. (C) Avidity did not correlate with the ability to control viral replication for either Gag-specific responses (left panel; n = 10) or Env-specific responses (right panel; n = 8). Correlation statistics were analyzed using the Spearman correlation.

resented greater than 80% of the overall HIV-specific CD8 T-cell responses in most cell lines (Fig. 3A). Moreover, a progressive increase in magnitude and specificity always resulted in greater antiviral efficacy for Gag-specific responses and diminished antiviral efficacy for Env-specific responses (Fig. 4).

These data provide a possible functional explanation for population-based studies showing that Gag-specific CD8 T cells, as measured by IFN-γ ELISPOT assays, are associated with lower viral load, whereas Env-specific responses are associated with higher viral load (20). Although we found it difficult to establish viable CTL clones and lines specific for many Env epitopes, we have been able to establish one Env

gp41-specific clone that displays robust neutralizing activity in this assay (18); however, this is the only Env epitope for which we have been able to generate antiviral clones and cell lines that have similar antiviral potency to what we routinely observe for many Gag-specific clones and cell lines (data not shown). The data are also consistent with the recent report that HIV Gag-specific CD8 T-cell responses are superior to Env-specific responses in control of viral load in immunized and subsequently challenged monkeys (28). The mechanism accounting for these differences is not clear but may have to do with the fact that preformed Gag protein introduced into the cytoplasm at the time of initial viral entry can be processed and presented for recognition within 4 h of infection, whereas Env protein has to be synthesized de novo before it can sensitize infected cells for lysis, a process that takes up to 24 h (29). Another possible contribution to differential neutralization is epitope- or antigen-specific differences in lytic granule loading (25). The pathway of epitope processing for Env may also play a role, in that Env is cotranslationally translocated into the endoplasmic reticulum and then undergoes posttranslational modification and reverse transport into the cytosol, where it finally gains access to a transporter associated with antigen processing-dependent class I processing pathway (13). Differential processing of HIV epitopes has been reported recently (22), and assessing the relative kinetics of Gag and Env epitope processing will be important for future studies.

One of the striking differences between Env- and Gag-specific responses is that the Env-specific responses generated in vivo by natural infection were consistently of lower avidity than Gag-specific responses, despite the fact that we used similar peptide concentrations to generate all of the lines. However, consistent with previous reports using laboratory strains of virus and immortalized CD4-bearing cell lines as target cells, we observed no significant correlation between functional avidity and antiviral activity, when either Gag-specific cell lines or Env-specific cell lines were assessed separately. These data support the finding by Yang and his colleagues that the suppression of HIV replication by CTLs depends more on antigen specificity than functional avidity (38) and support the concept of an avidity threshold for effective immune containment (4). Additional future studies will be needed to address functional avidity of CTL lysis of peptide-pulsed uninfected cells compared with CTL recognition of virus-infected cells.

There are a number of limitations to these studies that must be acknowledged and should guide future experiments. Although similar techniques were used to generate the Env- and Gag-specific cell lines tested, and these lines were comparable when assessed by IFN-γ ELISPOT, the abilities of these cell lines to subsequently expand in vitro and maintain effector functions may be different. Although testing of CTL clones used here revealed a lack of virus inhibition by an Env-specific clone compared to two Gag-specific clones, we have previously reported the marked antiviral activity of an HLA-B*14-restricted CTL clone specific for a relatively conserved epitope in gp41 (18), indicating that ineffective inhibition of virus replication is not a universal characteristic of Env-specific responses. Attempts to further address this issue were problematic, in that we found it extremely difficult and in most cases impossible to generate long-term CTL clones specific for the envelope epitopes targeted here, despite the fact that we could readily generate Gag-specific clones. This would suggest that Env-specific CTL clones might be more terminally differentiated. We have previously reported PD-1 as an exhaustion marker in a majority of HIV-specific CD8 T cells during progressive disease (10). Using quantitative RT-PCR, we evaluated Gag- versus Env-specific CTL lines from subject 013196g for expression of PD-1. PD-1 mRNA levels trended higher in the Env-specific CTL lines, but this was not significant for the small number of lines tested (unpublished data). These studies need to be further assessed in the context of survival and/or proliferation capacity of Gag- versus Env-specific CTL lines over the course of the virus inhibition assays. Sequencing of autologous virus revealed that we were using the same peptide for in vitro stimulation that was present in vivo, but it is possible that the circulating Env epitopes actually differed from the epitopes that induced these responses, which may have mutated previously. As shown in Fig. 5, the data that Env-specific cell lines were of significantly lower avidity than Gag-specific lines could be consistent with a scenario in which the tested epitope sequences represent escape (partial or complete) variants that have been selected in vivo for Env, but not for Gag. Along these lines, it is also possible that rapid escape from Env-specific responses, as has been reported in acute infection (7), leads to less effective induction of long-term memory responses with the ability to expand in vitro.

