

Persistent Low-Level Viremia in HIV-1 Elite Controllers and Relationship to Immunologic Parameters

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Background. Human immunodeficiency virus type 1 (HIV-1) elite controllers are able to control virus replication to levels below the limits of detection by commercial assays, but the actual level of viremia in these individuals is not well defined. Here, we quantify plasma HIV-1 RNA in elite controllers and correlate this with specific immunologic parameters.

Methods. Plasma HIV-1 RNA levels were quantified in 90 elite controllers with use of a real time reverse-transcriptase polymerase chain reaction assay with a sensitivity of 0.2 copies/mL. HIV-1-specific immune responses and longitudinal CD4⁺ T cell counts were examined.

Results. The median plasma HIV-1 RNA level was 2 copies/mL (interquartile range, 0.2–14 copies/mL). A longitudinal analysis of 31 elite controllers demonstrated 2–5-fold fluctuations in viremia in the majority of individuals; 6 had persistent levels below 1 copy/mL. Viremia correlated directly with HIV-1-specific neutralizing antibodies and Western blot reactivity but not with CD8⁺ T cell responses. Absolute CD4⁺ T cell decrease was more common among individuals with detectable viremia ($P = .04$).

Conclusions. Low-level viremia is present in the majority of elite controllers and is associated with higher HIV-1-specific antibody responses. Absolute CD4⁺ T cell loss is more common among viremic individuals, suggesting that even very low-level viremia has negative consequences over time.

In light of the recent failure of a prophylactic human immunodeficiency virus type 1 (HIV-1) vaccine trial [1], the rationale for current T cell-based HIV-1 vaccine approaches has been questioned. Understanding the relationships between low-level viremia, adaptive immune responses, and disease progression is of im-

portance, particularly in situations of natural HIV-1 control that are relevant to vaccine approaches based on protection from disease progression. Such situations of control of HIV-1 are seen in elite controllers, individuals who spontaneously control virus replication without antiretroviral drugs, to levels below the limits of detection by commercial assays [2]. Although some recent studies have indicated low-level viremia in these persons, the relationship to immunologic parameters, including CD4⁺ T cell decrease remains to be determined [3–5].

We previously reported that low-level viremia can be detected by ultrasensitive quantitative polymerase chain reaction (PCR) in elite controllers and is associated with the expression of immunoregulatory receptors that affect CD4⁺ T cell function [6]. In that study, the relationship between low-level viremia and other immunologic parameters was not examined. Low-level viremia and its relationship to HIV-1 antibody lev-

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els have recently been described in elite controllers, but semi-quantitative methods were used to measure both HIV-1 RNA and HIV-1 antibody levels, so the number of copies per mL of plasma was not defined [4]. Furthermore, the relationship between residual low-level viremia and Western blot reactivity, HIV-1-specific neutralizing antibodies, and CD8⁺ T cell responses remains unknown.

Here, we used an assay described elsewhere [7] for the detection of HIV-1 RNA with single-copy sensitivity to quantitatively characterize the level of viral RNA in plasma of elite controllers. We examined the relationship between virus load, absolute CD4⁺ T cell counts, and HIV-1-specific immune responses. We find that the majority of elite controllers maintain detectable levels of plasma viremia, with a median virus load of 2 copies/mL. Moreover, we show that this extremely low level of viremia correlates positively with HIV-1-specific neutralizing antibodies and full Western blot reactivity. We also show that CD4⁺ T cell decrease over time is more common among individuals with low-level viremia.

METHODS

Study subjects. HIV-1 elite controllers were randomly selected from a local cohort [8]. These subjects maintained plasma HIV-1 RNA levels below the limit of detection by commercial assays (<50 copies, by ultrasensitive PCR) without antiretroviral therapy. Definition of elite control was as described elsewhere [8]. The Massachusetts General Hospital Institutional Review Board approved all studies, and written informed consent was obtained from all participants prior to enrollment. Plasma and peripheral blood mononuclear cells (PBMC) were obtained as described elsewhere [8].

