Antiretroviral therapy interruptions result in loss of protective humoral immunity to neoantigens in HIV-infected individuals

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Objective: Sustained antiretroviral therapy (ART)-mediated viral suppression restores responses to vaccination in HIV-1-infected individuals. As ART interruption occur frequently in resource-constrained settings, we studied their effects on the ability to mount humoral immune responses against a neoantigen.

Design: Treatment-naive HIV-1-infected individuals were treated with stavudine, lamuvidine and lopinavir/ritonovir. Individuals who maintained viral load less than 50 copies/ml and $CD4^+$ T-cell counts more than 450 cells/μl for 6 months received three doses of rabies vaccine, and were randomized to 72 weeks of continuous ART (arm 1) or sequential 2, 4 and 8-week ART interruptions (arm 2). An additional vaccine dose was administered at study end.

Methods: Neutralizing antibody titers to rabies virus were assessed in plasma with a rapid fluorescent focus-inhibiting test.

Results: The proportion of participants achieving protective (>0.5 IU/ml) antibody titer after vaccination was similar (arm 1 = 92%; arm 2 = 91%), but over time the cumulative proportion of observations with protective titer was greater in arm 1 than arm 2 ($P = 0.0177$). From week 26 after vaccination, antibody titers were lower in arm 2 than arm 1, and volunteers in arm 2 lost protective antibody titers at a greater rate ($P = 0.0029$). After boosting, 100% of arm 1 and 95% arm 2 volunteers achieved protective antibody titer.

Conclusion: Our data indicate that individuals undergoing recurring ART interruption retain lower neutralizing antibody titers to a neoantigen, but maintain the ability to mount secondary responses upon boosting, suggesting that they might benefit from vaccine schedule intensification.

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Introduction

Sustained antiretroviral therapy (ART)-mediated viral suppression improves immune responses to vaccinations in HIV-1 infected individuals [1–4]. ART has been available in resource-constrained countries for several years through governmental and international funding programs [5], and adherence to ART in sub-Saharan

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Africa has been high [6]. However, supply chain interruptions, stock outs, power outages, employment migration, conflicts, and significant cultural stigma can disrupt adherence in these settings [7–9], with reported rates of therapy interruptions in regular therapy management in sub-Saharan Africa of 12.8 per 100 person years [10]. Qualitative studies have assessed the barriers to access to care [11,12] and demonstrated that instability and conflict result in lower adherence in children [13], whereas also pointing out that populations in conflict areas can be served effectively [14].

Although the negative correlates of protracted ART interruption have been characterized in a number of recent studies, ranging from increased rates of opportunistic infections, cardiovascular disease and ART resistance [15–19], it remains to be established how repeated, short-term interruptions, as are likely to occur in clinical settings, impact the levels and quality of overall immune reconstitution obtained while on ART. We recently reported that brief (up to 8 week) interruptions of ART do not result in permanent $CD4^+$ cell loss [20]; however, in our study, patients undergoing ART interruptions forfeited the gains in $CD4^+$ cell count observed in control individuals on continuous ART.

The relationship between viral replication and the establishment and maintenance of B-cell memory remains unclear. Early reports evidenced that chronic HIV infection causes polyclonal B cell activation, with resulting hypergammaglobulinemia [21]. B cell memory subsets are altered with expression of markers indicative of cell exhaustion, and responses to neoantigens are impaired (reviewed in [22]) as recently demonstrated in a cohort of viremic HIV-infected individuals with low $CD4^+$ cell count receiving rabies vaccination [23].

In primate models, Kuhrt *et al.* [24] demonstrated that naive B cells are lost quickly upon simian immunodeficiency Virus (SIV) infection, and their recovery after ART initiation is delayed as compared to the recovery of IgD^{neg} memory B cells.

