



Anti-HIV-1 ADCC Antibodies following Latency Reversal and Treatment Interruption

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ABSTRACT There is growing interest in utilizing antibody-dependent cellular cytotoxicity (ADCC) to eliminate infected cells following reactivation from HIV-1 latency. A potential barrier is that HIV-1-specific ADCC antibodies decline in patients on long-term antiretroviral therapy (ART) and may not be sufficient to eliminate reactivated latently infected cells. It is not known whether reactivation from latency with latency-reversing agents (LRAs) could provide sufficient antigenic stimulus to boost HIV-1-specific ADCC. We found that treatment with the LRA panobinostat or a short analytical treatment interruption (ATI), 21 to 59 days, was not sufficient to stimulate an increase in ADCC-competent antibodies, despite viral rebound in all subjects who underwent the short ATI. In contrast, a longer ATI, 2 to 12 months, among subjects enrolled in the Strategies for Management of Antiretroviral Therapy (SMART) trial robustly boosted HIV-1 gp120-specific Fc receptor-binding antibodies and ADCC against HIV-1-infected cells *in vitro*. These results show that there is a lag between viral recrudescence and the boosting of ADCC antibodies, which has implications for strategies toward eliminating latently infected cells.

IMPORTANCE The “shock and kill” HIV-1 cure strategy aims to reactivate HIV-1 expression in latently infected cells and subsequently eliminate the reactivated cells through immune-mediated killing. Several latency reversing agents (LRAs) have been examined *in vivo*, but LRAs alone have not been able to achieve HIV-1 remission and prevent viral rebound following analytical treatment interruption (ATI). In this study, we examined whether LRA treatment or ATI can provide sufficient antigenic stimulus to boost HIV-1-specific functional antibodies that can eliminate HIV-1-infected cells. Our study has implications for the antigenic stimulus required for antilateness strategies and/or therapeutic vaccines to boost functional antibodies and assist in eliminating the latent reservoir.

KEYWORDS ADCC, analytical treatment interruption, HIV-1 cure, latency, latency-reversing agent, panobinostat, SMART trial

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A major focus in the field of human immunodeficiency virus type 1 (HIV-1) cure research is to develop agents and strategies that reactivate HIV-1 from latency, induce killing of the reactivated cells, and achieve HIV-1 remission. Several latency-reversing agents (LRAs) have been examined both *in vitro* and *in vivo* (1–7). An emerging class of drugs that can potentially serve as LRAs is the histone deacetylase inhibitors (HDACi), which act epigenetically to upregulate gene expression by promoting the acetylation of DNA-bound histones (8). One HDACi that has shown promise in reversing HIV-1 latency *in vivo* is panobinostat, which was administered to participants three times per week, every second week, for 8 weeks (3). Panobinostat treatment resulted in a significant increase in cell-associated unspliced HIV-1 RNA and also induced plasma viremia in a proportion of subjects. A decrease in total integrated HIV-1 DNA, however, was not detected, and panobinostat treatment failed to prevent rebound of viremia during analytical antiretroviral therapy (ART) interruption. Consequently, reactivation of HIV-1 latency by panobinostat was not sufficient to significantly reduce the viral reservoir, suggesting that complementing modalities need to be harnessed to purge latently infected cells.

Elimination of reactivated cells expressing HIV-1 antigens on the surface could theoretically be achieved through antibody-dependent cellular cytotoxicity (ADCC). If viral protein expression is sufficiently induced, HIV-1-specific antibodies can target reactivated cells by binding to the HIV-1 envelope protein (Env) expressed on the cell surface (9). The fragment crystallizable (Fc) constant regions of these Env-bound antibodies can then cross-link Fc gamma receptor IIIa (Fc γ RIIIa) on innate effector cells, such as natural killer (NK) cells or monocytes, and mediate cytolysis of the infected target cells (10, 11).

A potential barrier for ADCC to eradicate the latent reservoir is that HIV-1-specific ADCC antibodies decline in subjects on long-term ART (12, 13). It is not known whether host ADCC antibodies will need to be boosted prior to latency reversal for ADCC to be effective in eliminating reactivated cells. If sufficient reactivation of the latent reservoir can be achieved, the resulting increase in viremia could potentially provide antigenic stimulation to boost ADCC antibody levels. The recrudescence of virus after an analytical treatment interruption (ATI) could provide a larger antigenic stimulus to trigger memory B cell responses and boost ADCC antibody levels as well.

To assess the boosting of HIV-1-specific ADCC antibodies after viral recrudescence, we analyzed plasma samples from the panobinostat clinical trial that were obtained pre- and post-panobinostat treatment and after a subsequent brief ART interruption (median, 21 days) (3). To analyze whether a larger antigenic stimulus from a longer period of ART interruption (2 to 12 months) could lead to a bigger boost in ADCC antibodies, we analyzed plasma samples from the SMART (Strategies for Management of Antiretroviral Therapy) trial, which compared the effects of continuous and episodic use of ART guided by CD4⁺ T cell counts (14). Our results show that neither panobinostat treatment nor a very short ATI boosted HIV-1-specific ADCC, and ART interruption of 2 months or more was required to boost HIV-1-specific ADCC.

RESULTS

Panobinostat treatment in ART-suppressed subjects does not boost HIV-1-specific antibody responses. We first studied 14 ART-suppressed HIV-1-infected subjects who received 20 mg of panobinostat orally three times per week, every second week, for a period of 8 weeks (3). Panobinostat disrupted HIV-1 latency *in vivo*, with an increase in cell-associated HIV-1 RNA and an increase in the proportion of plasma samples during panobinostat treatment testing positive for HIV-1 RNA (3). We hypothesized that this modest amount of reactivation could trigger memory B cell responses to boost HIV-1-specific antibodies. However, we found no difference in the concentration of gp120-specific IgG in the plasma samples pre- and post-panobinostat treatment (14.7 μ g/ml [6.6 to 51.9] versus 16.5 μ g/ml [5.9 to 49.3]; not significant [ns] [Fig. 1A]; median [interquartile range]).