These studies also need to be examined in the context of an increasing number of studies in humans and monkeys regarding the antiviral efficacy of CTL lines and clones. Monkey studies have shown differences in the antiviral ability of multiple CTL clones from a single individual and specific for the same epitope (9), but a similar consistent difference in Env- versus Gag-specific responses has not been reported. Our own earlier data using laboratory strains of virus and infected cell lines rather than primary lymphocytes and CTL clones revealed consistent differences in antiviral efficacy depending on the protein targeted, with responses to the RT protein consistently less effective than those to Gag or Nef (38). The mechanistic underpinnings of these differences remain to be defined.

Taken together, our findings support the hypothesis that, notwithstanding the existence of individual epitope-specific differences, overall CD8 T-cell-mediated control of HIV infection during steady-state viremia in chronic infection is protein specific. Moreover, they indicate that a sizeable fraction of responses detectable in infected persons have little antiviral efficacy. These results suggest that the antiviral efficacy of vaccine-induced HIV-specific CD8 T-cell responses should be assessed in terms of ability to neutralize HIV in vitro and the development of high-throughput mechanisms to assess functional activity of vaccine-induced CTL responses should be a priority.

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HLA-Associated Viral Mutations Are Common in Human Immunodeficiency Virus Type 1 Elite Controllers[▽]

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Elite controllers (EC) of human immunodeficiency virus type 1 (HIV-1) maintain viremia below the limit of detection without antiretroviral treatment. Virus-specific cytotoxic CD8$^+$ T lymphocytes are believed to play a crucial role in viral containment, but the degree of immune imprinting and compensatory mutations in EC is unclear. We obtained plasma *gag*, *pol*, and *nef* sequences from HLA-diverse subjects and found that 30 to 40% of the predefined HLA-associated polymorphic sites show evidence of immune selection pressure in EC, compared to approximately 50% of the sites in chronic progressors. These data indicate ongoing viral replication and escape from cytotoxic T lymphocytes are present even in strictly controlled HIV-1 infection.

Human immunodeficiency virus type 1 (HIV-1)-infected persons who control viremia to below the limit of detection (<50 RNA copies/ml plasma) without therapy have been called elite controllers (EC) (3–5, 25, 28). Understanding the mechanisms responsible for successful viral control should contribute greatly to understanding HIV-1 pathogenesis and vaccine development.

Current evidence supports the notion that virus-specific cytotoxic T lymphocytes (CTLs) play a crucial role in controlling AIDS virus replication (1, 17, 18, 20, 27–32). Many studies have indicated that broad Gag-specific CTL responses are associated with lower plasma viral loads and better clinical outcomes (14, 19, 28, 33). However, viral escape from CTLs is commonly seen in AIDS virus infection (1, 10, 15, 21, 29). Recently, we reported that the replication capacity of chimeric viruses encoding *gag-protease* derived from EC was significantly reduced, associated with distinct HLA class I alleles in EC (26), suggesting that escape mutations from alleles enriched in EC diminish viral replicative fitness. However, to date, no population studies have examined the extent to which HLA-associated mutations, indicative of CTL escape mutations, are present in viruses from EC. In this study, we evaluated HLA-associated mutations in HIV-1 protein sequences (54 Gag, 41 reverse transcriptase [RT], and 39 Nef) derived from plasma viruses from EC and compared these to sequences obtained from untreated chronic progressors (CP) similarly obtained from North America (567 Gag, 392 RT, and 686 Nef) (7, 9). The median plasma viral load of CP was 120,000 (interquartile range, 42,000 to 310,000) RNA copies/ml. These studies were guided by a comprehensive list of HLA-associated polymorphisms in HIV-1 clade B defined in a cohort of more than 1,200 individuals by phylogenetically informed methods (7–9, 16). Our objective was to define the relative extent of polymorphisms in circulating plasma viruses from EC that could be attributed to HLA class I selection pressure, namely, putative CTL-driven mutations. Since there is bias in the distribution of HLA class I alleles between EC and CP (28), we report results in terms of the proportion of HLA-associated polymorphic sites within a given individual's autologous HIV sequence exhibiting the predefined specific HLA-associated polymorphisms. For each subject, the total number of predefined HLA-associated polymorphic sites in autologous viral sequences was determined and divided by the potential number in the context of their specific HLA class I allotype.

