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Association of HIV neutralizing antibody with lower viral load after treatment interruption in a prospective trial (A5170)

Robert J. McLinden^{a,c}, Robert M. Paris^b, Victoria R. Polonis^c, Nicole C. Close^d, Zhaohui Su^e, Cecilia M. Shikuma^a, David M. Margolis^f, and Jerome H. Kim^c

^aUniversity of Hawai'i at Manoa, Honolulu, Hawaii ^bWalter Reed Army Medical Center, Washington, District of Columbia ^cUS Military HIV Research Program, Rockville ^dEmpiriStat, Inc., Mt. Airy, Maryland ^eCBAR, Harvard University, Boston, Massachusetts ^fUniversity of North Carolina, Chapel Hill, North Carolina, USA

Abstract

Objective—We investigated the impact of neutralizing antibodies (NAbs) on CD4 T-cell count and viral load in a cohort of HAART recipients who underwent extended structured treatment interruption.

Design—Substudy of NAb in the AIDS Clinical Trials Group 5170 trial.

Methods—Early plasma samples from 50 volunteers who discontinued HAART were evaluated in a peripheral blood mononuclear cell-based neutralization assay against a panel of four subtype B primary isolates.

Results—We found that high-titer (90% inhibitory dose > 500) NAb against two or more isolates was associated with reduced viral load ($P=0.003$ at 12-week posttreatment interruption). This effect faded with time, losing significance ($P=0.161$) by study conclusion. Participants possessing the highest NAb levels against individual isolates appeared more likely to have lower viral loads with the association gaining significance against the R5-tropic primary isolate US1 ($P=0.005$). There was no association between broader neutralization and CD4 T-cell slope over time.

Conclusion—The data suggest that high-titer NAb responses at the time of treatment interruption are associated with reduced viral load over time, but not CD4⁺ T-cell decline.

Keywords

CD4⁺ T cells; HAART; HIV; neutralizing antibodies; viral load

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Correspondence to Jerome H. Kim, US Military HIV Research Program (MHRP)/Walter Reed Army Institute of Research (WRAIR), 1600 East Gude Drive, Rockville, MD 20850, USA. Tel: +1 301 251 7764; fax: +1 301 762 7460; jkim@hivresearch.org.

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Conflicts of interest There are no conflicts of interest.

Introduction

HIV-infected patients on HAART are an informative population in the study of immune responses to HIV-1. Improvements in immunologic status, including increases in overall CD4⁺ T-cell counts and lymphoproliferative responses are observed in patients with suppressed viral replication [1–4]. Several studies have shown that cytotoxic T lymphocyte and neutralizing antibody (NAb) responses to HIV-1 improve, at least transiently, in most patients treated with HAART [5,6]. Although the mechanism remains unclear, HAART may slow virus evolution, allowing the immune response to catch-up [7].

Long-term administration of HAART may be associated with toxicity and drug resistance [8,9]. Treatment interruption has been proposed as a way to reduce HAART associated toxicity and boost anti-HIV immune responses [10]. To date, studies of treatment interruption have been equivocal with respect to virologic and clinical outcomes [11–14]. However, it may be important to distinguish HIV-positive individuals who initiate antiretroviral therapy (ART) shortly after infection while still possessing relatively intact immune systems from those initiating therapy after longer period of time wherein serious long-term damage has already occurred. In one recent study of HIV-1-positive individuals initiating ART within 6 months of infection [15], 40% ($N=73$) were able to maintain low HIV RNA concentrations (<5000 copies/ml) following treatment interruption (24 weeks) suggesting that early treatment may preserve the immune system and facilitate the development of more effective immune responses.

Cell-mediated immune responses appear to improve in breadth and magnitude during treatment interruption [12,16], whereas elevated NAb titers and broadly NABs have been observed in some patients with poor HAART adherence and in those with chronic, untreatment infection [17–20]. In one study, overall binding and NAb development appeared to be impaired in patients who were treated with HAART shortly after seroconversion. However, some of these same patients showed strong autologous NAb upon treatment interruption suggesting that some degree of B-cell priming likely occurred prior to HAART leading to a strong secondary NAb response to the 'fixed' virus population posttreatment [21].

In the present study we examined the neutralizing capacity of individual patient plasma taken at the time of treatment interruption against a panel of subtype B primary isolates. Although neutralization at the time of treatment interruption was not associated with CD4 cell count decline, there was a strong association with reduced viral load after treatment interruption. This effect waned over time, suggesting viral escape.