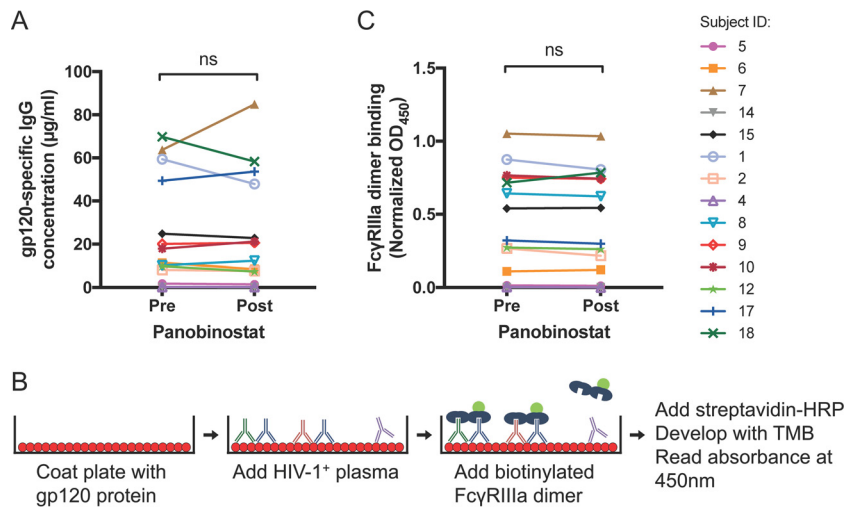


FIG 1 HIV-1-specific antibody responses following panobinostat treatment. (A) Concentrations of gp120-specific IgG antibodies pre- and post-panobinostat treatment. (B) ELISA to measure dimeric Fc γ RIIIa binding. Ninety-six-well ELISA plates were first coated with 50 ng/well of HIV-1_{BaL} gp120 prior to addition of diluted HIV-1⁺ plasma samples (containing HIV-1-specific IgG antibodies). Biotinylated recombinant dimeric Fc γ RIIIa was then added, followed by HRP-conjugated streptavidin. The wells were developed with TMB substrate, and absorbance was measured at 450 nm. (C) Levels of gp120-specific antibodies that bind dimeric Fc γ RIIIa pre- and post-panobinostat treatment. The cross-linking of recombinant Fc γ RIIIa was used as a surrogate measure of antibody Fc-mediated effector function. Statistical analyses between matched pairs were performed using the Wilcoxon signed-rank test.

HIV-1-specific antibodies could theoretically eliminate reactivated latently infected cells through Fc-mediated effector functions, including ADCC. As a surrogate measure of Fc effector function, we performed a novel enzyme-linked immunosorbent assay (ELISA) to examine the ability of gp120-specific antibodies within the plasma samples to cross-link dimeric Fc γ RIIIa, the primary Fc receptor responsible for ADCC (Fig. 1B). We show that panobinostat was not able to boost the level of gp120-specific antibodies capable of cross-linking dimeric Fc γ RIIIa (normalized optical density at 450 nm [OD₄₅₀], 0.430 [0.086 to 0.755] versus 0.421 [0.093 to 0.753]; ns [Fig. 1C]).

To validate our results with a functional ADCC assay, we performed an infected-cell elimination assay and assessed whether panobinostat treatment boosted HIV-1-specific ADCC. This assay examines the loss of HIV-1 p24⁺ cells; the gating strategy to analyze elimination of an HIV-1-infected cell line is shown in Fig. 2A. We found that panobinostat treatment did not increase HIV-1-specific ADCC (pre-panobinostat area under the curve [AUC], 16.9 [12.2 to 21.8], versus post-panobinostat AUC, 18.2 [14.3 to 23.5]; ns [Fig. 2B]). We found that the Fc γ RIIIa dimer-binding ELISA correlated significantly with the functional ADCC assay ($P = 0.0004$; $r = 0.58$).

A short ATI post-panobinostat treatment was also insufficient to boost HIV-1-specific antibody responses. As part of the panobinostat trial, 9 subjects underwent a short ATI to examine whether panobinostat could delay viral rebound in the absence of ART. All 9 subjects had rebound in HIV-1 viremia, and the median time for plasma viral load to exceed 50 copies/ml was 14 days (range, 10 to 45) (Table 1). We hypothesized that viral rebound from the short ATI following panobinostat treatment could provide a larger stimulus to boost HIV-1-specific antibodies. We examined plasma samples obtained immediately prior to ART restart from all 9 subjects during the ATI. The median length of ATI for the 9 subjects was 21 days (range, 21 to 59), while the median length of viremia during ATI (days from emergence of virus in plasma to ART restart) was 14 days (range, 7 to 18). The subjects had a median viral load of 2.5×10^4 copies/ml (range, 2.5×10^3 to 1.6×10^6) immediately before restarting ART (Table 1). We measured the concentration of gp120-specific IgG antibodies and found no significant difference between the post-panobinostat treatment and ATI time points (20.6 μ g/ml [7.6 to 50.8] versus 14.4 μ g/ml [9.3 to 37.2]; ns [Fig. 3A]). Similarly, as a surrogate