HIV-1 RNA determination. A single-copy assay for HIV-1 RNA detection was performed as described elsewhere [7], starting with 9 mL of plasma, which afforded a lower limit of detection of 0.2 RNA copies/mL. PCR amplification and sequencing of the HIV-1 *gag* region was performed for each subject from either plasma or PBMC samples, as described elsewhere [9], to rule out inefficient amplification by single-copy assay attributable to possible polymorphisms in the probe and/or primer sequences. Further details of optimum amplification conditions and performance characteristics, as well as quality control procedures to prevent artifactual amplification, are described elsewhere [7].

Assessment of HIV-1-specific CD8⁺ T cell responses. In 53 individuals with available fresh PBMCs, interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) assays were performed as described elsewhere [10, 11]; the assays included 410 peptides 16–19 amino acids in length, overlapping by 10 amino acids, spanning the entire HIV-1 proteome. The same time point was used for ELISPOT and HIV-1 RNA detection. Positive responses were confirmed in a second assay after cell incubation

for 24 h. Responses were considered to be positive if experimental wells had at least 3 times the mean number of spot forming cells (SFCs) in the 6 negative control wells, there were at least 5 spots present in the experimental well, and the number of SFCs per 1×10^6 PBMCs was >55. Responses to peptides were previously shown to be largely mediated by CD8⁺ T cells [11, 12].

Assessment of HIV-1-specific antibody responses. Western blot was performed according to the manufacturer's instructions (GS HIV 1 Western Blot; Bio-Rad) for qualitative detection of HIV-1 antibodies to specific viral proteins (gp160, gp120, p65, p55, p51, gp41, p40, p31, p24, and p18). Neutralizing antibody responses against HIV-1 Env-pseudoviruses were measured as described elsewhere [4, 10]. Briefly, 3-fold serial dilutions of plasma samples were performed in a 96-well flat bottom plate, 200 median tissue culture infective doses (TCID₅₀) of the virus was added to each well, and the plates were incubated for 1 h at 37°C. TZM.bl cells were then added. Following a 48-h incubation at 37°C, 100 μ L of Bright-Glo luciferase reagent (Promega) was added. The cells were allowed to lyse for 2 min, then the lysate was transferred to a 96-well black solid plate, and luminescence was measured using a Victor 3 luminometer (Perkin Elmer). The 80% inhibitory dose (ID₈₀) titer was calculated as the serum dilution that caused an 80% reduction in relative luminescence units, compared with the virus control wells, after subtraction of cell control relative luminescence units.

Statistical analysis. Statistical analyses and graphical presentations were performed using GraphPad Prism, version 5.0 (GraphPad Software). A nonparametric Spearman test was used to calculate correlations. Significance when comparing 2 groups was determined with a 2-tailed nonparametric Mann Whitney *U* test. Changes in absolute CD4⁺ T cell count over time were calculated using a linear regression model to calculate the value of the slope.

RESULTS

Low-level plasma viremia is detectable in the majority of HIV-1 elite controllers. HIV-1 RNA levels were measured at a single time point in 90 HIV-1 elite controllers. Amplification of *gag* sequences was successfully obtained in 62 individuals. Polymorphisms in the primer and/or probe sequences that could affect amplification were present in 7 (11%) of 62 persons, which is consistent with our experience with this assay in large datasets [13]. All individuals with confirmed primer and/or probe mismatches were excluded, leaving 83 individuals in the analysis. The level of plasma viremia varied over a wide distribution (figure 1A), with a median of 2.3 copies/mL (interquartile range, 0.2–14 copies/mL). In the majority of individuals ($n = 79$), the average HIV-1 RNA levels measured by single-copy assay were consistent with results of previous ul-