Recently, Gelinck *et al.* [25] demonstrated that patient undergoing ART and with a $CD4^+$ cell count more than 500 cells/μl had incomplete immune reconstitution, but recovered the ability to mount a full antibody response to $CD4^+$-dependent antigens and develop protective immunity upon receiving a course of rabies vaccination. In prior clinical studies [26] we demonstrated that T-cell-mediated responses to recall antigens were not affected by brief (up to 12 weeks) viremic episodes.

In contrast to B and T lymphocyte subsets, the effects of short-term viremic episodes on the maintenance of antibody titers and long-term B cell memory in ART-treated individuals (i.e. individuals who have recovered the capability of mounting a satisfactory B-cell-mediated response) is less clear.

To assess their impact on immune fitness, we studied the effects of recurring ART interruptions on the ability to maintain protective antibody titers against a neoantigen by comparing the antibody titers to a full rabies vaccination course in individuals who, after receiving the same ART regiment for 6 months and achieving a $CD4^+$ cell counts of 450 cells/μl, were randomized to continuous or intermittent ART.

Methods

Study design

A detailed description of the study design, population, patient disposition and primary outcomes has been reported [20]. Briefly, between 2006 and 2010 HIV-infected individuals with $CD4^+$ cell count 200–350 cells/μl and no reported history of antirabies vaccination from the Themba Lethu Clinic (Johannesburg, RSA) were treated with stavudine, lamuvidine and lopinavir/ritonavir for up to 40 weeks; nucleoside switch to zidovudine was allowed for stavudine toxicity. After successful completion of 6 months of treatment, qualifying volunteers (i.e. patients with HIV viral load <50 copies/ml and $CD4^+$ cell count ≥ 450 cells/μl) received three doses of rabies vaccine (Verorab, Sanofi-Aventis, Bridgewater, New Jersey, USA) at week 16, 17 and 22 after viral suppression; a recall vaccine dose was given at study end.

The use of rabies vaccination as a neoantigen has been reported in a number of prior studies in HIV-infected populations [27–29].

All participants were screened for Hepatitis B and C (serology) and Mycobacterium tuberculosis (sputum and radiography) infection at inception. Participants with positive tests were excluded from the study and referred for in-clinic treatment.

Fifty-three participants were randomized to two study arms (see also Fig. 1):

(1) arm 1 (control, continuous ART): 72 weeks of continuous ART; $n = 27$
(2) arm 2 (intermittent ART): sequential 2, 4 and 8-week ART interruptions, separated by 16 weeks of continuous ART; $n = 26$

The randomized group was composed of 69% women, with a mean age 35 ± 8 years; all participants were black Africans.

Details of the patient follow-up for the parent study have been reported [20]. For this study, 23 participants in arm 1

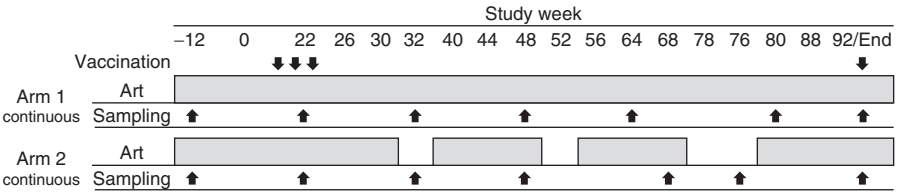

Fig. 1. Trial scheme. Schematic representation of antiretroviral therapy (ART) administration (grey boxes) and blood sampling (arrows) for the two study groups (Top line: arm 1, continuous ART; bottom line: arm 2, intermittent ART). Details of the clinical trial and its primary endpoints have been reported in [20].

and 25 in arm 2 had multiple samples tested for antirabies antibody titers. Of these, 20 and 23, respectively, had measurements after boosting (last assessment).

The study and informed consent procedure was approved by the University of Witwatersrand's Ethics Board (Medicine) and the Wistar Institute's Institutional Review Board.

Laboratory testing and neutralizing antibody titers to rabies virus

The $CD4^+$ cell counts and HIV viral load were tested at the Department of Hematology, University of the Witwatersrand, Johannesburg, ZA.

