Enhanced Antiretroviral Therapy in Rhesus Macaques Improves RT-SHIV Viral Decay Kinetics

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Using an established nonhuman primate model, rhesus macaques were infected intravenously with a chimeric simian immunodeficiency virus (SIV) consisting of SIV_{mac239} with the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase from clone HXBc2 (RT-SHIV). The impacts of two enhanced (four- and five-drug) highly active antiretroviral therapies (HAART) on early viral decay and rebound were determined. The four-drug combination consisted of an integrase inhibitor, L-870-812 (L-812), together with a three-drug regimen comprising emtricitabine [(−)-FTC], tenofovir (TFV), and efavirenz (EFV). The five-drug combination consisted of one analog for each of the four DNA precursors {using TFV, (−)-FTC, (−)-β-D-(2R,4R)-1,3-dioxolane-2,6-diaminopurine (amdoxovir [DAPD]), and zidovudine (AZT)}, together with EFV. A cohort treated with a three-drug combination of (−)-FTC, TFV, and EFV served as treated controls. Daily administration of a three-, four-, or five-drug combination of antiretroviral agents was initiated at week 6 or 8 after inoculation and continued up to week 50, followed by a rebound period. Plasma samples were collected routinely, and drug levels were monitored using liquid chromatography-tandem mass spectrometry (LC–MS-MS). Viral loads were monitored with a standard TaqMan quantitative reverse transcriptase PCR (qRT-PCR) assay. Comprehensive analyses of replication dynamics were performed. RT-SHIV infection in rhesus macaques produced typical viral infection kinetics, with untreated controls establishing persistent viral loads of $>10^4$ copies of RNA/ml. RT-SHIV loads at the start of treatment (V_0) were similar in all treated cohorts ($P > 0.5$). All antiretroviral drug levels were measureable in plasma. The four-drug and five-drug combination regimens (enhanced HAART) improved suppression of the viral load (within 1 week; $P < 0.01$) and had overall greater potency ($P < 0.02$) than the three-drug regimen (HAART). Moreover, rebound viremia occurred rapidly following cessation of any treatment. The enhanced HAART (four- or five-drug combination) showed significant improvement in viral suppression compared to the three-drug combination, but no combination was sufficient to eliminate viral reservoirs.

The current treatment for AIDS is highly active antiretroviral therapy (HAART), which includes a combination of antiretroviral drugs, usually three or more from two or more classes. HAART serves as a means to achieve long-term control of replication of human immunodeficiency virus type 1 (HIV-1) (1–6). Effective HAART can reduce viremia to below the detectable limits of conventional clinical assays (<50 viral-RNA [vRNA] copies/ml) in people living with HIV-1 who are able to adhere to the treatment regimen. However, viremia inevitably rebounds following cessation of HAART, likely due to established viral reservoirs (7–9). In addition, with more sensitive quantitative reverse transcriptase PCR (qRT-PCR) assays now available, it is clear that low-level viremia persists (10). Therefore, HIV-1 is not eradicated with current HAART.

Attempts to address mechanisms of viral persistence have been limited in people living with HIV-1, partly because comprehensive tissue sampling during suppressive HAART is not feasible, and HIV-1 eradication cannot be proven unless individuals can be removed from HAART without viral rebound (11). However, ethical concerns surround structured treatment interruptions (12). Nonhuman primate models, particularly simian immunodeficiency virus (SIV) infection of macaques, have provided robust animal models for AIDS, contributing significantly to the understanding of important aspects of pathogenesis, viral diversity, and long-lived reservoirs (13–17). Macaques infected with SIV often exhibit a fatal immunodeficiency disease similar to that in humans infected with HIV-1, but the disease course is accelerated, permit-

ting more rapid experimentation (18). A limitation to SIV as a model for HAART is that SIV is not susceptible to the nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs) that are widely used in current HAART. Nonhuman primate models that can utilize NNRTIs have been developed by us and others (18–21). One rhesus macaque model uses a virus consisting of the backbone of the pathogenic molecular clone SIV_{mac239} with the HIV-1 RT from clone HXBc2 (RT-SHIV) (21). RT-SHIV is sensitive to several nucleoside RT inhibitors (NRTIs), protease inhibitors (PIs), and NNRTIs (22–24). Studies in RT-SHIV-infected macaques support the relevance of this animal model for identifying potential reservoirs of latency/persistence during HAART.