As shown in Fig. 1A, the proportion of putative CTL escape sites observed in EC was substantial in the Gag, RT, and Nef proteins (37.5%, 30.8%, and 42.1%, respectively) but still significantly lower than that observed in CP (0.375 versus 0.500 [$P < 0.0001$], 0.308 versus 0.400 [$P < 0.0001$], and 0.421 versus 0.533 [$P < 0.0001$], respectively). The proportion of HLA-associated mutations remained high in EC even after HLA-B57 subjects were removed (Fig. 1B).

We repeated the analysis limited to HLA-associated sites inside (within ±3 amino acids [aa]) published (Los Alamos National Database) or predicted (Epipred tool; Microsoft Research) CTL epitopes. Limiting the analysis to these sites has been used as an indication of mutations that are likely to directly affect escape from CTLs (9, 23), as opposed to compensatory mutations, which are usually observed more distant

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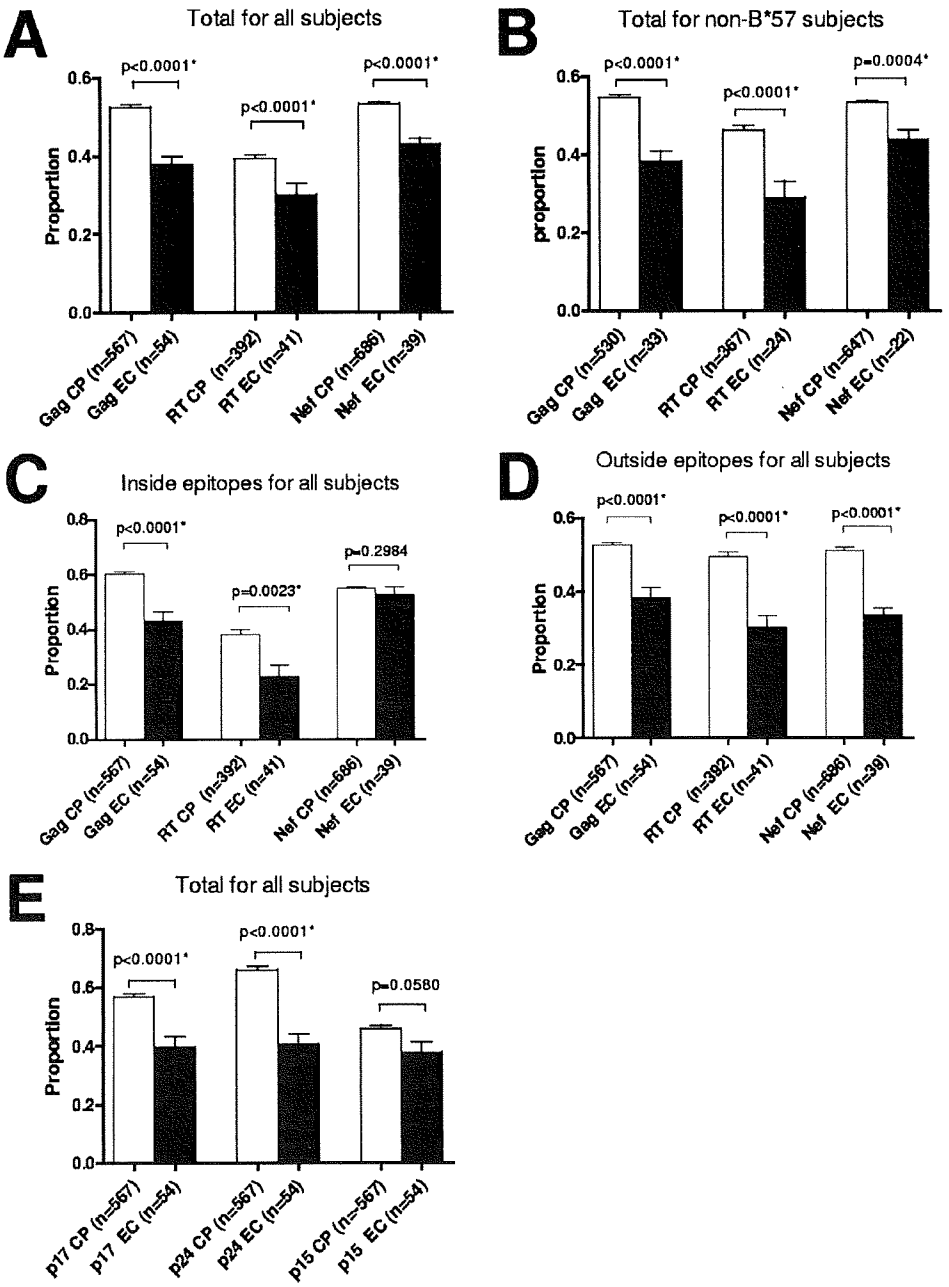