Methods

Clinical samples

Volunteers from 31 AIDS Clinical Trials Group (ACTG) sites were chosen to participate in an observational, prospective study (A5170) of the clinical outcome of antiretroviral treatment interruption [14]. The overall cohort consisted of 167 HIV-infected persons over the age of 18 who voluntarily agreed to discontinue ART for the duration of the study (96 weeks). Minimum requirements for study enrollment were a CD4⁺ T-cell count more than 350 cells/ μ l, plasma viral load less than 55 000 copies/ml and adherence to a combination of two or more predetermined antiretroviral agents for a minimum of 6 months. Immunologic and virologic markers of disease status, including CD4⁺ T-cell count and HIV-1 viral RNA copy number (viral load) were measured every 4 weeks during the period of treatment interruption. Clinical progression was defined as two, consecutive CD4⁺ T-cell counts less

than 250 cells/ μ l. Frozen plasma samples from 50 volunteers were obtained and heat-inactivated (56°C/30 min) prior to use. Volunteers were selected by CD4⁺ T-cell decline at 24-week posttreatment interruption and divided evenly into two groups: 'progressors' and 'nonprogressors' [22]. Progressors were defined by a CD4⁺ T-cell reduction of more than 40% at week 24. Nine of the 25 progressors reinitiated ART prior to study end. Nonprogressors were defined by a CD4⁺ T-cell decline of less than 20% over the same period. CD4 T-cell decline in this group was less than 50% of that observed in the progressor group at week 24. No one in this group re-initiated ART during the study. The two groups had similar CD4⁺ T-cell counts and viral loads at trial entry. Additional exploratory analyses were performed by assigning volunteers to groups by number of isolates neutralized, designated as 'restricted' neutralization if 0–1 isolate was neutralized with a titer (90% inhibitory dose) greater than 500 and 'expanded' neutralization, if volunteer plasma neutralized two or more isolates with an 90% inhibitory dose greater than 500.

Viruses

We tested volunteer plasmas against four subtype B primary isolates [US1, US4 and 89.6 as well as a recombinant B' (CM237) isolate from Thailand], all obtained from the NIH AIDS Reagent and Research Program (Germantown, Maryland, USA). All isolates utilize CCR5 (R5) as coreceptor and represent a range of neutralization sensitivity (Weak-89.6, CM237, US4, US1-Strong) to pooled HIV-positive sera and sCD4 [6,23–26]. Of note, isolate 89.6 is also known to use CXCR4 and is considered dual-tropic (R5/X4) [27]. A single stock of each isolate (sufficient for all experiments) was generated by infection of PHA/IL-2-stimulated peripheral blood mononuclear cells (PBMCs) from a single donor (SeraCare, Inc., Milford, Massachusetts, USA) [26]. To confirm stock virus identity, a segment of the envelope region (C2–V4) was sequenced from proviral DNA.

Virus titration assay

Titration assays were performed as described [26]. Briefly, stock virus was serially diluted in cRPMI/IL-2 media cRPMI/IL-2 [RPMI 1640 (Quality Biological, Gaithersburg, Maryland, USA), 15% fetal calf serum (Gemini, Woodland, California, USA), 1% L-glutamine (Quality Biological), 1% penicillin/streptomycin (Quality Biological), and 20 U/ml rhIL-2 (Roche, Indianapolis, Indiana, USA)] in a 96-well 0.5 ml plate. PHA/IL-2-stimulated PBMC (1.5×10^5 cells/well) were added to a final volume of 100 μ l and incubated overnight at 37°C/5%CO₂. Cells were washed thrice in cRPMI/IL-2 and transferred to a 96-well round-bottom plate. Supernatants were harvested for measurement of p24 core antigen by sandwich ELISA on days 4, 6, and 8 and lysed according to the kit manufacturer's protocol (Coulter, Hialeah, Florida, USA). Optimal virus dilution and harvest day (>10 ng/ml of p24) were determined for use in downstream virus neutralization assays.

