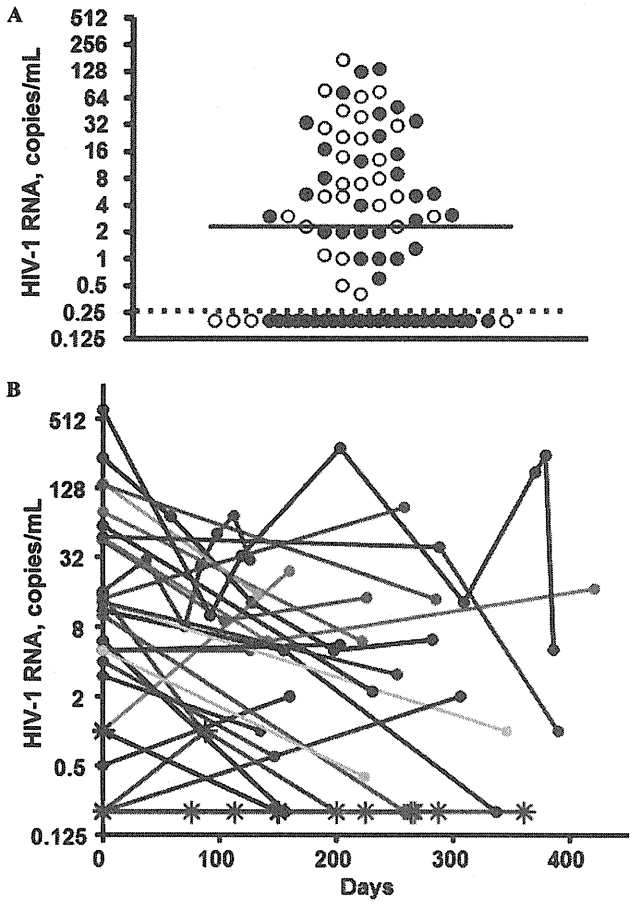

Figure 1. Plasma human immunodeficiency virus type 1 (HIV-1) RNA load distribution among 83 elite controllers *(A)*. The cut off level of 0.2 copies/mL is represented by the dotted red line. The median values are indicated with the black horizontal bar. The arithmetic mean HIV-1 RNA virus load for each 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 1*B*). 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 2*A*).

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

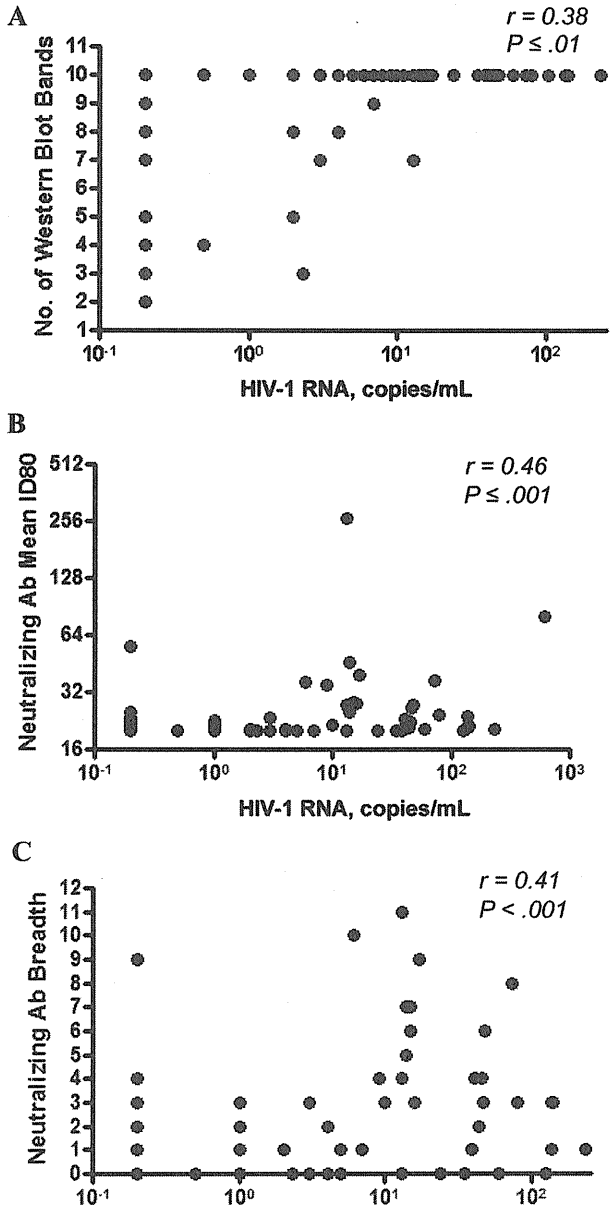

Figure 2. Antibody responses to human immunodeficiency virus type 1 (HIV-1) in elite controllers. Mean plasma virus load is plotted against *(A)* the number of virion proteins recognized by HIV-1 antibodies (Ab), as detected by Western blot; *(B)* the mean neutralizing antibody 80% inhibitory dose (ID80)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

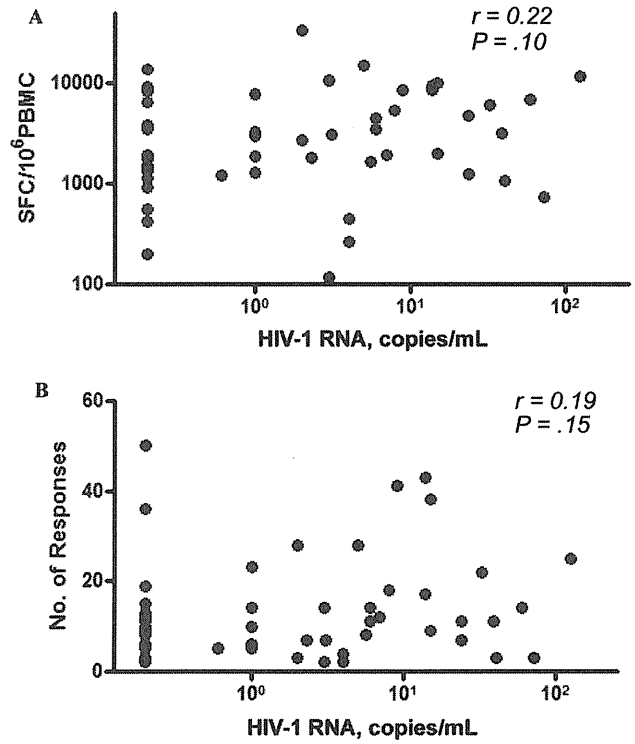

Figure 3. CD8⁺ T cell responses in elite controllers. *A,* Correlation between plasma virus load and total magnitude of the CD8⁺ T cell response, measured by enzyme-linked immunospot assay and presented in spot forming cells (SFC) per 1×10^6 peripheral blood mononuclear cells (PBMCs). *B,* Total breadth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