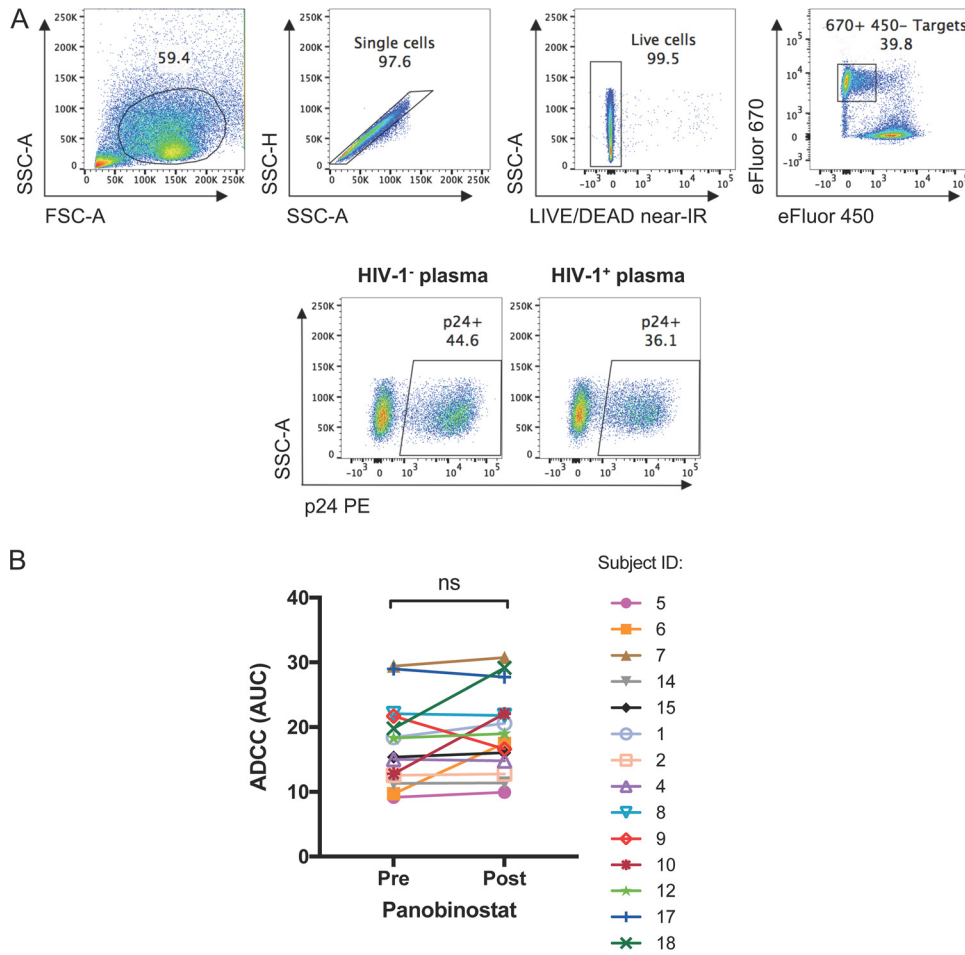


FIG 2 HIV-1-specific ADCC following panobinostat treatment. The infected-cell elimination assay was performed to assess ADCC against the HIV-1-infected 8E5/LAV cell line. Healthy donor PBMCs were used as effector cells and incubated with the target cells at an effector/target ratio of 1:1, with plasma samples added at 10-fold serial dilutions from 1:100 to 1:100,000. (A) Plots depict the gating strategy used to analyze flow cytometry data for the infected-cell elimination assay. Shown here is a representative sample with both effector and target cells. The first gate was set using a target cell-only control (based on forward and side scatter parameters) and applied to all samples. Next, gating was on single cells, live cells, eFluor 670⁺ eFluor 450⁻ target cells, and lastly, HIV-1-infected cells (determined by staining for HIV-1 p24). The bottom images are two representative plots showing elimination of p24⁺ cells in the presence of HIV-1⁺ plasma compared to HIV-1⁻ plasma. Percent ADCC was calculated and is plotted against log₁₀(dilution⁻¹) to determine the area under curve (AUC) for each subject at each time point. (B) Anti-HIV-1 ADCC for 14 subjects pre- and post-panobinostat treatment. Matched pairs were analyzed using the Wilcoxon signed-rank test. The ADCC data were averaged from 3 independent experiments.

of ADCC function, we found no difference in the level of gp120-specific antibodies capable of binding dimeric FcγR1IIa (normalized OD₄₅₀, 0.622 [0.238 to 0.764] versus 0.522 [0.225 to 0.768]; ns [Fig. 3B]). To further study ADCC after this short ATI, we performed the infected-cell elimination assay and found no difference in HIV-1-specific ADCC in these 9 subjects (post-panobinostat AUC, 20.6 [15.7 to 24.9], versus ATI AUC, 17.1 [15.0 to 22.9]; ns [Fig. 3C]).

HIV-1-specific antibody responses and ADCC increase significantly following 2 months of ATI. The data presented thus far demonstrate that neither panobinostat nor a short ATI post-panobinostat treatment was able to boost HIV-1-specific ADCC responses. Since the short ATI, a median of 21 days (range, 21 to 59 days), was not sufficient, we next assessed whether a longer ATI, 2 to 12 months, could stimulate HIV-1-specific ADCC. We obtained 60 samples from the SMART trial, with 30 subjects randomly assigned to the continuous use of ART and 30 assigned to undergo ART interruption (Table 2). The subjects in the ART group served as negative controls for the ATI samples, as we have previously shown that continuous ART results in a slow decline

TABLE 1 Characteristics of panobinostat trial subjects who underwent ATI^a

Subject identifier	Age (yrs)	No. of days from ATI to VL of >50 copies/ml	No. of days from VL of >20 copies/ml to ART restart	Length of ATI (days)	VL before ART restart (copies/ml)	CD4 before ART restart (cells/mm ³)
1	43	14	11	21	10,692	700
2	49	14	7	21	106,261	490
4	50	45	14	59	6,634	550
8	51	14	14	21	193,365	600
9	51	24	14	31	25,187	620
10	47	10	14	21	136,590	600
12	34	17	7	24	2,476	ND
17	49	10	18	28	1,573,968	1,180
18	28	14	7	21	8,595	1,180
Median (range)	49 (28–41)	14 (10–45)	14 (7–18)	21 (21–59)	25,187 (2,476–1,573,968)	610 (490–1,180)

^aAll subjects were male. VL, viral load; ND, not determined.

in ADCC responses (12, 13). Eight subjects in the ART group were excluded from our analyses because they either failed or stopped ART and had viral loads exceeding 500 copies/ml at one or more time points. The viral loads of subjects in the two groups are shown in Fig. 4A. Subjects in the ART group had viral loads suppressed below 500 copies/ml for all 12 months, whereas almost all subjects in the ATI group had viral rebound throughout the 12 months.