Virus neutralization assay

Virus neutralization assays were performed as previously described [26]. Briefly, sample plasmas and pooled HIV-1 subtype-specific plasma controls were initially diluted 1 : 5 in cRPMI/IL-2, followed by serial 1 : 4 dilutions. The final plasma dilution range including antibody, cells, and virus was 1/20–1/20 480. For baseline virus growth, plasma was replaced with cRPMI/IL-2 in a minimum of eight wells. Normal human plasma was used as a control for nonspecific neutralization (Gemini). An equal volume of appropriately diluted virus stock was added to each well. Serum and virus were incubated for 30 min at 37°C/5% CO₂ prior to the addition of PHA/IL-2 stimulated PBMC (1.5×10^5 cells/well) in a final volume of 100 μ l. The plates were incubated overnight at 37°C/5% CO₂. Cells were washed thrice in cRPMI/IL-2 and transferred to a 96-well round-bottom plate. The cells were incubated according to predetermined virus growth kinetics (see above). Supernatants were

harvested for measurement of p24 core antigen by sandwich ELISA and lysed according to the kit manufacturer's protocol (Coulter). Lysed supernatants (quadruplicate wells) were pooled prior to loading on the ELISA plate. Each plasma was assayed at least twice. If the titers between experiments were more than $0.5\log_{10}$, a third experiment was performed. Titers were averaged prior to data analysis. p24 antigen in pooled experimental wells (V_n) was compared with cRMPI/IL-2 containing positive control wells (V_o). The ratio, V_n/V_o was plotted versus the reciprocal dilution of serum. Ninety percent inhibitory dose titers were calculated using linear regression analysis as previously described [28]. Titers less than 20 [no significant neutralization compared to media controls at the lowest plasma dilution (1 : 20)] were replaced with a value of 19 for statistical analyses.

Statistical analysis

Primary analyses compared NAb titers to changes in CD4 T-cell number after interruption of antiviral treatment. We calculated individual slopes for CD4 cell counts between 8 and 96 weeks after treatment interruption, to account for the two-phase decline, as previously described by Skiest *et al.*, on the entire cohort [14]. We then compared slopes by Spearman correlation coefficient with neutralization titer, as well as by neutralization category (restricted neutralization versus expanded neutralization), using a nonparametric test (Mann–Whitney *U* test). For analysis of longitudinal viral load data in the 'restricted' neutralization versus 'expanded' neutralization groups, we used generalized estimating equations for linear regression to account for the nonindependence of repeated viral load measurements [29,30]. We also stratified 90% inhibitory dose neutralization titer by quartile for each virus and performed a one-way analysis of variance against setpoint, log-transformed viral load, determined by taking the mean of viral load measurements up to 12 weeks after treatment interruption [31,32]. Models were compared with the lowest quartile of neutralization titer as the reference group. For all longitudinal analyses, observations after resumption of ART were censored. A two-sided level of significance of 0.05 was used for all analyses. *P* values for multiple comparisons were corrected using the Bonferroni method. Analyses were performed using Stata SE v10.0 (Stata Corp., College Station, Texas, USA).

Results

Clinical and laboratory data

The cohort consisted of 50 predominantly male (96%) volunteers (Table 1). Whites represented 67%, African Americans 19%, Hispanics 10%, and Asian/Pacific Islanders 4%. Of the 50 volunteers, 76% (38) remained on study for the full 96 weeks. Ten of the remaining 12 restarted ART prior to study conclusion, and two were lost to follow-up. The median CD4⁺ T-cell number at study initiation was 855 (292–1402 cells/ μ l). HIV-1 RNA viral load at study entry ranged from less than 50 to 12 441 copies/ml. From study conclusion, the median CD4 T-cell count had fallen to 454 cells/ μ l. By this point, all volunteers had detectable viral loads with a mean of $4.27 \log_{10}$ copies/ml (SD 0.80 log). To examine the impact of neutralizing activity at baseline (prior to HAART discontinuation), volunteers with similar CD4⁺ T-cell number at study entry were separated into progressor and nonprogressor groups. Progressors were defined by a more than 40% reduction in CD4⁺ T cells through week 24, whereas nonprogressors displayed a less than 20% decline. Mean $\log_{10}$ viral loads in the progressor and nonprogressor groups were comparable at both the beginning and end of the study. Proportions of volunteers with respect to ethnicity and sex were similar between the two groups (data not shown).

Neutralization of subtype B viruses

Table 1 summarizes the mean 90% inhibitory dose against a panel of four well characterized subtype B, PBMC-derived, low-passage primary virus isolates. When comparing geometric

mean titer between progressors and nonprogressors, we found no statistical difference. Subsequent examination of 90% inhibitory dose at the individual isolate level also failed to show statistical significance (US1, $P=0.90$; US4, $P=0.51$; CM237, $P=0.83$; 89.6, $P=0.68$).