Cryopreserved serum samples were assessed for neutralizing activity against rabies virus (rapid fluorescent focus inhibiting test) up to 56 weeks after vaccination and after boosting.

Sera were tested for neutralizing antibodies to rabies virus strain CVS-11, which is antigenically closely related to the vaccine strain, as described previously [30]. Briefly, sera samples were heat inactivated and diluted with 10% Dulbecco modified Eagle medium containing 10% fetal calf serum in eight serial dilutions starting from 1 : 5 in flat bottom 96-well plates. WHO reference serum was used for comparison. Pretitrated rabies virus CVS-11 was incubated for 1 h, and 3×10^5 cells/μl Baby Hamster Kidney-21 cells were added; duplicate 10 μl samples were incubated at 37°C for 24 h in Terasaki plates, after which they were fixed with 80% cold acetone and air dried. Fluorescein isothiocyanate-conjugated antirabies monoclonal antibodies were added for 1 h, after which the plates were washed and analyzed with a fluorescent microscope. Titers are expressed in international units standardized based on results obtained with the reference serum.

Statistical analysis

Two-sided level α of 0.05 Student's *t*-tests were applied to assess differences between arms for mean $\log_{10}$ transformed antirabies antibody titer at each measured time point; mean $CD4^+$ cell count at each observed time point; and mean overall percentage of visits with protective ($\geq$0.5 UI) antibody titer. Differences in proportions of visits with protective ($\geq$0.5UI) antibody titer at each study point were assessed using Fisher exact tests. Overall differences in proportions of visits with protective antibody titer were assessed using a Student's *t*-test. Differences in time to failure between arms was tested using a log-rank test in which failure was defined as loss of protective neutralizing antibody titer (<0.5 IU/ml) maintained for all subsequent follow-up time points (up to week 56). The effect of baseline or vaccination $CD4^+$ cell count or baseline viral load on $\log_{10}$ neutralizing antibody titer or time to failure was assessed using a linear regression model (least squares method). All statistical analysis was performed using R version 2.10.0 and JMP [31].

Results

Effectiveness of vaccination

As summarized in Table 1, prior to vaccination most participants had low to undetectable levels neutralizing antibody to rabies virus (mean $\log_{10}$ titers were -1.75 ± 0.6 for arm 1 and -1.87 ± 0.36 for arm 2); these values were not significantly different between arms.

After three vaccine inoculations, 21 of 25 participants in arm 1 and 21 of 23 participants in arm 2 achieved protective neutralizing antibody titers ($\geq$0.5 IU/ml), as reflected by a mean $\log_{10}$ titer of 0.18 ± 0.57 for arm 1 and 0.09 ± 0.36 for arm 2.

As described in our prior report of the study endpoints [20], $CD4^+$ cell counts, which were similar at baseline and at the time of vaccination, were significantly lower in arm 2 than arm 1 during or immediately after an ART interruption (study week 32 and 76, $P = 0.0202$ and 0.0003 respectively, Table 1), but were not significantly different at other time points (e.g. study weeks 48, 64 and study end).

We assessed the effect of baseline $CD4^+$ cell count, baseline viral load and vaccination time $CD4^+$ cell count on $\log_{10}$ neutralizing antibody titer using a linear model; none of these variables had a significant effect.

Effect of antiretroviral therapy interruptions on antibody titers

To assess the effect of cyclic treatment interruptions on the retention of neutralizing antibody titers to rabies

Table 1. Antirabies antibody titers and CD4$^+$ cell counts.