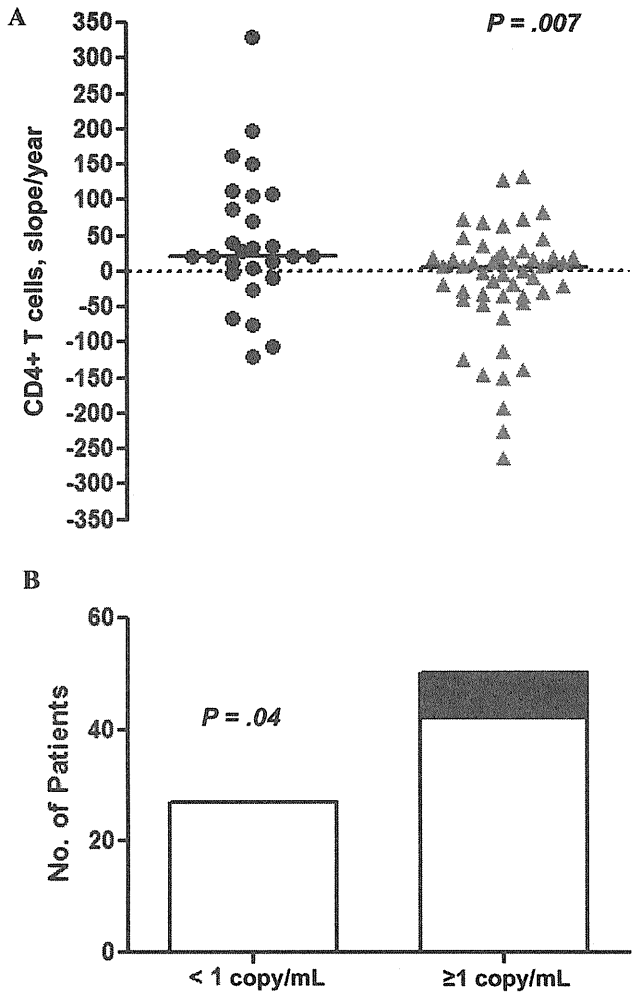

Figure 4. The change in absolute CD4⁺ T cell count per year was calculated by linear regression. *A,* The value of the slope is represented on the *Y* axis for subjects with human immunodeficiency virus type 1 (HIV-1) RNA levels <1 copy/mL (black circles) and for subjects with HIV-1 RNA levels ⩾1 copy/mL (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 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 $P \leqslant .05$) 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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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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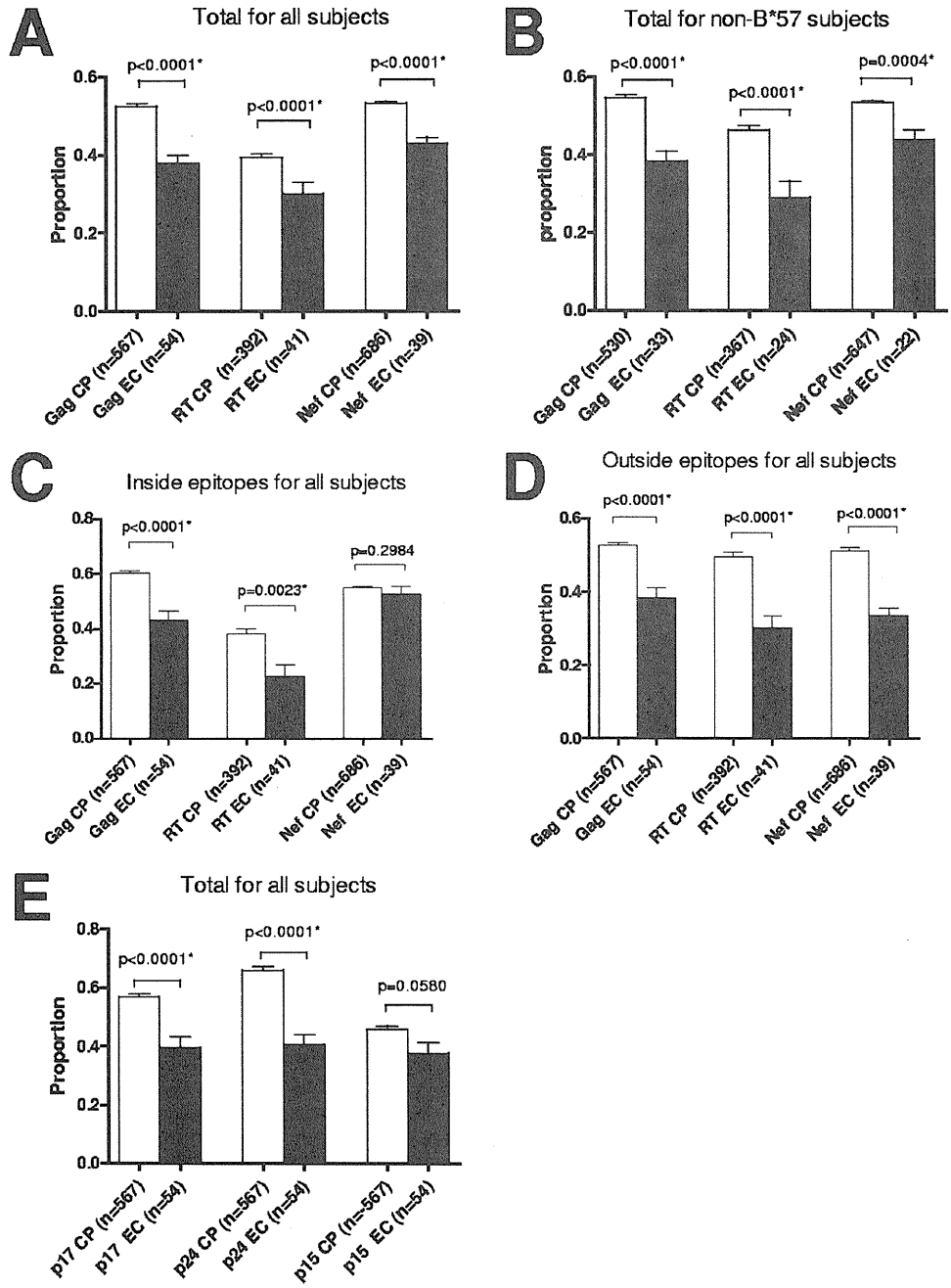