Efforts toward eradication of HIV have taken two approaches. One type, a sterilizing cure, requires that HIV be eradicated from the body of the infected person. The second, a functional cure, is less stringent in that it requires that the infected individual be able to stop taking antiretroviral therapy without suffering any adverse

consequences from the HIV that remains in the body. Attempts to reactivate latent virus during suppressive HAART to develop a sterilizing cure have had limited success (25). The alternative approach to achieve a functional cure is focused on more effective suppression of viral replication (26). In this study, we compare two enhanced-HAART regimens (a four- and a five-drug combination) to a current three-drug HAART in our animal model. Our comprehensive approach utilized the well-controlled RT-SHIV model of AIDS for evaluating antiviral strategies that aim to eliminate virus from reservoirs.

MATERIALS AND METHODS

Animals, virus stocks, and inoculations. Young adult rhesus macaques (*Macaca mulatta*; 1 to 3 years old, weighing 2 to 5 kg) were used for these studies. All macaques were from the type D retrovirus-free and SIV-free colony at the California National Primate Research Center (CNPRC) at the University of California (UC) Davis and were handled in accordance with the American Association for Accreditation of Laboratory Animal Care Standards. All protocols strictly adhered to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council. This study was approved by the UC Davis Institutional Animal Care and Use Committee.

RT-SHIV has an open *nef* reading frame, and the RT-SHIV stocks we use have the T-to-C substitution at position 8 of the SIV tRNA primer binding site, which is necessary for rapid replication of RT-SHIV *in vitro* and *in vivo* (27). Virus stocks were prepared by propagating viruses in CEMx174 cells (a T/B-cell hybrid tumor line), and concentrations were determined as previously described (18, 28). Importantly, levels of plasma vRNA in animals infected with RT-SHIV and not treated with antiretroviral drugs were comparable to the viral loads in untreated HIV-1-infected patients (18). Peak viremia in RT-SHIV-infected animals occurred 2 to 3 weeks postinoculation, and by 8 weeks, plasma viral loads approached set points.

HAART regimens in RT-SHIV-infected rhesus macaques. A standard three-drug combination regimen consisting of 2 NRTIs and 1 NNRTI was selected. In comparison, a four-drug regimen included the addition of a third class, an integrase inhibitor (INI), to the 2 NRTI and 1 NNRTI combination. A novel five-drug combination was designed to include 4 NRTIs (containing one nucleoside analog for each of the 4 natural nucleosides A, T, C, and G) together with 1 NNRTI. Specifically, the three-drug combination consisted of tenofovir (TFV) (30 mg/kg of body weight, subcutaneously [SQ], once a day [QD]; provided by Gilead Sciences, Inc., Foster City, CA), emtricitabine [(−)-FTC] (16 mg/kg SQ, QD; supplied by ST Pharm Co., Ltd., Seoul, South Korea), and efavirenz (EFV) (200 mg per day, orally [p.o.], QD; purchased from the Veterans Administration [VA] pharmacy, Atlanta, GA) and was initiated in a cohort of macaques (*n* = 8) at 6 weeks postinoculation, as previously reported (29). Similarly, a four-drug combination [using the identical three-drug combination of TFV, (−)-FTV, and EFV (98% pure; provided by Raymond Schinazi's Laboratory of Biochemical Pharmacology, Emory University, Atlanta, GA) with the addition of an INI, L-812 (provided by Merck, White House Station, NJ)] was initiated at 6 weeks post-RT-SHIV inoculation (*n* = 8). In addition, a five-drug combination {using the identical three-drug combination of TFV, (−)-FTC, and EFV with the addition of zidovudine (AZT) (30 mg/kg, SQ, twice a day [BID]; supplied by ST Pharm Co., Ltd., Seoul, South Korea), and amdoxovir (DAPD) (85 mg per day, p.o., QD; 98% pure; supplied by ST Pharm Co., Ltd., Seoul, South Korea)} was initiated at 8 weeks post-RT-SHIV inoculation in a third cohort of macaques (*n* = 7). The dosages of drugs were selected based on previous experience with the drugs and/or on previously published pharmacokinetic studies in rhesus macaques (18, 24, 30–34). Of note, the INI, L-812, has been previously reported to have potent antiviral activity *in vitro* against both HIV-1 and SIV (250 and 350 nM, respectively) (34). Untreated controls were averaged from RT-SHIV-infected macaques