Study week	Weeks from vaccination	Arm	n	Log_{10} antirabies Ab titer			Protective antirabies Ab titer				$CD4^+$ cell count		
				Mean	SD	Student's t-test P	N <0.5	N ≥0.5	Protective titer/total	Fisher's exact test P	Mean	SD	Student's t-test P
−12	−34	1	25	−1.75	0.60	0.3954	23	2	0.08	0.4902	263	56	0.2837
		2	23	−1.87	0.36		23	0	0.00		281	66	
22	0	1	25	0.18	0.57	0.5238	4	21	0.84	0.6681	492	96	0.3105
		2	22	0.09	0.36		2	21	0.91		525	133	
32	10	1	22	−0.41	0.75	0.0618	12	11	0.48	0.2214	506	79	0.0202[a]
		2	23	−0.88	0.90		17	6	0.26		447	95	
48	26	1	24	−0.58	0.70	0.0236[a]	15	9	0.38	0.3457	555	135	0.8112
		2	22	−1.13	0.88		17	5	0.23		564	114	
64/68[b]	Approximately 42	1	25	−0.74	0.76	0.0459[a]	16	9	0.36	0.0978	554	122	0.9559
		2	23	−1.21	0.83		20	3	0.13		552	123	
80/76[b]	Approximately 54	1	23	−0.25	0.66	0.0004[a]	6	17	0.74	0.0022[a]	578	121	0.0003[a]
		2	21	−1.2	0.92		16	5	0.24		452	81	
End	Approximately 4 from boost	1	22	1.57	0.55	0.1391	0	25	1.00	0.4444	597	146	0.1782
		2	20	1.25	0.81		1	19	0.95		544	107	

Ab, antibody.
[a]Significant result.
[b]Study week for arm 1/study week for arm 2.

virus, we assessed the overall percentage of observations with protective titer over the observation time until the vaccine boost (study weeks 32, 48, 64/68 and 80/76). As illustrated in Fig. 2a, the overall proportion of participants with protective titers was greater in control individuals than in patients with intermittent ART (arm 1, mean $0.47\% \pm 0.36$; arm 2, mean $0.22\% \pm 0.35$, $P = 0.0177$).

As antibody titers naturally decrease over time, we assessed whether the proportion of individuals with protective titers were different between arms at different postvaccination times. As summarized in Table 1, the proportion of individuals with protective titer was lower in the intermittent ART group, and this difference reached statistical significance at study week 80, when 74% of individuals in arm 1 had protective titers, as compared to 24% in arm 2 (Fisher exact test $P = 0.0022$).

Accordingly, a direct assessment of the antibody titers indicated that these were significantly higher control individuals at multiple postvaccination time points (mean $\log_{10}$ titer at week 48: arm 1 $-0.58\,\text{IU/ml} \pm 0.70$, arm 2 -1.13 ± 0.88, $P = 0.0236$. Weeks 64/68: arm 1, -0.74 ± 0.76, arm 2, -1.21 ± 0.83, $P = 0.0459$. Weeks 80/76, arm 1 -0.25 ± 0.66, arm 2 -1.2 ± 0.92, $P = 0.0004$), further supporting a loss of antibody titer over time.

Finally, we assessed the proportion of participants in each arm who, once they lost protective antibody titer, failed to recover it until booster immunization using a log-rank test. As illustrated in Fig. 2b, patients on intermittent ART (arm 2) lost protective titers at a significantly higher rate than control individuals ($P = 0.0029$), further confirming that individuals undergoing cyclical ART interruptions lose antibody titers to a neoantigen at a faster rate than individuals who maintain suppressive ART over time.

We also assessed the effect of baseline CD4$^+$ cell count, baseline viral load and vaccination time CD4$^+$ cell count on the time to failure using a linear model; none of these variables had a significant effect, suggesting that baseline conditions do not affect the loss of antibody titer during interruption.

Recall response

We sought to determine whether cyclical ART interruptions had lasting effects on antibody recall responses. To this purpose, qualifying participants (ART resumed, viral load <400) were inoculated with one dose of rabies vaccine at study end. Samples for this assessment were available for 20 participants in arm 1 and 22 in arm 2; individuals missing this time point had similar baseline titers as those assessed.