FIG. 1. Comparisons of the proportions of HLA-associated mutations between EC and CP. The mean and standard error of the proportion of sites with defined HLA-associated polymorphisms at which mutations were observed are shown. (A) Proportion of total HLA-associated sites at which mutations were observed 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

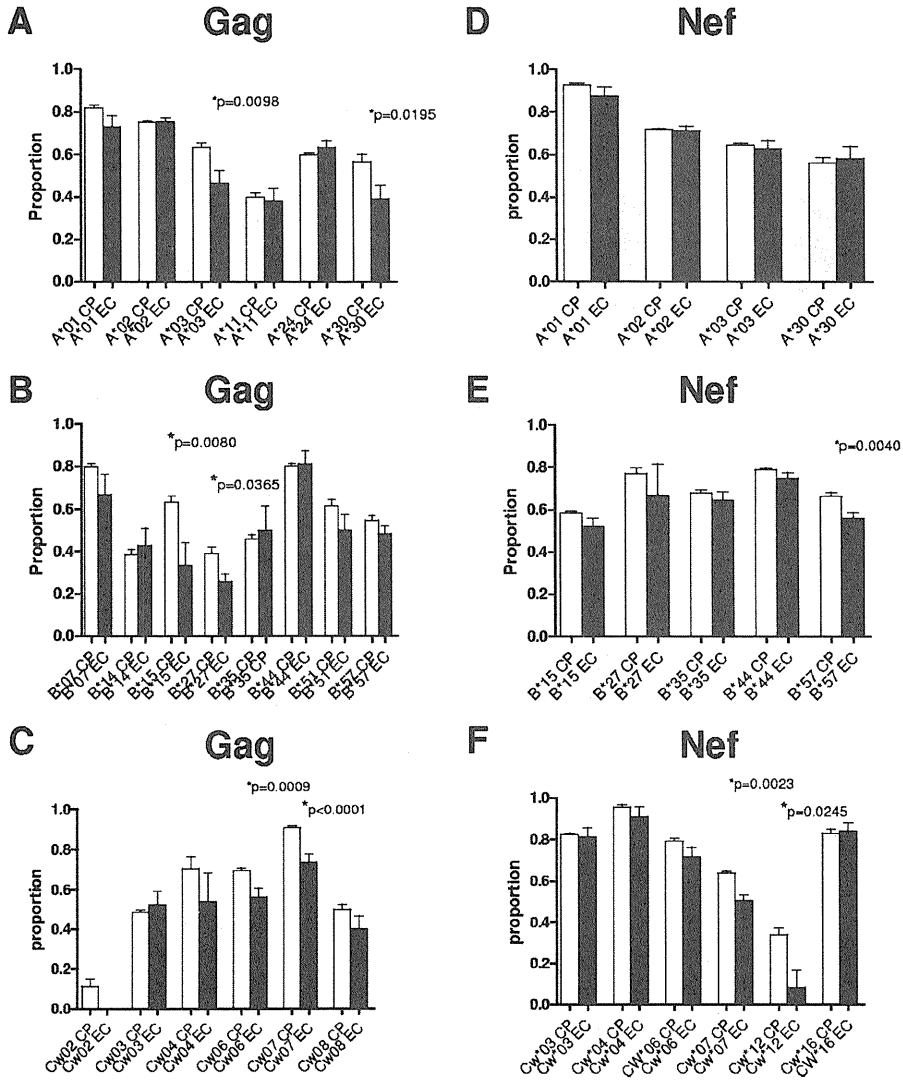

FIG. 2. Proportions of HLA-associated mutations in the Gag and Nef proteins by individual HLA class I alleles. The mean and standard error of the proportion of sites with defined HLA-associated polymorphisms at which mutations were observed are shown. HLA class I alleles present in more than four EC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

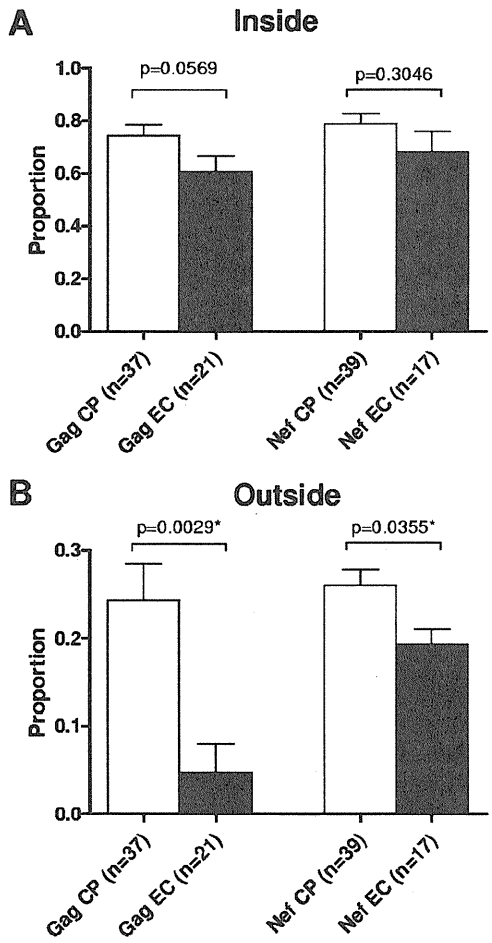

FIG. 3. Comparison of proportions of B*57-associated mutations between EC and CP. The mean and standard error of the proportion of HLA-associated sites at which mutations were observed are shown. (A) Proportion of B*57-associated sites falling within predicted B*57 CTL epitopes (inside epitopes and ±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

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AUTHOR'S CORRECTION

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

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Volume 83, no. 7, p. 3407–3412, 2009. GenBank accession numbers were erroneously omitted from the original publication. The GenBank accession numbers for the sequences derived from HIV elite controllers are EU517762 through EU517815 and EU517972 through EU518012 (as described in reference 25 of the original manuscript), EU873003 and EU873005 (as described in reference 26 of the original manuscript), and GU046566 through GU046604. The majority of the GenBank accession numbers for the sequences used to create the map of HLA-associated mutations appeared in references 7 and 9 of the original manuscript, and additional GenBank accession numbers are GQ303719 through GQ304249, GQ371216 through GQ372824, GQ398382 through GQ398387, and AY856956 through AY857186 (as described in a new publication [1]).