TABLE 1 HAART treatment scheme

Cohort	No. of animals	Drugs[a]	ART classes	Treatment initiation (wk)
3-drug	8	(−)-FTC, TFV, EFV	2 NRTI, 1 NNRTI	6
4-drug	4	(−)-FTC, TFV, EFV, L-812	2 NRTI, 1 NNRTI, 1 INI	6
5-drug	7	(−)-FTC, TFV, EFV, AZT, DAPD	4 NRTI, 1 NNRTI	8

[a] EFV, L-812, and DAPD were compounded into drug paste (1 dose/ml) from bulk powder using sucrose solution (63%).

from previous studies. The combination regimens are summarized in Table 1.

Plasma samples were collected at weeks 1, 2, 3, and 4 postinoculation and then, in general, at 2-week intervals thereafter. For the three-drug HAART regimen, drug therapy ceased at week 39, and viral rebound was measured up to week 64. For the four-drug enhanced-HAART regimen, drug therapy ceased at week 55 postinoculation, and viral rebound was measured in plasma up to week 74. For the five-drug enhanced-HAART regimen, drug therapy ceased at week 50 postinoculation, and viral rebound was measured in plasma up to week 66. The time to rebound, defined as the time from cessation of therapy to reach a viral load of >500 copies of vRNA/ml, was documented, and areas under the curve (AUC) of a plot of the viral load versus time were calculated.

Viral load. RT-SHIV RNA quantification, targeting the p27 *gag* region, was performed by real-time qRT-PCR utilizing TaqMan (Applied Biosystems) hydrolysis probes (35). For each cohort, the mean viral load at each time point was determined as vRNA copies/ml ± standard deviation (SD).

Drug extraction and quantification in plasma from RT-SHIV-inoculated rhesus macaques. Plasma aliquots were stored at −80°C. Antiretroviral drug levels were detected by liquid chromatography-tandem mass spectrometry (LC–MS-MS) (31, 32). Calibration curves were prepared using serially diluted standards added to plasma of drug-free donors. Briefly, acetonitrile was used to precipitate the plasma for (−)-FTC, DAPD, ZDV, and TFV detection, and methanol was used for EFV. In addition, a metabolite of DAPD, (−)-β-D-dioxolane guanosine (DXG), resulting from deamination by adenosine deaminase, was also quantified.

Viral-load dynamics. To simplify analysis, viral-load curves ($\log V$) were normalized by subtracting the starting $\log V_0$ from each $\log V$ at subsequent weekly intervals. This yielded curves of change in $\log V$ for a time range identified for each animal from treatment initiation (time zero) to undetectable values. Undetectable values were assumed when two consecutive samples were <50 vRNA copies/ml. The magnitudes of areas of displacement in $\log V$ [AUC $\log(V_0/V)$ versus time] of each animal were computed using a cubic spline routine (MESS Package; R Statistical Foundation, Vienna, Austria).

Statistics. Nonparametric AUC, box plots, and statistical tests were computed using the multcomp and ggplot2 packages in R (www.r-project.org; R statistical Foundation, Vienna, Austria). Other graphics were performed using GraphPad Prism (Graphpad Software, San Diego, CA, USA). Comparisons (*t* tests) between groups were performed using the Tukey correction for multiple comparisons, with a sandwich operator to provide consistent estimation of the covariance matrix, given apparent differences in variation (heteroscedascity) between groups.