We examined whether this longer ATI of 2 to 12 months could boost the level of gp120-specific IgG. For subjects in the ATI group, the concentration of gp120-specific IgG significantly increased 2, 4, and 12 months post-ATI (Fig. 4B). For subjects in the ART group, there was a gradual decrease in gp120-specific IgG after 4 to 12 months of continuous ART (Fig. 4B). This decline in gp120-specific antibodies is likely due to the lack of antigenic stimulation resulting from ART-mediated viral suppression. Next, as a surrogate measure of antibody Fc-mediated effector function, we measured the level of gp120-specific antibodies capable of cross-linking dimeric Fc γ R1IIa. We detected a significant increase in these Fc γ R1IIa dimer-binding antibodies after 2, 4, and 12 months of ATI and a significant decrease after 12 months of continuous ART (Fig. 4C). There was no significant difference in the baseline levels of Fc γ R1IIa dimer-binding antibodies between the ART group and the ATI group (normalized OD₄₅₀, 0.490 [0.181 to 0.751] for the ART group versus 0.331 [0.111 to 0.587] for the ATI group; ns).

To validate these results with a functional ADCC assay, we performed the infected-cell elimination assay with a subset of the subjects. There was a trend toward an increase in anti-HIV-1 ADCC for the ATI group at 4 months post-ATI and a significant increase 12 months post-ATI (baseline AUC, 29 [18.2 to 40.9], versus AUC at 4 months, 36.9 [31.1 to 46.4] [$P = 0.0507$], and AUC at 12 months, 39.6 [34 to 44.1] [$P = 0.028$] [Fig. 4D]). There were no significant differences in anti-HIV-1 ADCC for the subjects who remained on ART for 12 months. Similar to the case with panobinostat trial samples, we found that the Fc γ R1IIa dimer-binding ELISA correlated significantly with the functional ADCC assay for the SMART trial samples ($P < 0.0001$; $r = 0.58$).

The functional ADCC assay could detect a significant increase in HIV-1-specific ADCC only at 12 months post-ATI ($P = 0.028$), whereas the Fc γ R1IIa dimer-binding ELISA could detect a much more significant increase in ADCC antibodies earlier, at 4 months post-ATI ($P < 0.0001$). This suggests that the Fc γ R1IIa dimer-binding ELISA is more sensitive at detecting changes in ADCC antibodies and should be considered an alternative assay to examine the level of antibodies capable of mediating effector functions.

Modeling of the ATI-induced stimulus of ADCC antibodies. The lack of increase in ADCC antibodies after panobinostat treatment and the subsequent short ATI may have occurred for two reasons. Either (i) the viremia induced by panobinostat or encountered during ATI was insufficient to stimulate an increase in ADCC antibodies or (ii) there was a significant delay between viral recrudescence and the stimulation of

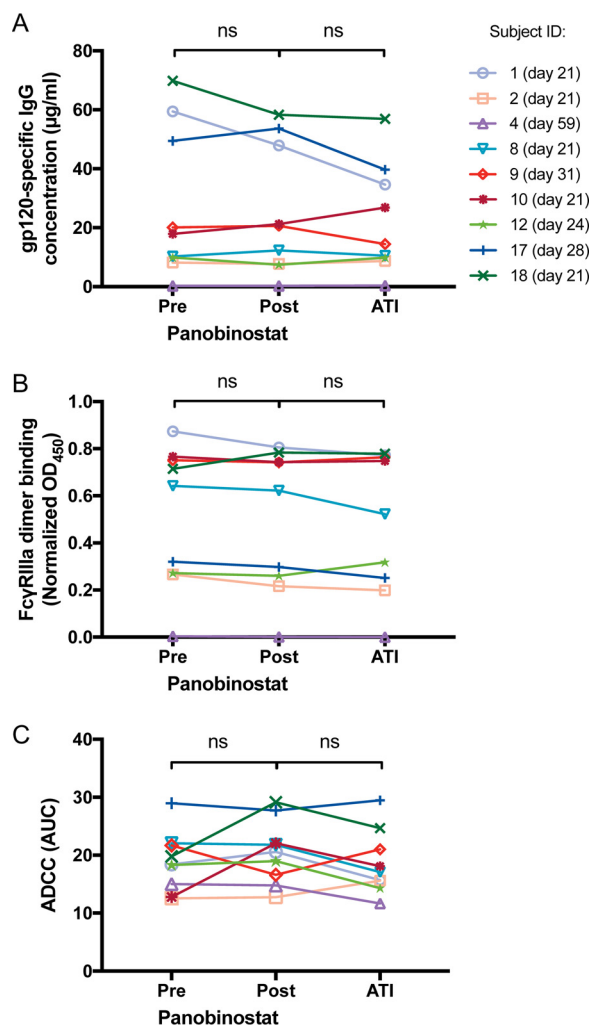


FIG 3 HIV-1-specific antibody responses and ADCC following a short ATI after panobinostat treatment. Nine of the 15 subjects underwent ART interruption for a median of 21 days (range, 21 to 59 days), with ART restarted following two consecutive viral load measurements of more than 1,000 copies/ml. The ATI load measurements of more than 1,000 copies/ml. The ATI samples were obtained immediately prior to ART restart, and the duration of ATI for each subject is listed in parentheses next to the subject IDs. (A) Concentrations of gp120-specific IgG antibodies for the 9 subjects pre- and post-panobinostat treatment and following a short ATI. (B) Levels of gp120-specific antibodies that bind dimeric FcγRIIIa pre- and post-panobinostat treatment and following a short ATI. (C) Anti-HIV-1 ADCC for 9 subjects pre- and post-panobinostat treatment and following a short ATI. Statistical analyses between multiple matched groups were performed using the Friedman test followed by Dunn's posttest. The ADCC data were averaged from 3 independent experiments.