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Retrovirology

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Short report

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Reactivation from latency displays HIV particle budding at plasma membrane, accompanying CD44 upregulation and recruitment

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Abstract

Background: It has been accepted that HIV buds from the cell surface in T lymphocytes, whereas in macrophages it buds into intracellular endosomes. Recent studies, on the other hand, suggest that HIV preferentially buds from the cell surface even in monocytic cells. However, most studies are based on observations in acutely infected cells and little is known about HIV budding concomitant with reactivation from latency. Such studies would provide a better understanding of a reservoir for HIV.

Results: We observed HIV budding in latently infected T lymphocytic and monocytic cell lines following TNF-α stimulation and examined the upregulation of host factors that may be involved in particle production. Electron microscopy analysis revealed that reactivation of latently infected J1.1 cells (latently infected Jurkat cells with HIV-1) and U1 cells (latently infected U937 cells with HIV-1) displayed HIV particle budding predominantly at the plasma membrane, a morphology that is similar to particle budding in acutely infected Jurkat and U937 cells. When mRNA expression levels were quantified by qRT-PCR, we found that particle production from reactivated J1.1 and U1 cells was accompanied by CD44 upregulation. This upregulation was similarly observed when Jurkat and U937 cells were acutely infected with HIV-1 but not when just stimulated with TNF-α, suggesting that CD44 upregulation was linked with HIV production but not with cell stimulation. The molecules in endocytic pathways such as CD63 and HRS were also upregulated when U1 cells were reactivated and U937 cells were acutely infected with HIV-1. Confocal microscopy revealed that these upregulated host molecules were recruited to and accumulated at the sites where mature particles were formed at the plasma membrane.

Conclusion: Our study indicates that HIV particles are budded at the plasma membrane upon reactivation from latency, a morphology that is similar to particle budding in acute infection. Our data also suggest that HIV expression may lead to the upregulation of certain host cell molecules that are recruited to sites of particle assembly, possibly coordinating particle production.

Findings

It has been thought that HIV particles assemble and bud at the plasma membrane (PM) in T lymphocytes and HeLa cells, but at the endosomes in macrophages, suggesting that such endosomal targeting may be essential for HIV budding in macrophages [1-6]. However, recent studies using the inhibitors of the endocytic pathway and membrane-impermeant dyes have revealed that the PM is the primary site for HIV assembly and particle budding even in macrophages and that particles accumulate at the endosomes through endocytosis [7-9]. Nevertheless, these studies are based on observations in acutely infected cells and little is known about HIV budding concomitant with reactivation from latency. Latently infected resting T cells are known to serve as a stable reservoir for HIV during anti-retroviral therapy and to produce infectious particles upon cell reactivation. Studies on HIV production from latently infected cells upon reactivation are necessary for a better understanding of HIV pathogenesis, although some studies have indicated intracellular accumulation of particles in chronically or latently infected cells [10,11]. Here, we employed J1.1 cells that were Jurkat T lymphocytic cells latently infected with HIV-1, and U1 cells that were U937 monocytic cells latently infected with HIV-1, and observed HIV particle budding following reactivation.

We initially tested the dose of TNF-α, and temporally monitored cell growth and HIV particle production after stimulation (Fig. 1A). J1.1 cells proliferated equally regardless of the dose of TNF-α, and the particle production levels increased to 50 ng/ml TNF-α. In contrast, proliferation of U1 cells was inhibited in a dose-dependent manner, and the highest level of particle production was observed at 50 ng/ml. We thus used 50 ng/ml TNF-α for further experiments. To avoid nonspecific stimulation by changing the medium, we added TNF-α directly to the culture medium, and this led to the higher dose of TNF-α required in our study than in other reports [12,13].

Electron microscopy was carried out to examine where particle budding occurred in J1.1 and U1 cells upon reactivation (Fig. 1B). Little or no particles were produced in either cell line before TNF-α stimulation (Fig. 1B, most left panels), consistent with previous reports [11-14]. Upon stimulation, nascent budding particles were visible on the surface of nearly all J1.1 cells, similar to the case with U1 cells (Fig. 1B, arrowheads). Unexpectedly, particles in intracellular vesicles were rarely seen in both J1.1 and U1 cells (Fig. 1B, arrow). The findings were confirmed by immunoelectric microscopy using anti-HIV-1 p17MA antibody (Fig. 1B, most right panels). Next, their parental cell lines, Jurkat and U937 cells, were infected with HIV-1, and particle production in acute infection were examined by electron microscopy. Particle budding was observed predominantly at the PM of both Jurkat and U937 cells (Fig. 1C, arrowheads) but some U937 cells displayed budding into intracellular compartments (Fig. 1C, arrows). Immunoelectric microscopy indicated similar results (Fig. 1C, most right panels). For quantification, we counted the number of cells containing particles at the PM alone or that of cells containing particles at both intracellular vesicles and the PM (Fig. 1D). Budding at the PM was prominent, regardless of whether cells were acutely or latently infected, or T lymphocytic or monocytic, suggesting that unlike chronically infected cells [10], HIV particles are most likely budded from the PM in latently infected cells, although it cannot be ruled out in this experiment that the particles observed in extracellular spaces might be released by exocytosis.