RESULTS

Efficacy of the enhanced-HAART regimen in rhesus macaques. Distinct cohorts of young adult macaques were each inoculated intravenously with cell-free RT-SHIV, as described previously (18). All animals became persistently infected (with viral loads reaching a peak at week 2), and antiviral drug combinations were initiated at 6 to 8 weeks postinoculation (Fig. 1). All of the treated
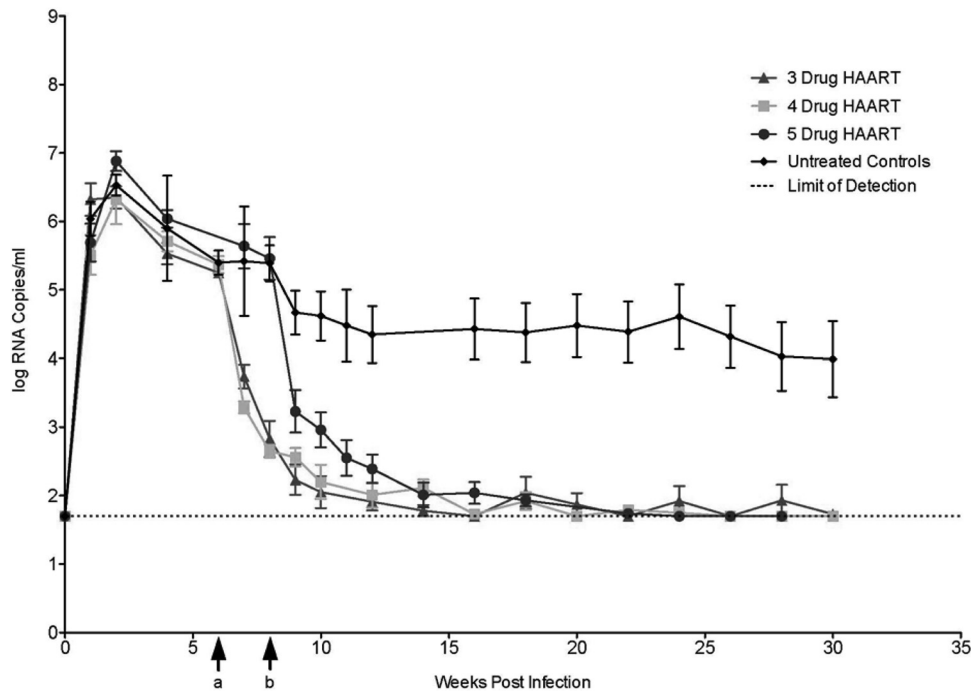
FIG 1 Summary of RT-SHIV infection and decay kinetics in rhesus macaques following a HAART (three-drug) or enhanced-HAART (four- or five-drug) regimen. Cohorts were treated with a three-drug [EFV, TFV, and (−)-FTC] ($n = 8$), four-drug [EFV, TFV, (−)-FTC, and L-812] ($n = 8$), or five-drug [EFV, TFV, (−)-FTC, AZT, and DAPD] ($n = 7$) regimen. Viral loads were monitored using qRT-PCR in macaques inoculated with RT-SHIV (week 0), followed by combination HAART initiated at 6 weeks (a) (three drugs and four drugs) or 8 weeks (b) (five drugs) after inoculation. The average viral load for each cohort, including an untreated control group (pooled historic data), is summarized as $\log_{10}$ vRNA copies/ml ± SD (error bars) for each time point (shown from weeks 0 to 30), with a limit of detection of ≥50 copies/ml.

macaque cohorts in these studies showed normal weight gains with moderate side effects that were reversible with early detection and dose modifications. Data from control (no antiretroviral drugs) RT-SHIV-inoculated animals from two other studies were included in this study as historic controls. The mean viral load ± SD for this group of RT-SHIV-infected control animals is shown in Fig. 1. In contrast to the drug-treated animals, many of the untreated RT-SHIV-inoculated control animals developed severe complications of simian AIDS and were euthanized during the experimental time course.