ADCC antibodies (which was not detected due to the early sampling time in the panobinostat study). To analyze the relationship between the level of viremia and the boosting of ADCC antibodies, we modeled the FcγRIIIa dimer-binding antibody data from the SMART trial. We first calculated the rate of change in ADCC antibodies (FcγRIIIa dimer-binding antibodies) from baseline to 2 months and plotted the rates against the viral loads at 2 months (Fig. 5). There was a significant correlation between the rate of increase in ADCC and viral loads at 2 months ($r = 0.67$; $P < 0.0001$). This suggests that the level of viral antigenic stimulus is an important driver of the ADCC response following ATI. We then performed orthogonal regression using the ATI samples to determine the threshold viral load needed to induce an increase in ADCC antibodies. The estimated threshold viral load (x-intercept of the regression line in Fig. 5) was rather low, at approximately 326 RNA copies/ml. While this low threshold may be sufficient to initiate an increase in the level of ADCC antibodies, our data from the panobinostat trial

TABLE 2 Characteristics of SMART trial patients who either remained on ART or underwent ATI for 12 months

Characteristic	Value for group	
	ART (<i>n</i> = 22)	ATI (<i>n</i> = 30)
Sex, no. (%)		
Male	17 (77)	22 (73)
Female	5 (23)	8 (27)
Age (yrs), median (range)	46.5 (36–58)	42.5 (31–58)
Baseline CD4 count (cells/mm ³), median (range)	683.5 (387–1,215)	837.5 (384–1,526)
Nadir CD4 count (cells/mm ³), median (range)	200 (11–720)	281.5 (14–601)
Baseline viral load (copies/ml), median (range)	50 (50–400)	50 (50–400)
Prior recorded highest viral load (copies/ml), median (range)	36,150 (320–545,136)	38,730.5 (50–742,932)
Duration from initiation of ART to study entry (yrs), median (range)	7 (5–16)	6 (5–13)

suggests that constant antigenic stimulus over longer periods will probably be required to drive a meaningful increase in ADCC antibodies.

To assess the effect of time on the increase in ADCC antibodies after ATI, we plotted the rate of change in ADCC antibodies against the difference in viral loads from baseline to 2 months (Fig. 6A) and from 2 to 4 months (Fig. 6B). There was a significant correlation between the rate of ADCC and difference in viral loads from baseline to 2 months ($r = 0.64$; $P < 0.0001$) but not from 2 to 4 months ($r = 0.09$; $P = 0.52$). This implies that the increase in ADCC antibodies from baseline to 2 months in the ATI group was due to the substantial increase in viral load within the first 2 months of ATI. The lack of correlation between 2 and 4 months likely reflects most subjects reaching a stable viral set point, with relatively small changes in viral loads from month 2 onwards and an equilibrium existing between constant antigenic exposure and the ADCC antibody response.

DISCUSSION

There is growing interest in utilizing immune-mediated effector functions to eliminate infected cells following latency reversal (15, 16). ADCC antibodies are of particular interest because they can potentially eliminate reactivated latently infected cells expressing HIV-1 Env (9). The LRA panobinostat induced an increase in cellular HIV-1 RNA and plasma viremia (3); however, we found that this level of HIV-1 antigen expression did not provide sufficient antigenic stimulus to boost HIV-1-specific ADCC immunity. Even a short ATI post-panobinostat treatment was unable to increase gp120-specific FcγRIIIa dimer-binding antibodies or ADCC against HIV-1-infected cells. However, for the SMART trial subjects who interrupted ART, a significant increase in ADCC antibodies occurred within the first 2 months of ATI. The level of recrudescence viremia correlated strongly with the increase in ADCC antibodies after ATI in the SMART trial, suggesting that a longer antigenic stimulus is a key driver of the ADCC response to an ATI.

Our study shows that LRA treatment alone was not sufficient to boost preexisting ADCC immunity against HIV-1, highlighting the need for immunological interventions alongside latency reversal to eradicate the latent reservoir. A number of potential therapeutic vaccines have improved anti-HIV-1 immune responses *in vivo* (17–20) or delayed viral rebound during ATI in a macaque model (21) and could theoretically be administered prior to latency reversal to prime the immune system for killing of reactivated cells. A recent trial examined the combined effects of administering the therapeutic vaccine Vacc-4x (22) and granulocyte-macrophage colony-stimulating factor (GM-CSF) along with the LRA romidepsin (23). While a decrease in total HIV-1 DNA after immunization and romidepsin treatment could be detected, this combined “prime and shock” strategy was unable to prevent viral rebound after the interruption of ART. Further optimization of combinatorial approaches will likely be needed to measurably affect the latent reservoir.