Gene expression analysis based on cDNA microarrays has extensively been employed and has provided evidence for the modulation of host cellular gene expression upon HIV infection (replication and latency) [15-20]. Although numerous host genes are modulated upon HIV infection, it is conceivable that expression levels of host membrane components may change by feedback regulation upon HIV reactivation, as HIV requires host cell membrane for particle budding. A membrane contains a number of microdomains, enriched in cholesterol (i.e., rafts) and in tetraspanins (e.g., CD63 and CD81), which accumulate at sites of HIV budding [7,21-26]. It has been shown that TSG101, a component of endosomal sorting complex required for transport (ESCRT) is recruited to the sites of particle assembly and is responsible for HIV particle budding [27,28]. Thus we chose endosomal (EEA1, CD63, HRS, TSG101, and Syntaxin12) and PM (CD44 and SNAP23) markers and quantified their mRNA levels by qRT-PCR (Fig. 2A and 2B) using the primer sets shown in Additional File 1. Their properties and functions are as follows: EEA1 is a marker molecule for early endosome; HRS is an initial molecule for the ESCRT pathway; Syntaxin12 is a SNARE molecule for endosomal membrane fusion; CD44 is an adhesion molecule implicated in cell migration; SNAP23 is a SNARE molecule for PM fusion in the exocytic pathway. When the mRNA levels in J1.1 cells stimulated with TNF-α were compared with those in unstimulated J1.1 cells, CD44 gene expression was increased, but the other genes tested were largely unaltered. No significant upregulation of CD44 was observed when cells of its uninfected parental line, Jurkat, were similarly stimulated with TNF-α, indicating that the CD44 upregulation was not simply due to cell stimulation (Fig. 2A, upper). CD44 has been reported hardly expressed even at mRNA level in unstimulated Jurkat cells [29]. A similar analysis was carried out for U1 cells. Downregulation of CD44 has been reported for chronically infected monocytic cells [30]. Upon reactivation, CD44 upregulation was apparent but the endocytic molecules (CD63

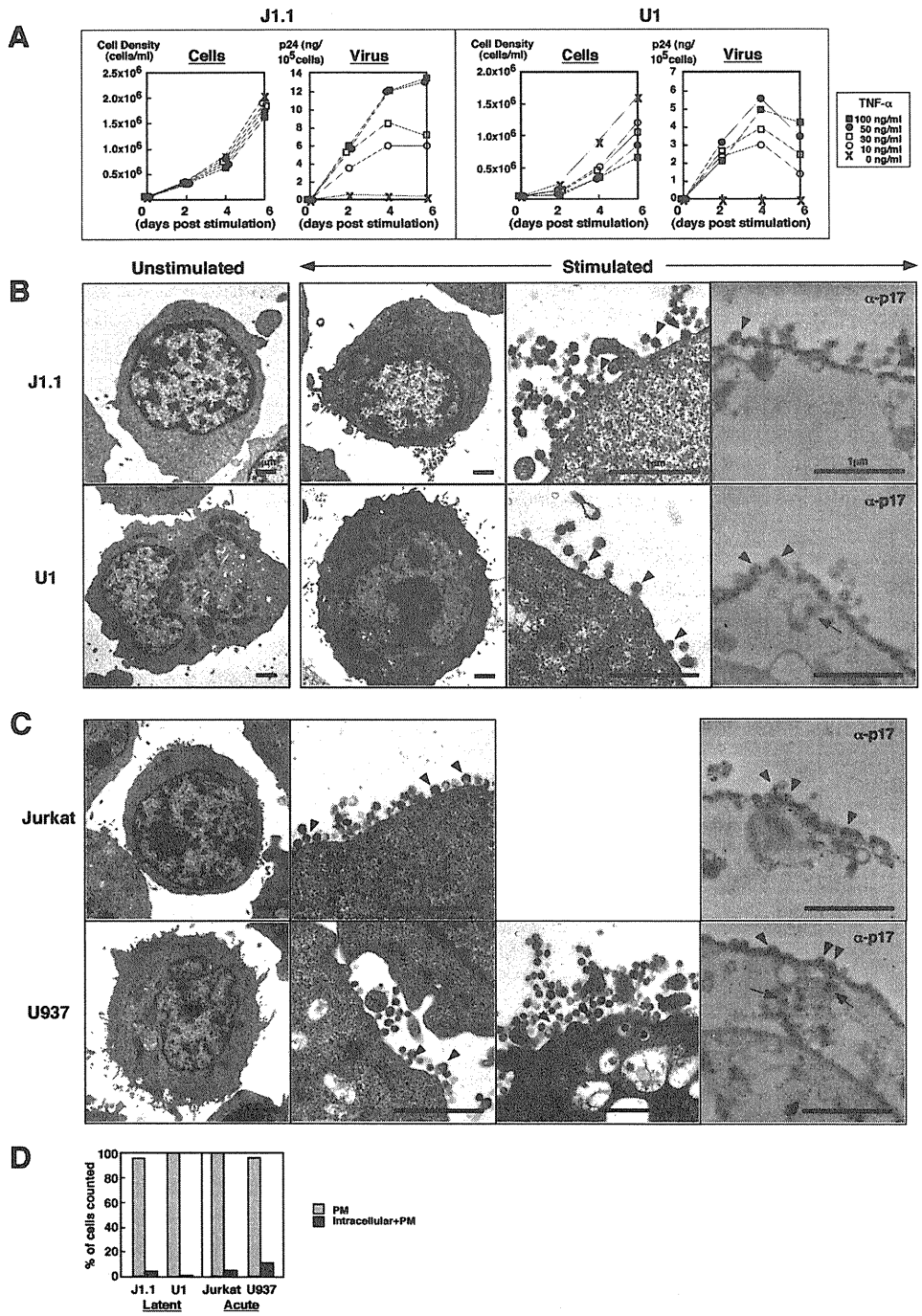

Figure 1

Reactivation of latently infected J1.1 and U1 cells displays HIV particle budding at the PM. (A) HIV production from J1.1 and U1 cells upon TNF-α stimulation. J1.1 and U1 cells were stimulated with TNF-α (~100 ng/ml). Levels of particle production were measured by p24 antigen ELISA. (B) HIV particle budding from J1.1 and U1 cells upon TNF-α stimulation. J1.1 and U1 cells stimulated with 50 ng/ml TNF-α were subjected to conventional electron microscopy and immunoelectric microscopy using anti-HIV-1 p17MA antibody. (C) HIV particle budding from acutely infected Jurkat and U937 cells. Jurkat and U937 cells were infected with HIV-1 (LAV strain) corresponding to 100–200 ng of p24CA antigen and were analyzed by electron microscopy. Arrowheads indicate budding particles and arrows indicate particles into intracellular vesicles in (B) and (C). (D) Semi-quantification of HIV-1 particle localization. Approximately 300 of particle-positive cells observed by conventional electron microscopy were sorted into the categories indicated.