Multi-isolate neutralization

A central question surrounding the relationship between NAb and clinical outcome is whether a high-titer heterologous response is associated with slower disease progression. To assess this in the context of the study, the number of participants with 90% inhibitory dose at least 500 for 1 virus or less (Restricted Neutralization-RN) and at least 2 virus (Expanded Neutralization-EN) were compared (Fig. 1a). Twenty of 25 participants (80%) in the Progressor group exhibited restricted neutralization. Eighteen of 25 (72%) in the nonprogressor group fell into the restricted neutralization category. Expanded neutralization was limited to nine individuals in the Progressor group and seven in the nonprogressor group. Based on the study definition, expanded neutralization was not associated with slower disease progression.

Similarly, we looked at the impact of expanded neutralization on the RNA viral load over the course of the study. At study entry both groups had a similar number with suppressed viral load on treatment, and mean $\log_{10}$ RNA viral loads were similar between the restricted neutralization and expanded neutralization groups (restricted neutralization: 2.05 ± 0.659 versus expanded neutralization: 1.84 ± 0.557 ; $P=0.35$, Student's t test). In contrast, Fig. 1b showed that the expanded neutralization group possessed a significantly lower mean $\log_{10}$ RNA viral load posttreatment interruption (~4-week posttreatment interruption) (restricted neutralization: 4.06 ± 1.24 versus expanded neutralization: 3.02 ± 1.45 ; $P=0.018$, Student's t test). At study endpoint, the expanded neutralization group advantage was no longer significant (restricted neutralization: 4.33 ± 0.909 versus expanded neutralization: 4.06 ± 0.52 ; $P=0.35$, Student's t test), suggesting that neutralization of multiple heterologous isolates at treatment interruption was associated with lower viral load and that this effect was transient.

We next evaluated posttreatment interruption viral RNA by quartile of neutralization titer with one-way analysis of variance (Fig. 2). A statistically significant difference in mean $\log_{10}$ viral loads at 12-week posttreatment interruption by neutralization titer was observed against US1 ($P=0.005$) (Fig. 2a). Examination of the distribution of viral load with quartile of NAb titer appeared to be nonlinear, suggesting that there may be a discrete neutralization threshold necessary for a significant reduction in viral load. This was significantly different between the third and fourth quartiles (Bonferroni corrected P value, 0.036). A nonlinear trend test was also performed using a Wald-type test for a nonlinear hypothesis testing, which yielded an overall Bonferroni-corrected P value of 0.05 [33]. A similar pattern was also observed for the other three viral isolates. However, these differences did not reach statistical significance (Figs. 2b–d).

Duration of neutralization antibody effect

As the principal measure of progression in this study was limited to the rate of $CD4^+$ T-cell decline 24 weeks after treatment interruption, we expanded the analysis to all $CD4^+$ T-cell observations between restricted neutralization and expanded neutralization groups over the entire study (96 weeks or the last $CD4$ T-cell count prior to resumption of ART if the volunteer resumed therapy prior to 96 weeks). The expanded neutralization group ($n=12$) had a higher, but not statistically significant $CD4$ T-cell count compared to the restricted neutralization group ($n=38$; $P=0.19$). Similarly, comparison of $CD4$ slope between 8 and 96 weeks was not different ($P=0.22$). Examination of Lowess-smoothed regression plots suggested no difference over the period of study, which was confirmed by generalized estimating equation analysis comparing the two groups over time ($P=0.98$) (Fig. 3a). In

contrast, examination of Lowess-smoothed regression plots for $\log_{10}$ viral RNA showed a lower rate of viral replication after treatment interruption for the expanded neutralization group (Fig. 3b). Using a model that limited observations to 12, 24, 48, and 96-week intervals, we found the strongest association at 12-week posttreatment interruption ($P=0.003$), which decreased over time, losing statistical significance by week 48 ($P=0.023$ at week 24, $P=0.071$ at week 48, and $P=0.161$ at week 96).

Discussion

Although cell-mediated immunity has been associated with control of viremia, the role of NAb is less clear [34–39]. A number of studies investigating the contribution of NAbs in vertical transmission, nonhuman primate models, vaccines, long-term nonprogressors and highly exposed persistently seronegative cohorts have provided varying results with regard to acquisition and disease progression [40–45]. In this study, we compared NAb titers against a panel of heterologous subtype B HIV-1 primary isolates at time of HAART cessation to changes in viral load and $CD4^+$ T-cell number during a clinical trial of volunteers undergoing long-term structured treatment interruption [14]. There did not appear to be significant association between baseline NAb titers and either $CD4$ T-cell count or viral load at study conclusion. However, stratification of NAb into quartiles revealed that higher titers were associated with lower viral load, and lower NAb titers may be linked with higher viral load. In addition, a more careful analysis of high-titer multiple isolate neutralization at baseline versus viral load changes over time suggested that NAb were associated with nondurable viral load control.