As indicated in Table 1, after receiving the boost, similar proportions of study participants in both study arms (arm

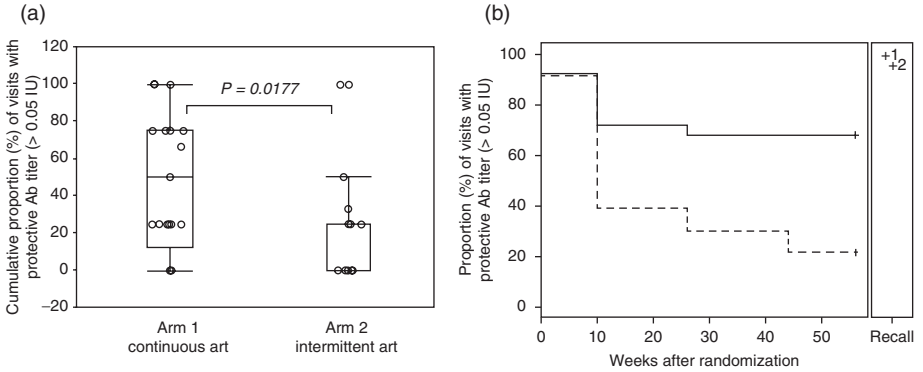

Fig. 2. Proportions of participants with protective antirabies antibody titers. (a) Boxes represent the proportion of visits with protective antibody (Ab) titer (antirabies Ab titer >0.5 IU) in the two study arms (see also Fig. 1 legend), including study weeks 32, 48, 64/68 and 80/76 (postvaccination, preboost). The Student's *t*-test *P* value for the difference between arms is reported. (b) The Kaplan–Meier plot represents the proportion of participants maintaining protective Ab titer in arm 1 (continuous ART, solid line) and arm 2 (intermittent ART, dotted line). Failure is defined as loss of protective neutralizing Ab titer (<0.5 IU/ml), maintained for all subsequent follow-up time points up to week 56. The proportion of participants achieving protective titer after boosting is reported in the right box(+) for arm 1 and 2. Log rank test $P = 0.0029$.

1: 25/25 participants; arm 2: 19/20 participants, $P = 0.4444$, Fig. 2b, right box) achieved protective neutralizing antibody titer, indicating that the ability to mount a recall response to the antigen was not lost in individuals undergoing cyclical ART interruptions.

Discussion

We have assessed antibody titers to a neoantigen (rabies vaccine) over the course of a cycle of short-term ART interruptions in HIV-infected patients.

Our data indicate that upon undergoing sustained (6 months) viral suppression and achieving a partial immune reconstitution ($CD4^+$ >450 cells/μl), most individuals in our cohort developed a satisfactory B-cell-mediated response to the neoantigen. This observation is in keeping with reports that ART-mediated viral suppression results in increased vaccine responses [32]. Because the vast majority of individuals had similar neutralizing antibody titers, we conclude that adequate B and $CD4^+$ T-cell responses existed in both arms at the time of vaccination.

The titers of neutralizing antibody to rabies virus were lower, at most time points (including after a 2-week interruption), in the intermittent ART arm as compared to controls on continuous ART. Importantly, the rate of decline of the protective titers was also significantly greater in the intermittent ART group.