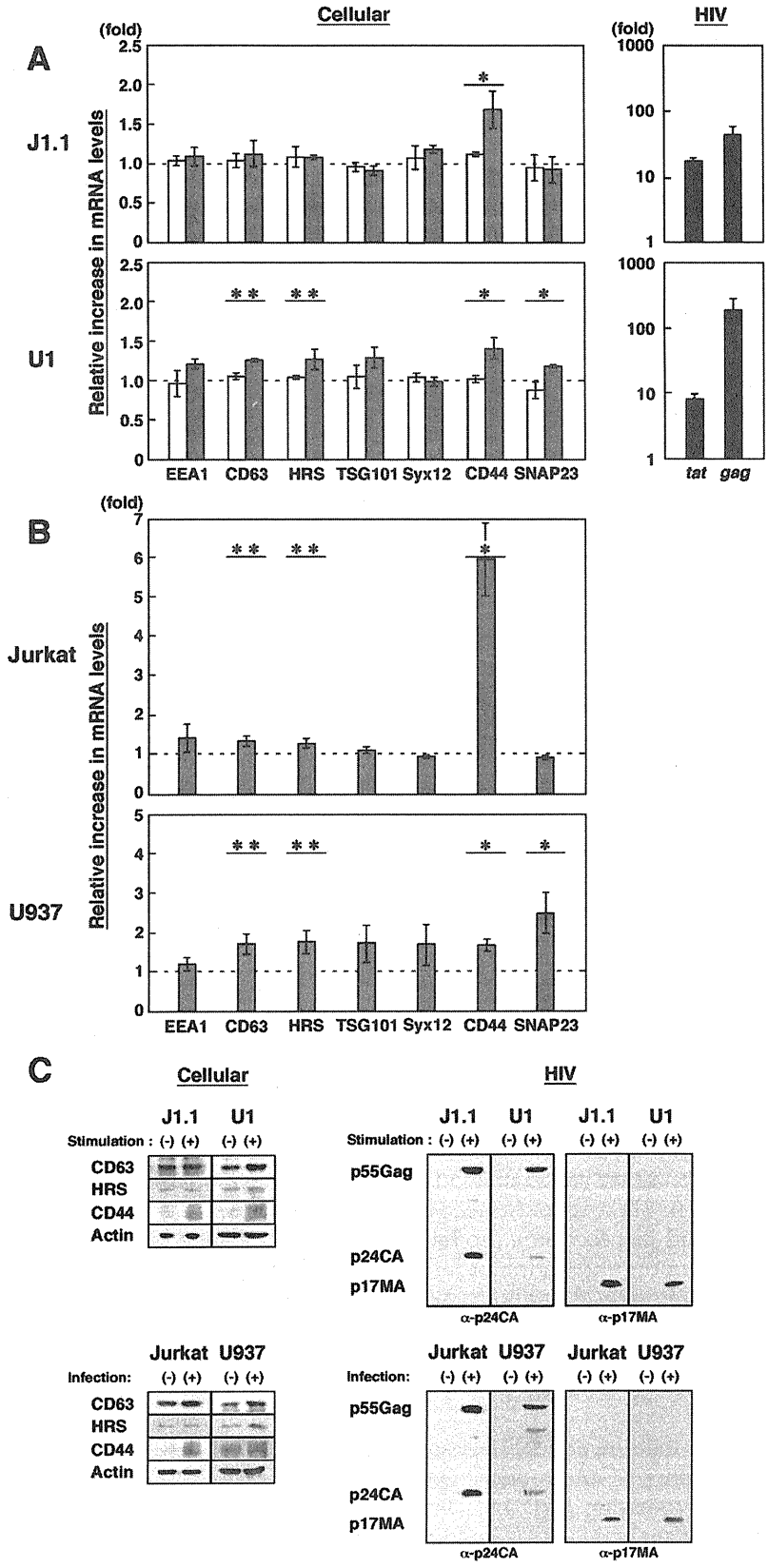

Figure 2 *(see legend on next page)*

Figure 2 *(see previous page)*
HIV particle production is accompanied by mRNA upregulation of CD44 and endocytic molecules. (A) Differential gene expression upon reactivation. J1.1 and U1 cells were either unstimulated or stimulated with 50 ng/ml TNF-α (gray columns). For comparison, uninfected Jurkat and U937 cells were similarly stimulated (white columns). Expression of each gene was quantified by qRT-PCR and normalized to the level of GAPDH. The fold increase of expression of each gene upon stimulation was shown. *, $p < 0.01$; **, $p < 0.05$. Expression levels of HIV-1 *gag* and *tat* mRNAs were quantified using specific primers (HIV-1 nucleotide positions 701–720 and 787–806 for *gag* and 5965–5987 and 8389–8411 for *tat*, respectively) (black columns). (B) Differential gene expression upon acute infection. Jurkat and U937 cells were infected with HIV-1 and subjected to qRT-PCR. The fold increase of each gene expression upon infection was shown (gray columns). *, $p < 0.01$; **, $p < 0.05$. (C) Protein expression of J1.1, U1, Jurkat, and U937 cells. TNF-α stimulation and infection were similarly performed. Cells were subjected to Western blotting using anti-CD44, anti-CD63, anti-HRS, anti-actin, anti-HIV-1 p17MA, and anti-p24CA antibodies.