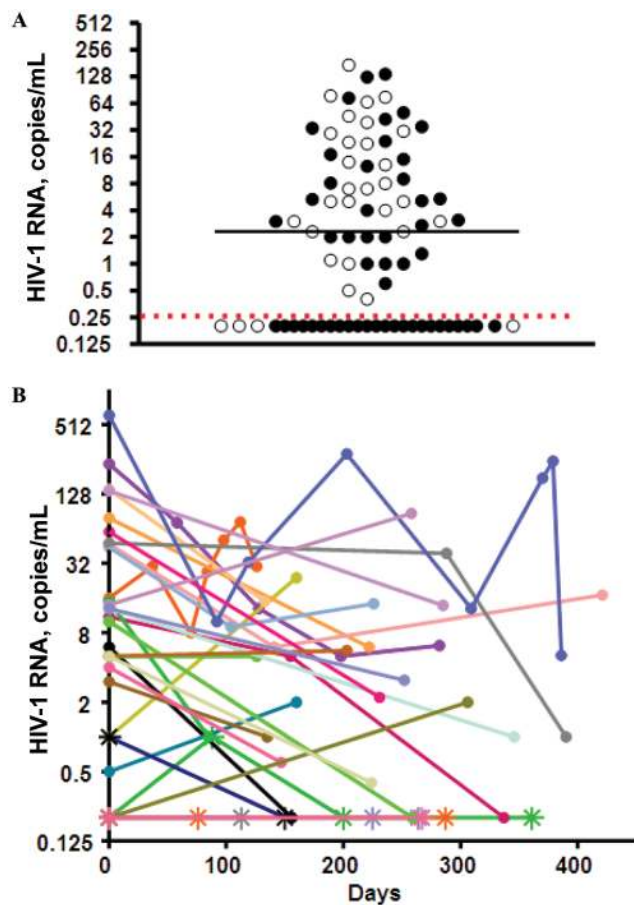


Figure 1. Plasma human immunodeficiency virus type 1 (HIV-1) RNA load distribution among 83 elite controllers (A). The cut off level of 0.2 copies/mL is represented by the dotted red line. The median values are indicated with the black horizontal bar. The arithmetic mean HIV-1 RNA virus load for each subject is shown. Open circles represent patients with longitudinal HIV-1 RNA values; filled circles represent subjects with single time point virus load determinations. B, Longitudinal HIV-1 RNA virus load in 31 elite controllers. Each line represents 1 study subject. Subjects with all HIV-1 RNA measurements <1 copy/mL are shown with asterisks ($n = 6$).

trasensitive commercial assays, whereas 4 individuals had apparent “blips” >50 RNA copies/mL at the time the plasma was obtained for the single-copy assay. Thirty (36%) of 83 individuals had HIV-1 RNA levels of ≤ 1 copies/mL; in 25 (30%) individuals, virus could not be detected with a single measurement (<0.2 copies/mL).

We next used the single-copy assay to measure longitudinal plasma samples from 31 of these individuals, including 11 in whom the initial viral load measurement result was <0.2 copies/mL. Follow-up ranged from 85 to 420 days (mean duration, 232 days) and included 2–8 measurements (mean, 3 measurements) per subject (figure 1B). The majority of individuals showed fluctuations in plasma HIV-1 RNA levels, 5 had transient viral “blips” >50 copies/mL, as described elsewhere in elite controllers [8]. Of 11 persons with RNA levels below the limit

of detection by the single-copy assay who were studied longitudinally, 6 had levels <1 copy/mL at all time points measured. For 4 of these, the values were all <0.2 copies/mL, whereas 2 subjects had arithmetic mean RNA values of 0.4 and 0.6 copies/mL, respectively. Of the 4 with undetectable RNA levels in this assay (<0.2 copies/mL), we were able to sequence virus from 2 and confirm that there were no primer mismatches. These data show that viremia can be detected in the majority of elite controllers, but there are rare individuals in whom the level of plasma virus is persistently below the ability to measure, even by an assay that is 250-fold more sensitive than current commercial assays.

The breadth and potency of HIV-1 antibody responses inversely correlates with plasma viremia. We previously demonstrated that elite controllers have very low levels of heterologous neutralizing antibodies, compared with individuals with higher virus loads, suggesting that HIV-1 replication drives the production of heterologous neutralizing antibodies [14]. To determine the effect of extremely low-level viremia on HIV-1-specific antibody responses, Western blot was performed in all individuals, and detection of antibodies to gp120, gp160, gp41, p18, p24, p31, p40, p51, p55, and/or p65 was recorded. All elite controllers had detectable responses to multiple proteins (range, 2 to 10 proteins), and the majority of individuals (77%) had antibodies against all proteins tested. The most common antibodies detected were gp120 and gp160, which were detected in all individuals, followed by p24, p40, and gp41, which were present in 98%, 96%, and 93% of individuals, respectively. The least commonly detected antibodies were p18, p31, and p65, which were found in 80%, 84%, and 86% of individuals, respectively. One individual had only 2 antibodies detected (gp120 and gp160) in 2 tests performed over a 76-day period, at a time when the individual’s level of plasma viremia was <0.2 copies/mL. Analysis of all individuals together indicated that the number of bands detected correlated directly with plasma HIV-1 RNA levels and that full Western blot reactivity was present in all persons with plasma viral loads of >13 copies/mL ($r = 0.38$; $P \leq .01$) (figure 2A).