FIG. 1. Comparisons of the proportions of HLA-associated mutations between EC and CP. The mean and standard error of the proportion of sites with defined HLA-associated polymorphisms at which mutations were observed are shown. (A) Proportion of total HLA-associated sites at which mutations were observed in all of the subjects. (B) Proportion of total HLA-associated sites at which mutations were observed in non-B*57 subjects. (C) Proportion of HLA-associated sites falling within predicted CTL epitopes at which mutations were observed (inside epitopes and ± 3 aa) in all subjects. (D) Proportion of HLA-associated sites outside of predicted CTL epitopes (outside of predicted epitopes and ±3 aa) at which mutations were observed in all subjects. (E) Proportion of total HLA-associated sites at which mutations were observed in Gag subunits in all subjects.

from the epitope (6). In this analysis, the proportion of HLA-associated mutations remained high in EC (Fig. 1C).

Intriguingly, significant differences in HLA-associated polymorphisms between EC and CP were also evident in regions outside of CTL epitopes in all three proteins, with even stronger *P* values (Fig. 1D), which may suggest the presence of fewer compensatory mutations among EC. Thus, accumulation of compensatory mutations may also characterize disease progression (6). The high proportion of HLA-associated mutations in EC was seen regardless of the Gag subprotein (p17, p24, or p15) (Fig. 1E).

We next compared the proportion of HLA-associated polymorphisms present in the Gag and Nef proteins on an HLA-allele-specific basis. RT was excluded because of the small