and HRS) and SNAP23 were also upregulated in U1 cells. The modulation of others such as TSG101 was not statistically significant. These upregulations were not observed when uninfected U937 cells were stimulated (Fig. 2A, lower). The gene expression profiles upon reactivation were consistent with protein expression levels of the molecules when analyzed by Western blotting (Fig. 2C). We cannot simply compare the data of J1.1 and U1 cells, since expression levels of individual genes differ between the cell lines, but these significant upregulations were not observed in their parental but uninfected cells, suggesting that the upregulations might be linked with HIV expression. To test this possibility, we quantified expression levels of the same genes in acutely infected Jurkat and U937 cells and compared them with the levels in uninfected Jurkat and U937 cells. Upregulation of CD44 was observed in acutely infected Jurkat cells (Fig. 2B, upper), and this magnitude fold of upregulation was likely due to a very low level of CD44 expression in uninfected Jurkat cells [29]. In acutely infected U937 cells, besides CD44 upregulation, upregulation of other genes (CD63, HRS, and SNAP23) was observed (Fig. 2B, lower). Together, the results indicate that the upregulation of host molecules observed here was likely to be linked with HIV production. Higher levels of *gag* mRNA than *tat* mRNA observed in this study were possibly because we analyzed at a late stage of HIV replication. Western blotting confirmed HIV antigens, p55Gag precursor and its processing products, p24CA and p17MA, appeared upon reactivation or infection and showed that unlike anti-p24CA antibody, anti-p17MA antibody used in this study (against the C-terminal region of p17MA) recognized the mature p17MA domain but not the unprocessed p55Gag (Fig. 2C).

Confocal microscopy revealed that the anti-p17MA antibody specifically detected mature p17MA produced upon HIV protease-mediated Gag processing (Fig. 3A). Since Gag processing occurs concomitant with particle budding, the p17MA signal obtained with the antibody most likely represents the sites of particle budding [4,31]. No p17MA signal was seen on the day after infection, indicating that it was not derived from residual HIV (Fig. 3A). When J1.1 and U1 cells were reactivated, the p17MA antigens were observed at the cell periphery, likely at the PM, but no signals were seen in unstimulated cells (Fig. 3B). Similarly, the p17MA antigens were observed at the PM in acutely infected Jurkat and U937 cells (Fig. 3C). For quantification, we counted the numbers of cells based on p17MA distribution patterns (PM, intracellular+PM, or negative) and confirmed that HIV particles were preferentially formed at the PM (Fig. 3D), consistent with the data obtained by electron microscopy (Fig. 1D).

To understand the significance of the upregulation of host molecules observed here, we examined intracellular localization of the molecules by immunostaining. No CD44 staining was found in unstimulated J1.1 and U1 cells, consistent with previous reports indicating CD44 downregulation during latency [30,32]. Following reactivation, CD44 was visible and colocalized with the p17MA antigens at the PM. Similarly, CD63 and HRS stainings were rarely seen in unstimulated cells but became visible and colocalized with the 17MA signals, especially in U1 cells (Fig. 3B). These findings were very apparent in acutely infected cells (Fig. 3C): CD63 recruitment, as reported previously for acutely infected Jurkat T cells and macrophages [7,25,33,34], and HRS and CD44 accumulations to the sites where mature particles were formed. Together, our data suggest that HIV expression may lead to the upregulation of certain host molecules that are recruited to the sites of particle assembly, possibly to coordinate particle production. Because CD44 is a cell adhesion molecule that mediates lymphocyte aggregation and homing [35,36], it is conceivable that the CD44 recruitment to HIV assembly sites may lead to an efficient cell-to-cell transmission of HIV and infected cell migration to lymph nodes.

In conclusion, despite numerous literature on HIV budding to intracellular compartments especially in macrophages, our data indicate that upon reactivation from latent infection, HIV predominantly buds at the PM, a