Marked heterogeneity in neutralizing antibody responses among elite controllers has also been described elsewhere, with some individuals having broad responses and others having minimal or no neutralizing antibodies [8]. To determine whether the level of plasma HIV-1 RNA was associated with differences in neutralizing antibodies, we tested plasma samples from all subjects against a standard reference panel of 12 primary clade B HIV-1 viruses [15]. Despite high-level inhibition of the neutralization-sensitive laboratory strain SF162 (data not shown), elite controllers’ plasma exhibited limited cross-neutralization against nearly all of the primary reference viruses. ID80 titers against a MuLV-negative control pseudovirus were below the cut off of <20 in all individuals. The average ID80

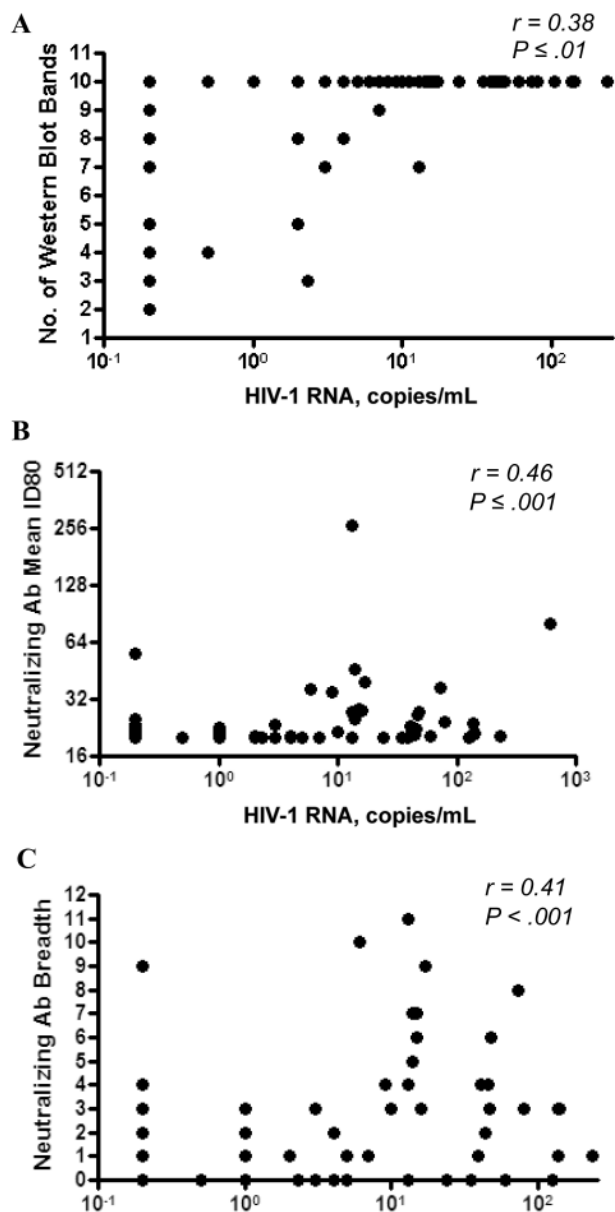


Figure 2. Antibody responses to human immunodeficiency virus type 1 (HIV-1) in elite controllers. Mean plasma virus load is plotted against (*A*) the number of virion proteins recognized by HIV-1 antibodies (Ab), as detected by Western blot; (*B*) the mean neutralizing antibody 80% inhibitory dose (ID80) 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.