Interestingly, the NAb titer relationship to viral load appeared to be nonlinear, a pattern that was consistently observed for the four viruses tested. Although the differences in mean $\log_{10}$ viral load were only significant for US1, these and other data suggest that there may be a neutralization 'threshold' necessary to control viral replication [46–48]. The unexpected finding that low-to-moderate NAb titers were associated with higher viral load raised interesting questions. These were not autologous isolates nor was the study performed in the setting of transmission. Specifically, this may speak to the complex relationship between pathogen and host, where circulating, but nonneutralizing anti-HIV antibodies may have a deleterious effect on viral load [49,50].

The clinical impact of this finding is unknown, as most HIV-infected individuals do not develop broadly NAb over the course of infection [49,50]. Others have observed that antibodies coated on virions or infected cells were bound directly, or through Fc-bound complement proteins, to potential target cells (e.g., macrophages and dendritic cells) resulting in enhanced transmission [51–53]. Similarly, it has been reported that complement-mediated antibody enhancement is specifically associated with increased viral load [54]. It is unclear whether this is a function of the virus/antibody interaction, quantity of antibody present or if antibodies to specific epitopes on the viral envelope are responsible for enhancement [55–57].

When observing the effect of neutralization over time, there appeared to be a positive but nonsignificant association between expanded neutralization and less rapid decline in $CD4$ T cells. This was, perhaps, expected as several viral and cellular factors influence $CD4^+$ T-cell death [22,58,59]. Consistent with earlier studies, we found higher titer multi-isolate reactive NAbs were associated with reduced viral load over time and that this effect was transient [60]. We did not attempt to determine whether this was a primary effect of the antibody or a surrogate for the health of the host immune system, the latter perhaps supported by previous work describing heterologous NAb in viremic controllers [60,61]. Alternatively, persons who neutralize multiple isolates may have a greater quasispecies diversity, hence greater

epitope recognition. Previous studies have suggested that the gradual loss of an effective NAb response may be attributed to the rapidity of HIV mutation/selection that prevents the development of effective autologous neutralizing responses [62,63].

In conclusion, we found that high-titer, heterologous HIV-1 NAb were associated with a reduction in HIV-1 viral load in individuals possessing a more extensive neutralization phenotype while there appeared to be minimal impact on CD4 T-cell decline. Of note, the time from infection to ART is unknown for these A5170 participants, and it is possible that the maturation of NAb responses seen during the natural history of infection may have been interrupted by early treatment [64]. However, the likelihood that more than a handful of these participants initiated therapy during acute or early infection is low. Similarly, the impact of pretreatment interruption ART on immune responses is complex, but in cases where undetectable viral load is achieved, antigen-driven cellular and humoral responses are ultimately diminished [65–67].

Additional studies using isolates from non-B subtypes and donors with diverse HLA haplotypes are needed to support this finding in the context of global pandemic. Further exploration of the interaction between breadth of neutralization and viral load or CD4 cell count could best be obtained from prospective cohort studies of HIV disease progression, ideally in the absence of HAART.

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Fig. 1. Baseline neutralization and serial viral loads stratified by neutralization status
 (a) Number of progressors and nonprogressors exhibiting the restricted neutralization or expanded neutralization neutralization phenotype. (b) Mean log₁₀ plasma viral load in restricted neutralization versus expanded neutralization groups at study entry, 4-week posttreatment interruption and study conclusion.

Fig. 2. Distribution plots of $\log_{10}$ HIV-1 RNA setpoint by quartile of neutralizing antibody titer
Points represent average of viral load measurement up to 12 weeks for each volunteer.

Fig. 3. Lowess-smoothed regression plots

(a) CD4 and (b) viral load trajectories based on two or more 90% inhibitory dose neutralization titers greater than 500.

Table 1

Volunteer clinical, virologic, and immunologic data.