Following initiation of the enhanced five-drug combination, drug concentrations in plasma were measured using LC–MS-MS. As expected, all five antiretroviral drugs were detected in plasma (data not shown). In addition, a major metabolite of DAPD (DXG) was also detected (data not shown). Plasma viral loads rapidly declined in all macaque cohorts following initiation of HAART or enhanced HAART (Fig. 1), with a pattern similar to that observed with HAART in HIV-1-infected humans (36). However, the four- and five-drug cohorts showed a more rapid decline than the three-drug cohort (as shown by the individual-animal curves for each group). By 22 weeks postinoculation, plasma viral loads were below the level of detection of the standard assay (<50 vRNA copies/ml) in all drug-treated animals, and the viral loads remained suppressed for the duration of therapy, with only occasional blips (Fig. 1). Although plasma vRNA was not detected (<50 vRNA copies/ml) with our standard viral-load assay during prolonged enhanced-HAART administration, persistent low-level viremia was detected with a more sensitive viral-load assay that used larger volumes of plasma available at

necropsy (data not shown). Treatment was stopped after 18 to 49 weeks of combination drug administration, depending on the experimental design of the cohort. Upon cessation of therapy, some of the animals in each cohort were maintained for an additional 16- to 25-week period of observation to measure viral-rebound dynamics.