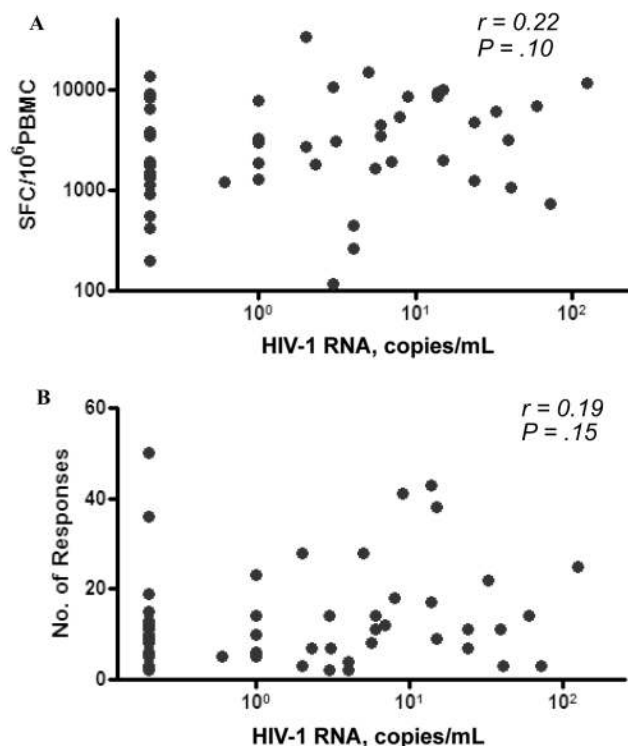


Figure 3. CD8⁺ T cell responses in elite controllers. *A*, Correlation between plasma virus load and total magnitude of the CD8⁺ T cell response, measured by enzyme-linked immunospot assay and presented in spot forming cells (SFC) per 1×10^6 peripheral blood mononuclear cells (PBMCs). *B*, Total breadth 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.

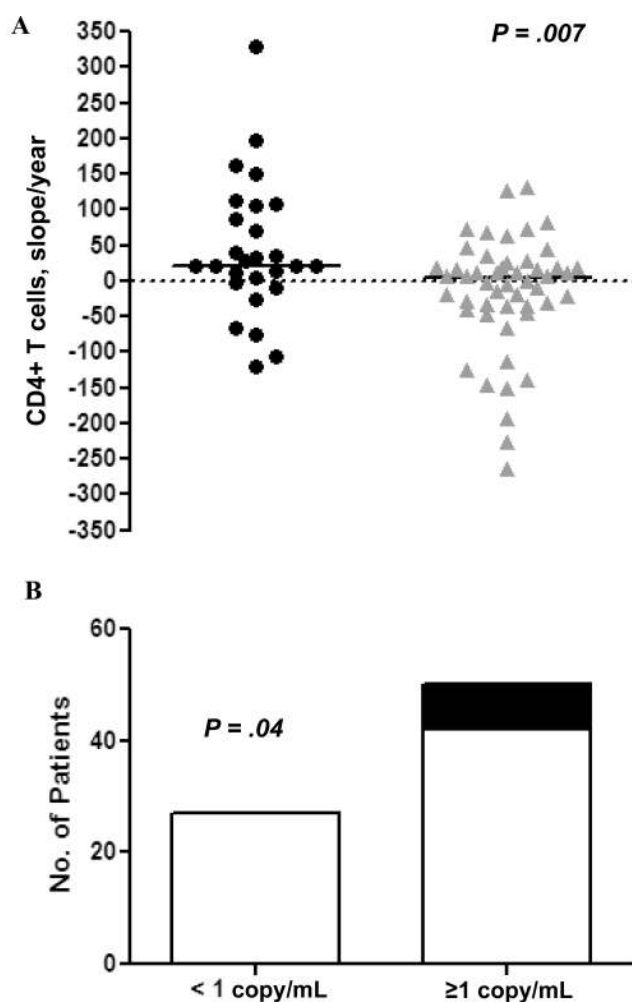


Figure 4. The change in absolute CD4⁺ T cell count per year was calculated by linear regression. *A*, The value of the slope is represented on the Y axis for subjects with human immunodeficiency virus type 1 (HIV-1) RNA levels <1 copy/mL (black circles) and for subjects with HIV-1 RNA levels ≥1 copy/mL (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 4A).

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 non-zero 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* ≤ .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 4B). 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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