A detailed analysis of the pathogenetic mechanisms underlying the observed loss of antibody titers is beyond the scope of this work; however, it's important to remark that although $CD4^+$ T cells are necessary to establish a full response to the rabies vaccine [25], previous work failed to report a correlation between $CD4^+$ cell counts and long-term serum antibody titers to vaccinia [33] or measles [34]. In addition, our own analysis failed to demonstrate a significant effect of initial $CD4^+$ cell count and viral load, as well as the $CD4^+$ cell count at the time of vaccination on the antibody titers achieved in response to vaccination. Thus, the temporary loss of $CD4^+$ due to acute viremia during ART interruption is unlikely to be directly responsible, but the effect of short-term viremia (and subsequent inflammation and T-cell activation) on the bone marrow microenvironment should be considered. Although memory B cells residing in the marginal zone of spleen and the subepithelial regions of lymph nodes [35] are considered the initiators of recall responses, protective antibody titers are maintained by long-lived antibody secreting plasma cells that largely home to the bone marrow (reviewed in [36]). A number of factors derived from bone marrow stromal cells have been implied in promoting the survival of plasma cells (e.g. IL-6, VLA-4 and CD44) [37]. In addition, long-lived plasma cell survival in the bone marrow requires signaling through the tumor necrosis factor (TNF) receptor family member B cell maturation (BCMA) [38]. Prior findings indicating that expression of TNF-receptor family members BCMA, B Lymphocyte Stimulator (BLyS) receptor B cell-activating factor receptor (BAFF-R) and CD95 is altered in viremic HIV-infected individuals [39] leading to a proapoptotic bone marrow environment.

We postulate that the reported alterations of the levels of BCMA, BLyS receptor (BAFF-R) and CD95 in viremic individuals [39], which is believed to result in a pro-apoptotic state, may contribute to deplete long-lived plasma cells from bone marrow even during short-term viral replication, contributing to the observed loss of protective immunoglobulin (Ig) titer, but not B-cell memory.

Polyclonal B cell activation may also contribute to the loss of antibody titers in individuals experiencing brief viremic episodes [21,40]. A loss of memory B cells [24], which has been associated with loss of specific Ig titers [41], did not seem to occur in our study, as recall responses to vaccine boost appeared to be similar in both groups. Further clinical studies incorporating bone marrow sampling will be required to determine if BCMA regulation and plasma cell apoptosis are indeed causative for the observed loss of antibody titers.

Administration of a recall vaccine dose at the end of the trial resulted in a protective titers in most individuals in both arms, and no significant difference in mean antibody titer was observed between arms at this point. This may be related with the rapid recovery of B cell memory subsets observed by Kuhrt *et al.* [24] in primate models of SIV infection and suggests that B cell memory is not irreparably lost upon brief episodes of viremia, and can be rapidly restored upon viral resuppression. Taken together, these data suggest that the adverse effect of brief viremic episodes may be limited to plasma cells and/or circulating antibody titers, the level of which appears to decay faster in individuals experiencing ART interruptions.

If confirmed in larger cohorts, our results have potential clinical implications, suggesting that the loss of vaccine-induced protective antibody titers should be considered when assessing individuals' adherence, and in the course of treatment interruptions dictated by the management of adverse events and other clinical situations. If a vaccination (e.g. influenza) is administered in individuals that subsequently interrupt ART or have poor adherence, the titers might need to be reconfirmed over time, so that recall vaccination may be scheduled as needed to avoid the loss of protective antibody titers.

Importantly, our study does not address the potential effects on poor adherence and/or ART discontinuations on the effectiveness of childhood immunizations administered according to predetermined priming and boosting schedules; further studies will be required to assess the effect of ART discontinuation in these individuals.

This study has some limitations. In the first place, the study was limited to a single T-cell-dependent immunization, to leverage the low frequency of prior exposure to rabies vaccination (neoantigen) in the target population. Further studies, beyond the scope of this report, should address multiple neo and pre-existing responses (e.g. to seasonal flu vaccine and cytomegalovirus), comparing the respective antibody titers to address time-dependent fluctuations.

Moreover, the number of individuals analyzed is limited. Although we consider this number sufficient for the exploratory nature of this study, we cannot exclude that small differences in antibody titers after primary or recall vaccination might be detected in larger studies.

Finally, our cohort started treatment with a $CD4^+$ cell count of 250–350 cells/μl, and was, therefore, not very immune compromised at the outset. Further studies may benefit from a stratification of individuals with lower and higher $CD4^+$ cell counts as reflective of more or less advanced disease.

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Conflicts of interest

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