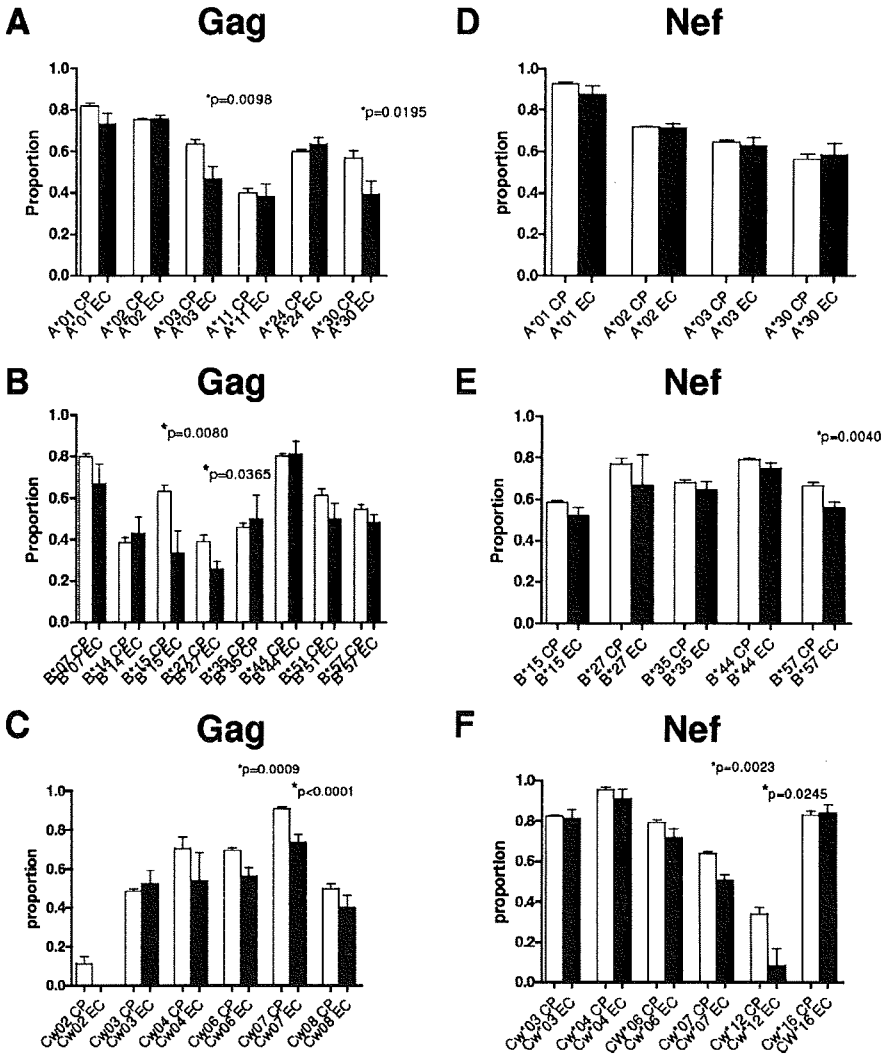

FIG. 2. Proportions of HLA-associated mutations in the Gag and Nef proteins by individual HLA class I alleles. The mean and standard error of the proportion of sites with defined HLA-associated polymorphisms at which mutations were observed are shown. HLA class I alleles present in more than four EC are shown. (A) HLA-A-associated mutations in the Gag protein. (B) HLA-B-associated mutations in the Gag protein. (C) HLA-C-associated mutations in the Gag protein. (D) HLA-A-associated mutations in the Nef protein. (E) HLA-B-associated mutations in the Nef protein. (F) HLA-C-associated mutations in the Nef protein.

numbers of HLA-associated polymorphisms identified. A high proportion of allele-specific mutations were observed in EC regardless of the HLA class I allele type in both the Gag and Nef proteins (Fig. 2). Of importance, for the majority of the alleles, EC viruses carried numbers of allele-specific mutations comparable to those of CP viruses. However, a significantly lower proportion of HLA-associated polymorphisms was observed in EC compared to CP for certain alleles, including HLA-A03, A30, B15, B27, Cw06, and Cw07 in Gag and for HLA-B57, Cw07, and Cw12 in Nef (Fig. 2A to F).

We next repeated this analysis for HLA-B57, which is overrepresented in EC and is associated with a large number of HLA allele-specific polymorphisms (28), allowing sufficient numbers to evaluate mutations inside and outside of epitopes separately (Fig. 3). B57 EC viruses tended to encode a smaller proportion of B57-associated changes inside predicted CTL epitopes in Gag than did B57 CP viruses; however, the difference did not reach statistical significance ($P = 0.0569$) (Fig. 3A). Such a trend was not seen for the Nef protein ($P = 0.3046$). As suggested by our earlier analyses, we observed significant differences in the frequency of B57-associated polymorphisms occurring outside of predicted CTL epitopes between EC and CP for both Gag and Nef ($P = 0.0029$ and $P = 0.0355$, respectively, Fig. 3B). Assuming that B57-associated changes outside of predicted CTL epitopes represent compensatory mutations, these data further indicate that the frequency of compensatory mutations may help to explain significant differences in the clinical disease course between B57 EC and B57 CP and may help explain why simple within-epitope sequence analysis has not shown any association (24). This model is consistent with recent results demonstrating the impact of escape and compensation on viral