Dynamics of viral suppression of enhanced HAART. The viral decay kinetics of the four- and five-drug regimens appeared multiphasic, as has previously been reported for rhesus macaques treated with the three-drug regimen (29). Due to the complex and often oscillatory viral decay profiles and limited permissible samplings (restricted to weekly intervals), the data in this study were analyzed using nonparametric approaches to avoid overfitting of data (Fig. 2). Viral response curves for the first 10 weeks in cohorts administered enhanced HAART (a four- or five-drug combination) were compared to previously reported data from HAART (three-drug) administration (29), now expressed as $\log_{10}$ copies of SIV RNA/ml plasma. Overall, viral dynamic curves appeared multiphasic and sometimes oscillated (Fig. 2A). Therefore, we did not assume linear functions. Instead, AUC were computed using cubic spline curves. To ensure a dynamic range of >4 log units, we restricted inclusion of animals with initial viral loads ($\log V_0$) of >5 × 10⁴ vRNA copies/ml prior to treatment. Median starting viral loads ($\log V_0$) at treatment initiation (week 0 on HAART) were similar for all three treatment groups (Fig. 2B) ($P > 0.5$; Tukey multiple-comparison test), and therefore, it was presumed that any changes in viral-load dynamics following antiretroviral administration could be ascribed to differences in the treatment regimens (i.e., enhanced HAART versus HAART).
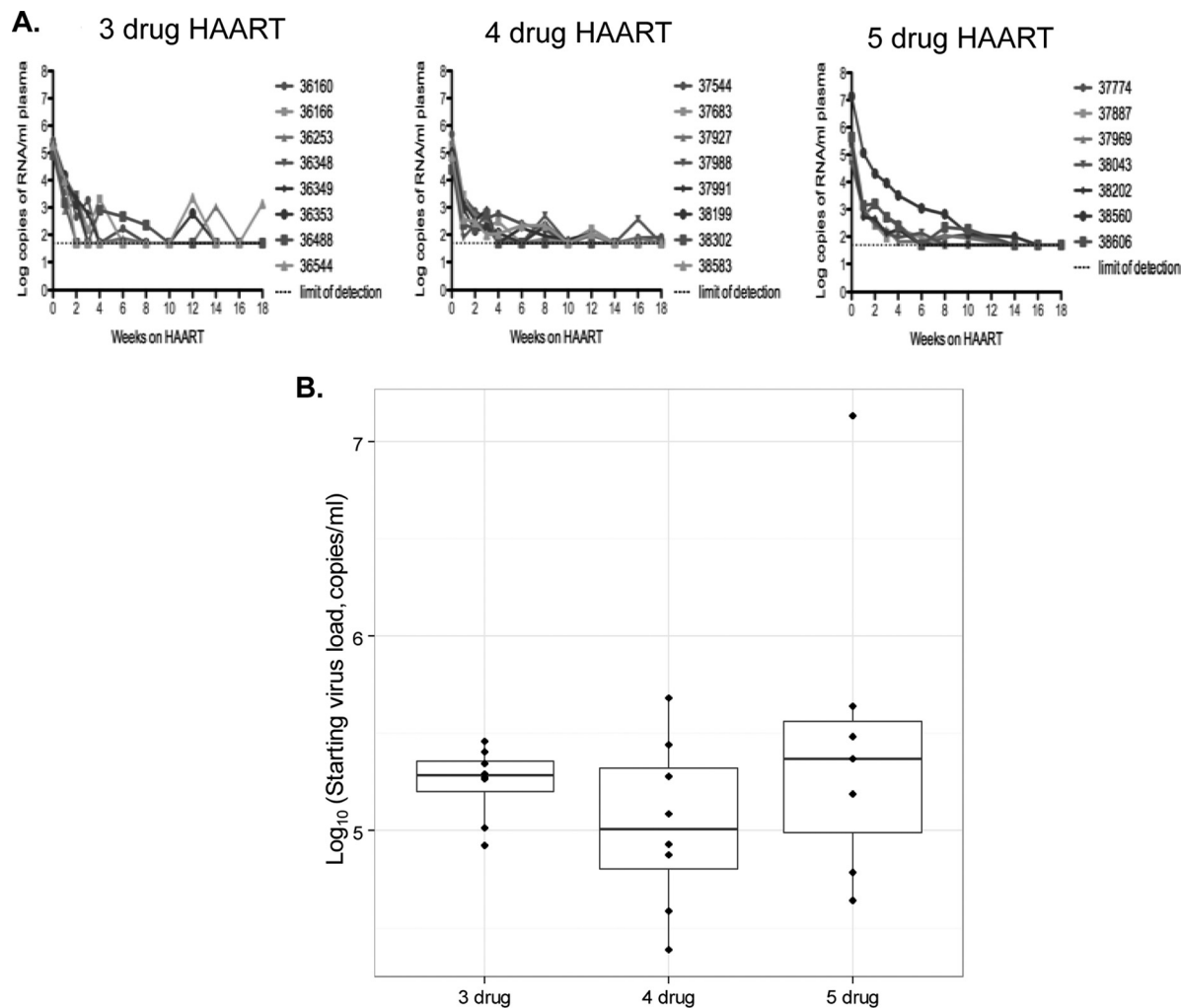
FIG 2 Comparative kinetics of RT-SHIV in rhesus macaques was assessed following enhanced HAART (four- or five-drug combination HAART) versus three-drug combination HAART. (A) Viral loads for individual animals within each treatment cohort (log vRNA copies/ml) over 10 weeks, monitored using RT-PCR. (B) AUC values computed for starting viral loads ($\log V_0$) prior to initiation of each HAART regimen. The median $\log V_0$ values for each treatment cohort were similar ($P > 0.5$). Horizontal lines and boxes represent median and interquartile ranges (IQR). Observations $>1.5\times$ IQR from the median are not linked with horizontal lines.

Three-drug treatment (HAART) produced a 1.4-fold reduction in the median $\log(V/V_0)$ after only 1 week. In comparison, the four-drug or five-drug (enhanced-HAART) treatment regimens produced 2.15- and 2.25-fold reductions, respectively, in the median $\log(V/V_0)$ during the same time interval (Fig. 3A). These data suggested improved viral-load reduction dynamics with four- and five-drug enhanced HAART compared to three-drug HAART ($P < 0.01$). Of note, there was no statistical difference in the viral decay dynamics between the two enhanced-HAART regimens. Similarly, the median displacement AUC of $\log(V_0/V)$ versus time following 10 weeks of enhanced HAART indicated >2-fold viral suppression ($P < 0.03$) compared with the three-drug treatment (Fig. 3B). In short, the greater AUC demonstrated a more pronounced decrease in viral-load dynamics over the initial 10-week time interval, suggesting that enhanced HAART is more effective at reducing the initial RT-SHIV load in macaques upon initiation of antiretroviral treatment.