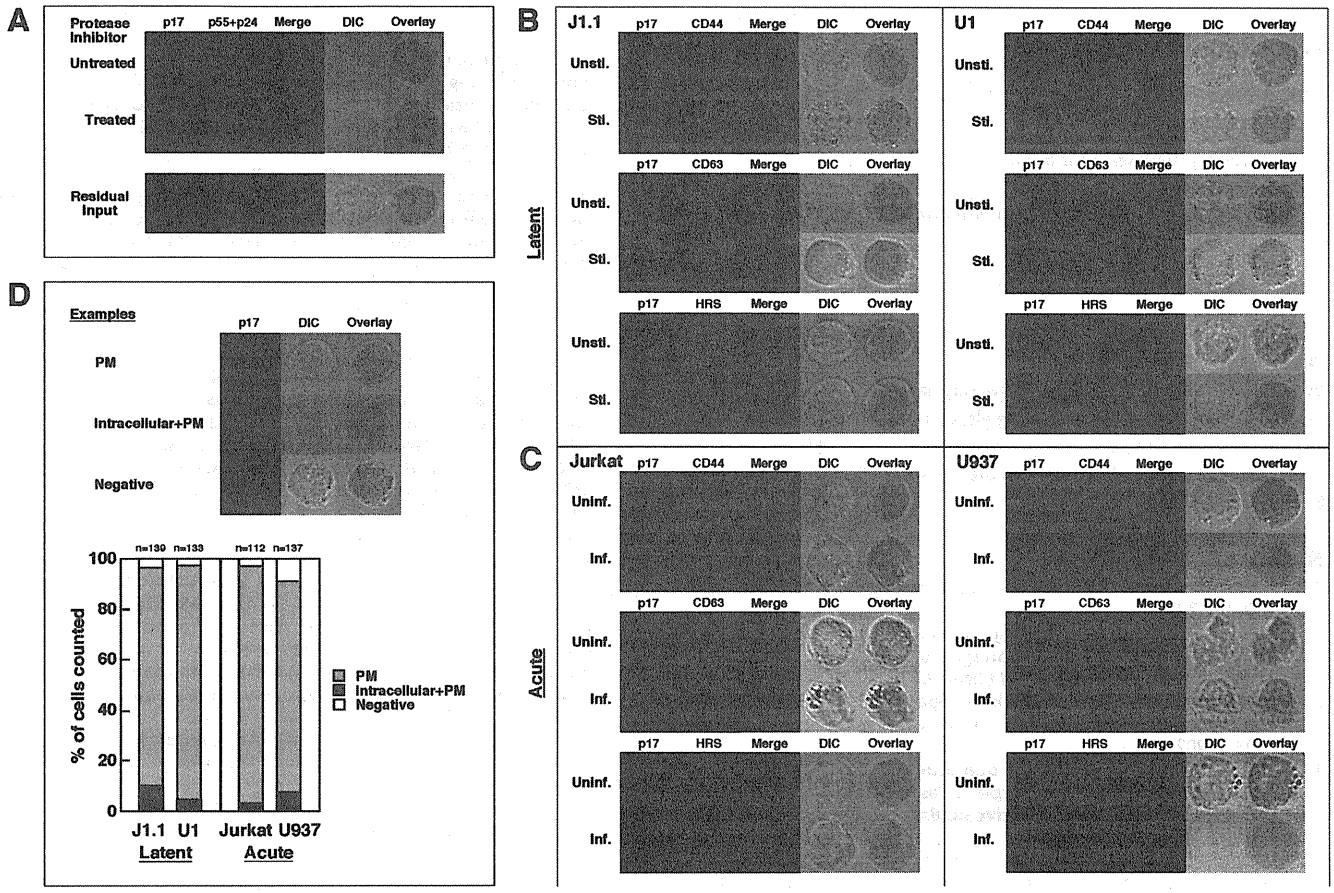

Figure 3
Upregulated molecules are accumulated to sites of HIV-1 particle budding. (A) Inhibition of Gag processing in J1.1 cells stimulated but treated with 1 μM ritonavir (upper) and residual HIV in Jurkat cells after infection (lower). For confocal microscopy, the cells were stained with anti-HIV-1 p17MA (green), p24CA (red) antibodies and with TOPRO-3 (blue). (B) Intracellular localization of upregulated molecules upon reactivation. J1.1 and U1 cells were either unstimulated (Unsti.) or stimulated (Sti.) with TNF-α and were immunostained with anti-p17MA antibody (green) and antibodies for CD44, CD63, and HRS (red). (C) Intracellular localization of upregulated molecules upon acute infection. Jurkat and U937 cells were infected with HIV-1 and immunostained. Inf., infected; Uninf., uninfected. (D) Semi-quantification of sites for HIV-1 particle production. Examples of cells exhibiting PM staining alone, intracellular+PM accumulations, and no signals (negative) (upper). Based on p17MA localization (PM, intracellular+PM, or negative), approximately 100–150 cells were sorted into the categories (lower).

morphology that is similar to particle budding in acute infection, suggesting that HIV latency have a potential for robust production of HIV observed for acute infection.

Abbreviations

HIV: human immunodeficiency virus; TNF-α: tumor necrosis factor-α; CA: capsid; MA: matrix; qRT-PCR: quantitative RT-PCR; TSG101: tumor susceptibility gene-101; HRS: hepatocyte growth factor regulated tyrosine kinase substrate; EEA1: early endosomal antigen 1; SNAP23: synaptosome associated 23 kDa protein.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MS performed the qRT-PCR analysis and confocal study. ED, TG, and KS carried out the electron microscopy analysis. YM designed the experiment and wrote the manuscript.

Additional material

Additional file 1

Sequences of primer sets used in the study.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1742-4690-6-63-S1.doc]

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Neutralizing antibodies in SIV control: Co-impact with T cells

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) and pathogenic simian immunodeficiency virus (SIV)-infected naïve hosts experience a characteristic absence of early and potent virus-specific neutralizing antibody (NAb) responses preceding establishment of persistent infection. Yet conversely, we have recently shown that NAbs passively immunized in rhesus macaques at early post-SIV challenge are capable of playing a critical role in non-sterile viremia control with implications of antibody-enhanced antigen presentation. In a current follow-up study we have further reported that NAbs mediate rapid elicitation of polyfunctional virus-specific CD4+ T-cells in vivo. The NAb-immunized macaques mounting these responses exhibited sustained viremia control for over 1 year, accompanied with robust anti-SIV cellular immunity. Perspectives obtained from the results are discussed.

1. Introduction: NAb absence in HIV-1/SIV acute infection

Absence of potent neutralizing antibody (NAb) responses in the very acute phase of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) infections is one major manifestation of defective adaptive immune responses in naïve hosts, generally failing in containment of virus replication unless privileged with certain genetic polymorphisms. HIV-1-specific NAb responses are unusually delayed in orders of months and hardly detected near peak infection. The primary humoral immune responses against these viruses are instead dominated by non-neutralizing virus-specific IgMs and IgGs [1] along with signs of aberrant polyclonal B-cell activation [2]. This initial failure is followed by a discordant array of NAbs appearing in the subacute to chronic phase, each reaching considerable titers yet being permissive of continuous neutralization escape by the autologous virus [3–6]. A preferential and possibly consequent exhaustion of HIV-1-specific B-cell responses has also been indicated in the chronic phase [7]. With these backgrounds a prophylactic induction of NAbs, particularly via pursuit of an optimal immunogen design eliciting a broadly neutralizing spectrum, has been a major aim in AIDS vaccine development [8].

Along with molecular analyses of NAbs and the HIV-1/SIV envelope proteins known for their skewed antigenicity, protective activities of monoclonal and polyclonal NAbs in vivo have been assessed by passive immunization mainly in nonhuman primates. To date, vaccine regimens inducing satisfactory NAb titers even against homologous challenge strains have not yet been developed. Passive immunization currently is a first choice surrogate for NAb analysis, but they do hold certain advantageous aspects, such as being suited for examining their impact within a certain time zone of interest. Initial studies showed that NAbs reaching a sufficient pre-challenge (or very early post-challenge) plasma or mucosal neutralizing titer typically render complete protection from chimeric simian/human immunodeficiency virus (SHIV) challenged via the same route [9–12], whereas titers to be attained for the viral inoculum sterilization had been a demanding one. On the other hand, it had been rather difficult to reach a consensus in determining whether NAbs can exhibit anti-HIV-1/SIV activity in established infections. This was partly because the rapid memory CD4+ T-cell destructive nature of CCR5-tropic HIV-1 and pathogenic SIV had been clarified only recently [13,14], which turned out to differentially validate the moments of NAb infusion in each study retrospectively. For example, NAbs passively administered in the chronic phase of HIV-1/SIV infection did not exert any impact on disease course even as a sequel to antiretroviral therapy in humans [15–16], while anti-SIV IG infusion at day 1 and day 14 post-SIVsmE660 challenge provided divergent viremia outcomes in infected macaques [17]. In HIV-1-inoculated human peripheral blood leukocyte-reconstituted SCID mice (hu-PBL-SCID mice), no suppressive effect was observed by NAb cocktail infusion past day 6 infection [18]. A common niche of these studies did however exist, which was the evaluation of the direct impact of NAbs on pre-peak viral replication and what we had designed to assess in our system.

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