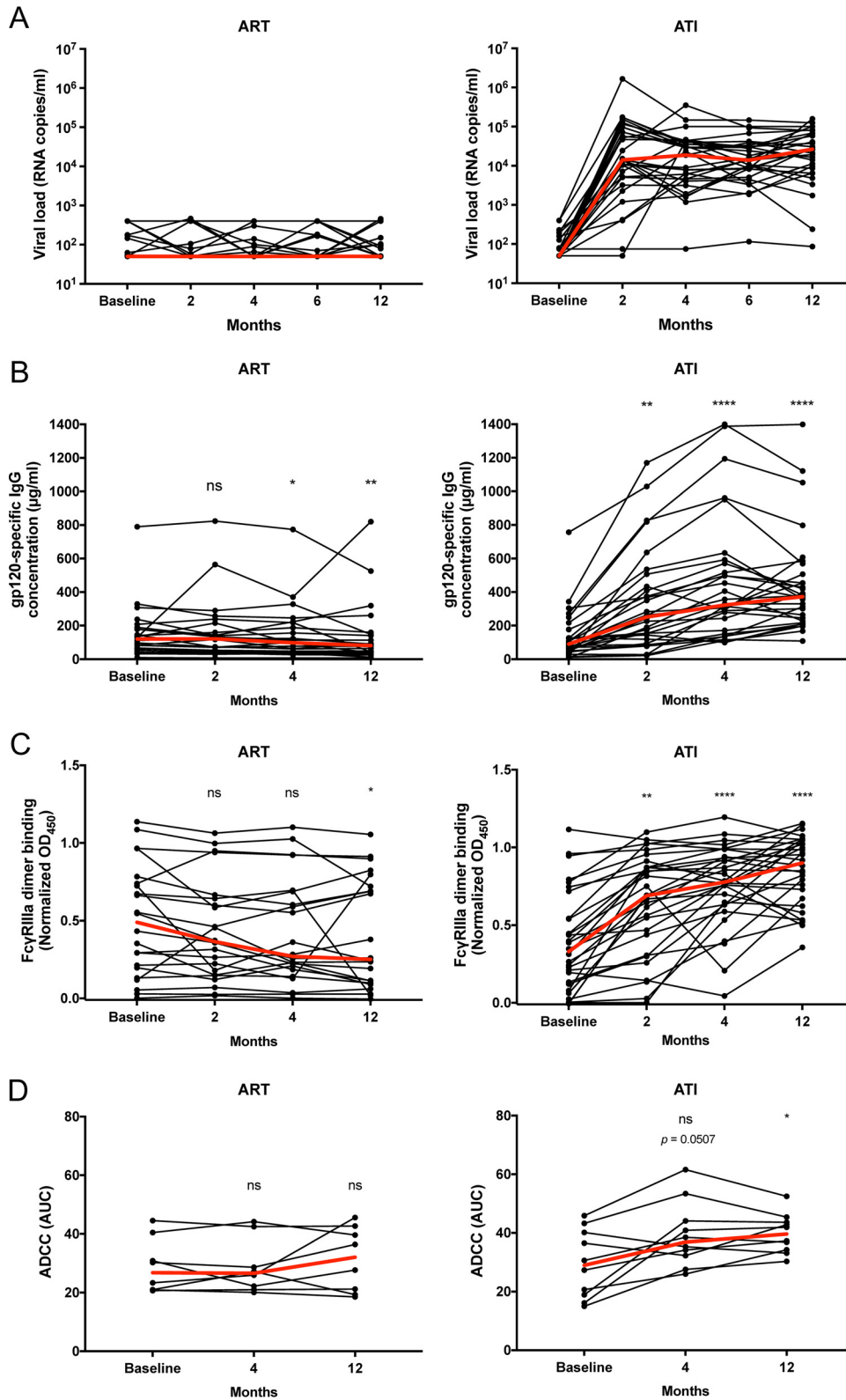


FIG 4 HIV-1-specific antibody responses and ADCC following 2 to 12 months of ATI. We obtained plasma samples from subjects in the SMART trial who either continued receiving ART (ART; $n = 22$) or interrupted ART (ATI; $n = 30$) for 12 months. (A) HIV-1 viral loads. (B) Concentrations of gp120-specific IgG antibodies. (C) Levels of gp120-specific antibodies that bind dimeric FcγRIIIa. (D) Anti-HIV-1 ADCC responses in a subset of subjects who continued receiving ART ($n = 8$) or underwent ATI ($n = 10$) for 12 months. The ADCC data were averaged from 2 independent experiments. Red lines depict the medians of each group. Statistical analyses between baseline and the different time points were performed using the Friedman test followed by Dunn's posttest. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

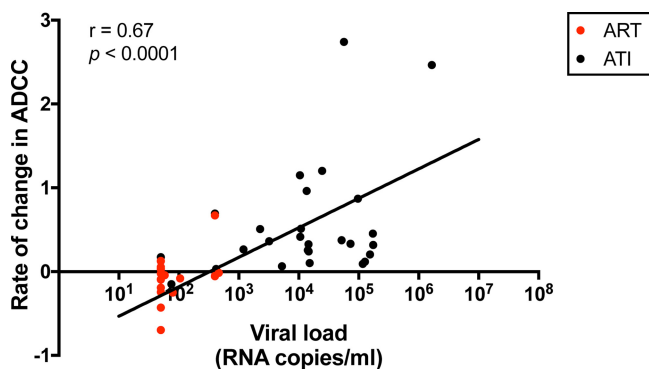


FIG 5 Modeling of the ATI-induced stimulus of ADCC antibodies. The rate of change in ADCC antibodies (dimeric Fc γ R1IIa-binding antibodies) was calculated for the SMART trial subjects from baseline to 2 months and plotted against the viral loads at the 2-month time point. The red dots represent subjects who were receiving ART, whereas the black dots represent subjects who interrupted ART. Orthogonal regression was performed on the ATI samples (black line) to estimate the threshold viral load required to induce an increase in ADCC antibodies. The correlation was performed with the nonparametric Spearman correlation.

Despite viral rebound in all 9 panobinostat trial subjects who underwent a short ATI (median of 21 days) post-panobinostat treatment, there was no increase in gp120-specific IgG or ADCC antibodies. Eight of the 9 subjects had an ATI of only 21 to 31 days, when their sample to measure ADCC was taken, and the actual duration of viremia was even shorter, at 7 to 18 days. The one panobinostat-treated subject with an ATI of 59 days (subject 4) only had viral rebound for 14 days, was treated with ART during acute infection, and had a very low baseline level of gp120-specific antibodies. In contrast, the first sample in the SMART trial was obtained at 2 months, by which time almost all subjects had a rise in ADCC antibodies. This suggests that a substantial lag exists between viral recrudescence and the stimulation of ADCC immunity after ATI. This is consistent with the observation of delayed CD8⁺ T cell responses after simian immunodeficiency virus (SIV) challenge in macaques, where even after vaccination, virus-specific CD8⁺ T cells are thought to do “too little too late” to alter the course of early infection (24). Anti-HIV-1 binding or ADCC antibodies might well have increased at a later time point after the panobinostat trial ATI, but plasma samples were not available for study. If future studies of HIV-1 control after LRA and/or immune modulation wish to take advantage of virus-induced immunity after ATI, it will be necessary for ATIs to be longer than that in the panobinostat trial. Our results are in line with a previous study that examined the kinetics of anti-gp120 antibodies following ATI (25), in which anti-gp120 antibody titers did not increase following 4 consecutive ATI cycles of 2 weeks off ART and 8 weeks on ART, despite a brief viral rebound every ATI cycle. Anti-gp120 antibody titers increased only following a fifth, longer ATI, 12 weeks. Another study examined the effect of ATI on heterologous neutralizing antibody responses in subjects who initiated ART during acute/early HIV-1 infection. The investigators found that neutralizing antibodies developed against some isolates only after 20 weeks of ATI (26).