Viral rebound. For each of the treatment groups, the plasma viral loads, in general, remained undetectable (<50 vRNA copies/ml) during the continued administration of drug regimens, with occasional blips, as mentioned above. Following cessation of therapy, we evaluated viral-load rebound in selected animals from each regimen group for an additional period (16 to 25 weeks). As expected, rebound occurred immediately (within 1 to 3 weeks) after cessation of the three-drug regimen. However, rebound also occurred with both the four- and five-drug regimens (also within 1 to 3 weeks), suggesting even enhanced HAART alone was unable to eliminate viral reservoirs. As a limited number of animals were available (as some were used for other studies) for the rebound phase, statistical analyses were limited. Again, all animals rebounded, with equivalent peaks within 3 weeks. These results are in agreement with our previous study of viral rebound after cessation of HAART (37).

DISCUSSION

In this study, we compared two enhanced-HAART regimens to the widely used three-drug HAART [(−)-FTC, TFV, and EFV] to determine whether enhanced-HAART regimens can provide
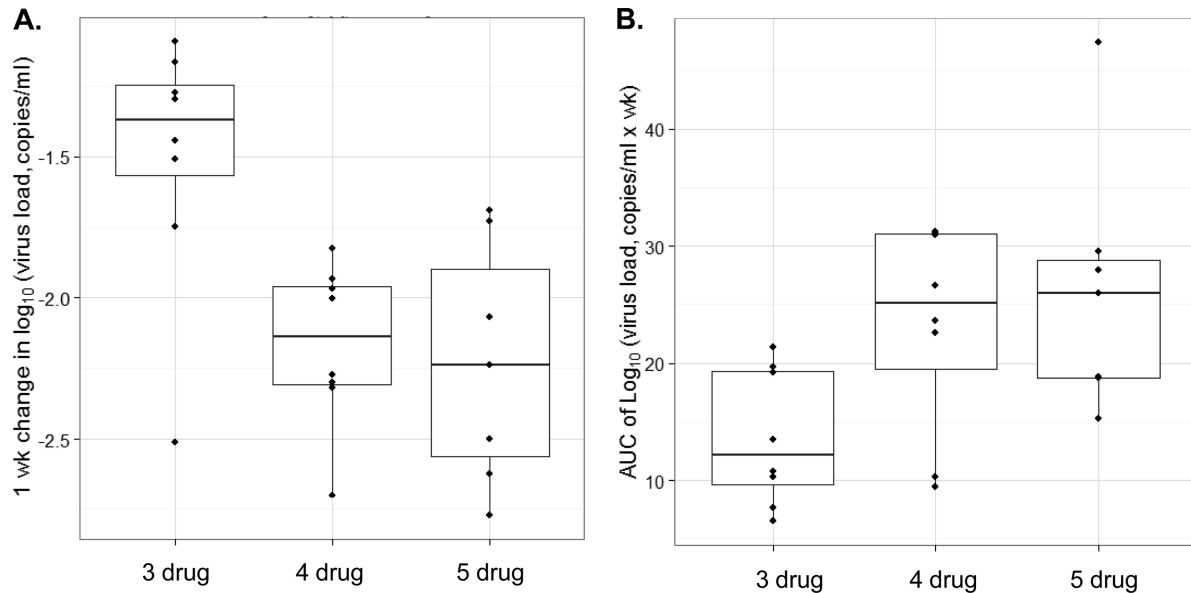
FIG 3 Comparison of RT-SHIV dynamics following a HAART (three-drug) or enhanced-HAART (four- or five-drug) regimen in rhesus macaques. Nonparametric analysis was performed on the viral-load data sets from each treatment cohort, assuming nonlinear decay kinetics. The AUC was determined for each individual animal and normalized by subtracting the initial viral load ($\log V_0$) from each subsequent viral load during treatment ($\log V$). (A) Box plots of the median log decrease in the viral load after 1 week of treatment for each cohort (enhanced HAART or HAART). Both enhanced-HAART regimens resulted in significantly greater reduction in the viral load than HAART ($P < 0.01$). (B) Median AUC of $\log(V_0/V)$ versus the time from the start of treatment (time zero) to the point at which two consecutive samples were at undetectable levels (<50 copies/ml). Again, both enhanced-HAART regimens resulted in significant increase in the AUC compared to HAART ($P < 0.05$).