Patient no.	CD4 cell count (entry)	log ₁₀ RNA (posttreatment interruption)	CD4 cell count (96 weeks)	log ₁₀ RNA (96 weeks)	Geometric mean titer ^d			Restart ART	
					US1	US4	CM237		
Progressors (<i>n</i> =25)									
1	887	5.76	446	4.52	176	158	<20	397	Yes
3	1009	3.80	432	4.93	36	<20	<20	61	No
5	1115	4.63	387	3.97	<20	22	19	<20	No
7	681	4.00	167	3.81	<20	<20	<20	23	Yes
8	1006	5.95	588	4.29	247	214	203	244	No
9	899	5.28	112	5.82	60	149	92	74	Yes
10	922	3.74	304	3.52	25	60	61	133	No
11	585	2.43	277	4.46	<20	<20	<20	40	No
13	1155	3.30	648	3.64	<20	35	43	70	No
18	1311	4.12	696	3.58	<20	<20	<20	<20	No
19	1118	5.78	445	4.07	180	139	373	215	No
22	671	3.91	200	4.55	62	111	54	62	Yes
23	706	5.50	539	4.52	<20	23	<20	<20	No
24	1191	2.05	454	3.80	457	1497	44	676	No
25	847	5.52	500	5.93	55	157	146	74	Yes
27	985	4.61	148	5.92	<20	<20	<20	<20	Yes
28	598	5.78	237	4.97	63	<20	126	<20	Yes
30	1141	4.16	621	3.33	<20	<20	<20	<20	No
32	707	4.73	353	4.42	1075	3917	1724	1330	No
33	649	4.70	435	5.85	<20	<20	<20	<20	Yes
41	1068	5.83	293	5.79	35	218	174	130	Yes
43	1204	5.56	722	4.40	1075	2955	859	1134	Yes
46	855	4.65	421	4.25	268	1225	592	1134	No
47	1200	1.69	384	4.34	23	<20	<20	<20	No
48	817	1.69	374	4.52	677	1900	775	1021	No
Summary ^b	922 (462)	4.37 (1.32)	421 (297)	4.53 (0.80)	65 (63–67)	96 (94–99)	64 (62–66)	91 (89–93)	10 (40%)

Patient no.	CD4 cell count (entry)	log ₁₀ RNA (posttreatment interruption)	CD4 cell count (96 weeks)	log ₁₀ RNA (96 weeks)	Geometric mean titer ^a			Restart ART
					US1	US4	CM237	
Nonprogressors (<i>n</i> =25)								
2	533	4.06	373	4.24	1725	5554	1530	No
4	652	6.31	454	4.63	<20	26	<20	No
6	856	5.18	726	4.82	<20	36	24	No
12	1306	2.53	1058	2.36	92	189	192	No
14	1117	1.69	1111	3.22	<20	<20	33	No
15	810	2.32	463	3.88	57	139	117	No
16	989	1.69	810	4.08	446	798	2008	No
17	586	2.43	434	4.02	197	48	31	No
20	1297	3.30	897	4.06	46	75	<20	No
21	851	1.69	715	2.88	1983	3002	1421	No
26	695	1.69	548	4.52	890	3617	845	No
29	1047	2.80	888	3.31	<20	48	24	No
31	833	4.74	493	4.45	<20	35	33	No
34	756	3.61	533	4.53	274	2962	1450	No
35	732	4.39	75	4.69	33	24	39	No
36	710	4.30	462	4.58	<20	20	<20	No
37	676	3.58	346	4.49	<20	<20	<20	No
38	911	2.07	903	2.93	<20	<20	<20	No
39	699	3.81	894	3.15	<20	35	<20	No
40	922	3.23	809	2.72	<20	<20	<20	No
42	292	4.76	310	4.75	52	49	22	No
44	432	1.69	375	3.23	2607	5026	1252	No
45	963	3.07	702	4.06	775	2015	783	No
49	986	4.45	454	5.44	262	39	42	No
50	693	4.08	423	4.87	<20	<20	<20	No
Summary ^b	810 (396)	3.34 (1.27)	533 (449)	4.00 (0.80)	85 (83–88)	123 (120–126)	84 (82–87)	0 (0)

ART, antiretroviral therapy.

^aGeometric mean titers (GMT) based on an infectious dose for 90% neutralization (90% inhibitory dose). Titers <20 represent a titer that is below the detection limit of the assay system.

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^bSummary statistics are mean (standard deviation) for log10 viral RNA; median (interquartile range) for CD4 T-cell counts, mean (95% confidence interval) for GMT, and sum (percentage) of those re-starting ART.