From our modeling studies, we determined the level of viremia required to drive an increase in ADCC antibodies to be 326 HIV RNA copies/ml. While this low threshold viral load may seem promising for latency reversal studies, romidepsin, one of the most potent LRAs tested *in vivo*, was able to induce only transient viral blips of 46 to 103 copies/ml in 5 out of 6 subjects in the first clinical trial (4) and 21 to 619 copies/ml in 7 out of 16 subjects in a subsequent trial (23). This transient low-level viremia induced by current LRAs will likely be insufficient to stimulate an increase in ADCC antibodies. In addition, our modeling does not take into account the lag between viral rebound and the stimulation of ADCC immunity. Despite viral rebound exceeding 326 HIV RNA copies/ml in all panobinostat-treated subjects who underwent ATI, we could not detect

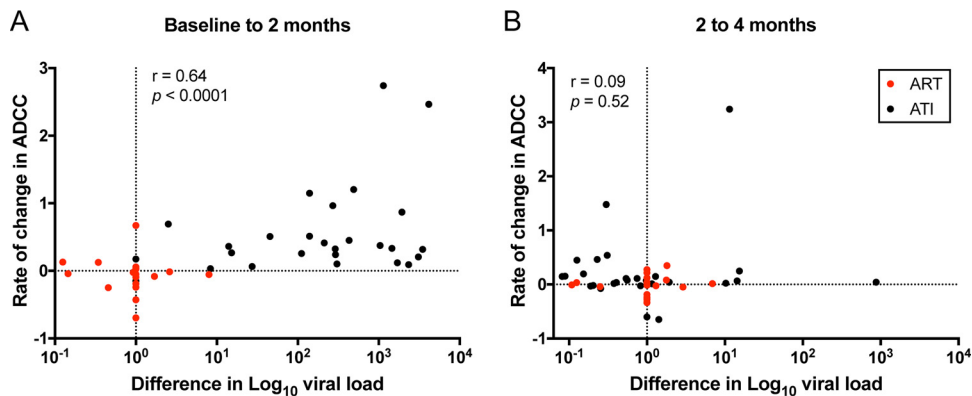


FIG 6 Correlations between the rate of change in ADCC versus the difference in viral load from baseline to 2 months (A) and 2 to 4 months (B). The red dots represent subjects who were receiving ART, whereas the black dots represent subjects who interrupted ART. Correlations were performed with the nonparametric Spearman correlation.

a significant increase in HIV-1-specific ADCC antibodies in the short period (21 to 59 days) these subjects were monitored.

An issue with stimulating preexisting ADCC responses to control reactivated latent infection is that viral escape from ADCC antibodies might have occurred in the earlier stages of infection (27), as has been described for cytotoxic T lymphocytes (28). Furthermore, a high proportion of ADCC antibodies in HIV-1-infected sera recognize CD4-induced epitopes on Env (29), which may be concealed on infected cells due to HIV-1 Nef- and Vpu-mediated downregulation of CD4 (30). This could potentially be overcome by using CD4 mimetic compounds that can expose CD4-induced epitopes on Env, enhance binding of serum ADCC antibodies, and improve the killing of reactivated cells (9, 31, 32). Additionally, the passive transfer of ADCC-mediating broadly neutralizing antibodies (bNAbs) after latency reversal might also be a feasible approach. While ADCC has not been formally proven to be a mechanism of viral control in subjects treated with bNAbs, many bNAbs have been shown to mediate ADCC *in vitro* (33–35) and *in vivo* in HIV-1-infected humanized mouse models (36–38). The passive transfer of single bNAbs in the setting of ATI in chronically infected individuals has also been examined, with VRC01 and 3BNC117 delaying viral rebound by several weeks compared to that in historical controls (39, 40). However, in most individuals, viral suppression was not maintained and bNAb-resistant rebound viruses emerged as bNAb levels waned. Careful consideration will need to be taken for bNAbs to be used as an antilatenacy approach, as preexisting viruses that are resistant to some bNAbs may reside within the latent reservoir (39, 41), and broad reactivation of genetically diverse HIV-1 proviruses can be induced by LRAs *in vivo* (42). The complex task of prescreening individuals for bNAb-resistant viruses and the combinatorial use of multiple bNAbs targeting different epitopes on Env may be necessary for bNAbs to be effective as an antilatenacy approach (36).

In summary, we show that panobinostat treatment or a short ATI following panobinostat treatment did not stimulate ADCC. However, a longer ATI, 2 to 12 months, robustly boosted ADCC antibodies and ADCC against HIV-1-infected cells, suggesting that there may be a lag between viral recrudescence and the stimulation of ADCC immunity after ATI. These data have implications for the antigenic stimulus required for antilatenacy strategies or therapeutic vaccines to boost ADCC in the hope of eradicating the latent reservoir.

MATERIALS AND METHODS

Clinical samples. The panobinostat trial enrolled 15 HIV-1-infected subjects on ART, 14 of whom had samples available and were included in this study. The subjects received 20 mg of panobinostat orally three times per week, every second week, for a period of 8 weeks (3). Nine of the 15 subjects underwent ART interruption after completion of panobinostat treatment for a median of 21 days (range, 21 to 59 days), with ART restarted following two consecutive viral load measurements of more than 1,000 copies/ml. We analyzed plasma samples collected 4 weeks before the first dose of panobinostat and 5

weeks after completion of panobinostat dosing. For the 9 subjects who underwent ART interruption, we analyzed samples obtained immediately prior to ART restart following interruption.