more rapid and/or more complete suppression during therapy in an animal model system. The enhanced regimens were a four-drug [(−)-FTC, TFV, and EFV plus L-812] and a five-drug [(−)-FTC, TFV, and EFV plus AZT and DAPD] combination. We demonstrated that enhanced HAART does provide improved results, with more rapid suppression of virus (29). Despite the enhanced suppression of these regimens, virus eradication was not achieved over the time studied, as virus was detectable at the end of the experiments in all animals.

In this study, we observed multiphasic viral decay curves similar to what has been reported in persons living with HIV-1 and receiving HAART. In HIV-1 decay, the first phase primarily occurs in productively infected CD4$^+$ T cells, typically with a half-life of 1 to 2 days. The second phase may reflect decay of HIV-infected macrophages and related cells or activation of preintegration latency with a half-life of 2 to 3 weeks (38). Additionally, we found a third phase of decay that is less pronounced, as low-level viremia persists in RT-SHIV-inoculated macaques despite a HAART regimen commonly used in humans (10, 29). Therefore, eradication of long-lasting or latently infected cells may require decades of suppressive therapy (39).

In these studies, we found more rapid viral decay kinetics following enhanced HAART (four- or five-drug regimen) than with the three-drug regimen. Viral loads for each of the treatment groups (HAART or enhanced HAART) were reduced to below 50 vRNA copies/ml by 10 weeks of treatment. A reduction in the viral load corresponded to confirmed detection of systemic drug concentrations of each antiretroviral drug following the enhanced five-drug HAART regimen. Despite improved viral decay kinetics in the cohorts receiving enhanced HAART (either the four- or five-drug regimen), rebound rapidly occurred following treatment cessation. Therefore, increasing the combination of drugs

administered may not be sufficient to eradicate viral reservoirs. Recently, a randomized open-label study of three- versus five-drug PI-based combination HAART in newly HIV-1-infected individuals also found no significant long-term impact on virologic or immunologic responses at 48 weeks beyond those achieved with standard three-drug PI-based HAART (40). Unlike this recent clinical study, which was limited to monitoring the viral load after 12 weeks of treatment, our studies demonstrated an immediate impact on viral decay kinetics during the first 10 weeks of treatment, which arguably could be of benefit to the infected individuals in preserving their functional immunity.

The persistence of HIV infection despite HAART is a major challenge. Animal models for viral persistence during antiviral therapy may be important and necessary for HIV eradication strategies. We and others have achieved long-term viral-load suppression in the RT-SHIV models (16, 19, 41). Shytaj et al. have also achieved impressive long-term suppression using a highly intensified multidrug ART in SIVmac$_{251}$-infected rhesus macaques. Their regimen consisted of TFV, (−)-FTC, and raltegravir, initially for 1.5 months and then intensified with the protease inhibitor darunavir (pharmacokinetically enhanced by ritonavir) for 80 days and, lastly, reinforced with the addition of the CCR5 antagonist maraviroc (42). Likewise, Kline et al. monitored RT-SHIV$_{mne}$ in rhesus macaques over 20 weeks and found persistence of viral reservoirs in lymphoid tissues, despite undetectable plasma viremia at the time of necropsy (43). Unfortunately, eradication was not achieved in any of these studies. In all cases where therapy was terminated, the viral load rebounded after cessation of therapy. Although eradication was not achieved, these models are valuable to evaluate strategies for HIV eradication.

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