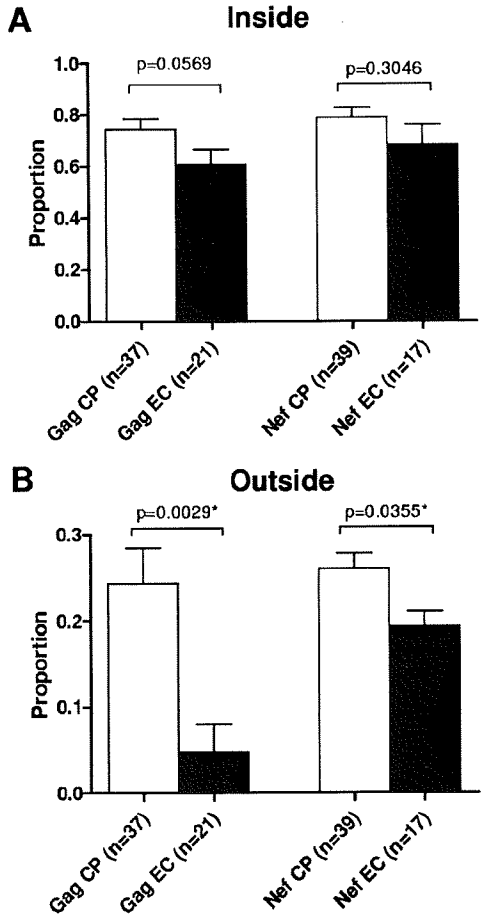

FIG. 3. Comparison of proportions of B*57-associated mutations between EC and CP. The mean and standard error of the proportion of HLA-associated sites at which mutations were observed are shown. (A) Proportion of B*57-associated sites falling within predicted B*57 CTL epitopes (inside epitopes and ± 3 aa) at which mutations were observed in the Gag and Nef proteins. (B) Proportion of B*57-associated sites outside of predicted B*57 CTL epitopes (outside of predicted epitopes and ±3 aa) at which mutations were observed in the Gag and Nef proteins.

replication capacity for the HLA-B57-restricted Gag epitope TW10 (6).

These results add considerably to currently available data (2, 4) in that they are based upon a substantially larger number of EC viral sequences and include multiple coding regions, they assess putative escape from CTLs in the context of multiple HLA class I alleles, they make direct comparison to CP viruses, and they use EC plasma viral sequences rather than proviral sequences, the latter of which do not represent actively replicating viruses in vivo in EC.

Why is it that escape from CTLs occurs in the context of such profound control of viremia? There are several feasible explanations. Firstly, CTLs targeting epitopes without escape may be contributing to the prevention of breakthrough viremia in EC. A few studies have suggested that subdominant CTL responses have an important role in controlling viremia (12, 13). Secondly, impaired viral pathogenicity due to CTL escape mutations may play a major role in controlling viremia. Recent studies demonstrating reduced viral replication capacity by HLA-B57 CTL escape mutations and recovery by putative compensatory mutations that occur outside of epitopes support this explanation (6, 22). As expected, we saw a stronger difference in the number of B57-associated mutations outside of predicted HLA-B57 epitopes than inside them. The role of compensatory mutations in HIV-1 disease progression remains unclear in non-B57 subjects. However, we also observed greater differences between EC and CP in the proportion of HLA-associated changes outside of CTL epitopes rather than within epitopes in B57-negative subjects (data not shown), suggesting that this mechanism might be applied to patterns of escape and disease progression for non-B57 alleles. Thirdly, de novo CTL responses targeting escape variants may contribute to the prevention of breakthrough viremia. Recognition of escape variants by HIV-specific CTLs has been reported (4, 11), yet the association with disease outcome is unknown. Finally, as observed in a different cohort in which individuals who subsequently achieved a low virus set point had experienced high viremia during the acute phase (our unpublished data), there is the possibility that a certain level of escape from CTLs is introduced during acute/early infection regardless of the subsequent viral set point. Similarly, there might be a concern that a longer duration of infection in EC than in CP increased the chance of viral evolution in EC regardless of the cause of viremia control. However, the important finding here is that, despite frequent evidence of escape from CTLs, viremia is still under control in EC. This suggests that escape per se is not necessarily detrimental, perhaps because of fitness constraints imposed.

There are limitations to the present study. HLA-associated polymorphisms outside of predicted CTL epitopes may represent false-positive associations, peptide processing mutations, or escape mutations in as-yet-undefined epitopes, so it will be important to investigate these mutations with larger cohorts and improved approaches to differentiate compensatory mutations from CTL escape mutations. Another limitation is that the list of HLA-associated polymorphisms used here was generated based upon viral sequences derived from chronic progressive infection and may have missed unique escape mutations present only in EC, if such mutations occur. Finally, the allele-specific mutations observed here are interpreted to be escape from CTLs, yet this has not been shown experimentally. Indeed, current assays using synthetic peptides to sensitize target cells in order to evaluate escape from CTLs are of limited value, since they do not assess potential impacts on antigen processing and presentation. Since these HLA allele-specific mutations are observed in plasma virus, the most likely interpretation is that they represent escape, but infection of cells with mutated viruses will be required to fully resolve this issue.

In conclusion, despite viral loads of <50 RNA copies/ml, EC plasma viruses display a substantial number of HLA-associated polymorphisms regardless of HLA class I allele types, indicating that viral escape from HIV-specific CTLs is common in EC. Further studies will be important to reveal the mechanisms of viremia control despite apparent escape from CTLs in persons who are able to maintain durable control of HIV infection.