In the SMART trial, HIV-1-infected subjects with CD4 cell counts above 350 cells/mm³ were randomly assigned to the continuous use of ART or the episodic use of ART (ATI) (14). For subjects who underwent ART interruption, ART was restarted when CD4 cell counts decreased to less than 250 cells/mm³. For the purposes of our study, we obtained plasma samples from 30 subjects in the ATI group who interrupted ART for at least 12 consecutive months and plasma samples from 30 subjects in the ART group who continued receiving ART for 12 months. The plasma samples were collected from these 60 subjects at baseline and 2, 4, 6, and 12 months after randomization. Additional selection criteria for our study required subjects to have at least 5 years of total ART use prior to randomization and excluded subjects who had viral loads exceeding 500 copies/ml in the year prior to randomization.

ELISA to assess gp120-specific IgG concentration and dimeric FcγR1IIa binding. ELISAs to determine the concentration of antibodies against HIV-1_{BAL} gp120 (obtained from the NIH AIDS reagent program [NIH-ARP]) within HIV-1⁺ plasma were performed as previously described (43), with the following modification. Serial dilutions of the anti-gp120 monoclonal antibody 2G12 were included to construct a standard curve, from which the concentration of gp120-specific antibodies within HIV-1⁺ plasma was determined using nonlinear regression analysis.

The level of gp120-specific antibodies that can cross-link dimeric FcγR1IIa was assessed as previously described for influenza virus-specific ADCC antibodies (Fig. 1B) (44). Briefly, ELISA plates were coated with 50 ng/well of HIV-1_{BAL} gp120 and blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). HIV-1⁺ plasma samples were then added at a 1:100 dilution (panobinostat trial) or a 1:1,000 dilution (SMART trial). Biotinylated recombinant dimeric FcγR1IIa was added, followed by horseradish peroxidase (HRP)-conjugated streptavidin (Thermo Fisher Scientific). The wells were developed with TMB substrate (Sigma-Aldrich) and the reaction was stopped with hydrochloric acid. The absorbance values (450 nm) of the HIV-1⁺ plasma samples were PBS background subtracted and normalized to HIVIG (immune globulin prepared from plasma of HIV-1-infected donors) (NIH-ARP).

Infected-cell elimination ADCC assay. A modified version of the infected-cell elimination assay (45, 46) was performed to assess ADCC mediated by HIV-1⁺ plasma obtained from the panobinostat and SMART trials. Cells from the HIV_{LAV}-infected 8E5/LAV cell line (NIH-ARP) was used as targets, with approximately 50% of these cells expressing intracellular HIV-1 p24 (47). Healthy donor peripheral blood mononuclear cells (PBMCs) were used as effector cells. Target 8E5/LAV cells were first stained with the viability dye LIVE/DEAD near-IR (Life Technologies) and the cell proliferation dye eFluor 670 (eBioscience), whereas effector cells were stained with the cell proliferation dye eFluor 450 (eBioscience). The effector and target cells were added to a 96-well V-bottom cell culture plate (Corning) at a 1:1 ratio in the presence of HIV-1⁺ plasma, HIV-1⁻ plasma, or no plasma. The plasma samples were first heat inactivated at 56°C for 1 h and added at 10-fold dilutions from 1:100 to 1:100,000. The plate was centrifuged at 300 × *g* for 1 min before a 5-h incubation at 37°C with 5% CO₂. Cells were then fixed with 1% formaldehyde and permeabilized with 1× fluorescence-activated cell sorting (FACS) permeabilization buffer (BD). An antibody against HIV-1 p24 (clone KC57-RD1; Beckman Coulter) was then added to stain for HIV-1-infected target cells. Lastly, cells were fixed with 1% formaldehyde and acquired on an LSRFortessa flow cytometer (BD). Percent ADCC was calculated using the following formula: percent p24⁺ cells in $\frac{[(\text{targets} + \text{effectors}) - (\text{targets} + \text{effectors} + \text{plasma})]}{[\text{targets} + \text{effectors}]} \times 100$. Percent ADCC values were plotted against log₁₀(dilution⁻¹) for each time point of a subject, and the area under curve (AUC) was calculated using GraphPad Prism 7. The resulting AUC values incorporate the percent ADCC values for all four dilutions of each sample. The ADCC data shown in Results were averaged from 2 or 3 independent experiments.

Modeling of the ATI-induced stimulation of ADCC antibodies. To model the level of viremia required for an increase in ADCC antibodies, we first calculated the rate of change in ADCC antibodies (*r*) using the formula

$$r = \frac{\ln(y_2) - \ln(y_1)}{t_2 - t_1}$$

where *y*₁ and *y*₂ are the dimeric FcγR1IIa-binding antibody levels (ADCC antibodies) at the first and second time points, respectively, with *t*₁ and *t*₂ being the first and second time points. Next, the viral load or difference in log₁₀ viral load was plotted against the rate of ADCC. Orthogonal regression was then performed in R (version 3.1.2) using the *onls* function from the *onls* library (version 0.1-1) to take into account the measurement error in both the *x* axis (viral load) and *y* axis (rate of change in ADCC). The threshold value to determine the level of viral load (VL_{threshold}) required for an increase in ADCC antibodies was defined as

$$VL_{\text{threshold}} > 10^{\frac{\text{intercept}}{\text{slope}}}$$

Statistics. Statistical analyses were performed with GraphPad Prism version 7. Comparisons between matched groups were analyzed using the nonparametric Wilcoxon signed-rank test, and comparisons between unmatched groups were analyzed using the nonparametric Mann-Whitney test. Comparisons between multiple matched groups were analyzed using the nonparametric Friedman test followed with Dunn's multiple comparison posttest. Correlations were performed using the nonparametric Spearman correlation test. *P* values of less than 0.05 were considered significant. Statistics given in Results are presented in the following format: (median [interquartile range] versus median [interquartile range]; *P* value of statistical test).

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