

Persistence of Genital Tract T Cell Responses in HIV-Infected Women on Highly Active Antiretroviral Therapy^{∇†}

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Received 9 March 2010/Accepted 13 July 2010

Initiation of highly active antiretroviral therapy (HAART) for HIV-infected individuals is associated with control of viremia, improved CD4 counts, and declining systemic HIV-specific immune responses. While HAART effectively reduces plasma viremia, it remains unclear how effectively antiretroviral drugs reach mucosal surfaces, such as those of the genital tract. The aim of this study was to determine the effect of HAART on genital tract CD4 T cell reconstitution, HIV shedding, and HIV-specific T cell responses. Cervical cytobrush and blood specimens were obtained from 35 HIV-infected, HAART-naïve women and 27 women on HAART in order to investigate HIV Gag-specific T cell responses by intracellular gamma interferon (IFN- γ) staining. Interleukin 1 β (IL-1 β), IL-6, and IL-8 concentrations were measured by enzyme-linked immunosorbent assays (ELISA). We show that for HIV-infected women, HAART is associated with significantly improved CD4 T cell counts both in blood and at the cervix. While HAART effectively suppressed both blood and cervical viremia, HIV-specific CD8 T cell responses in blood were lost, while those at the cervix were preserved.

South Africa currently has the largest number of individuals in the world infected with HIV (5.7 million), and the risk of HIV infection for young South African women is almost 6-fold that of young men (28). Highly active antiretroviral therapy (HAART) suppresses HIV replication and restores peripheral CD4 T cell counts in most HIV-infected individuals (2, 4, 57), and wide access to HAART has greatly reduced HIV-related morbidity and mortality rates worldwide (26). In South Africa, the eligibility criteria for the initiation of antiretroviral therapy are a blood CD4 count of less than 350 cells/ μ l or stage IV (AIDS) (59), and the current national recommendations for first-line treatment include the nucleoside reverse transcriptase inhibitors (NRTIs) lamivudine (3TC) and stavudine (d4T) combined with the nonnucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (EFV) or nevirapine (38). In 2007, it was estimated that of the ~889,000 HIV-infected individuals in South Africa needing HAART, only 371,731 (42%) were receiving treatment (50).

While HAART clearly reduces plasma viremia, it is unclear how various antiretroviral drugs influence HIV replication, virus shedding, and host immune reconstitution at mucosal surfaces of the genital tract, where HIV transmission occurs. NRTIs have been shown to reach the female genital tract more efficiently than protease inhibitors (PIs) and NNRTIs (16, 33). Although individuals on HAART (HAART⁺ individuals) shed

fewer HIV virions from mucosal surfaces than women not on HAART (HAART⁻ women) (13, 18, 20), it is apparent that HAART, even when administered early during infection, does not reliably result in reconstitution of T cell populations at these surfaces (22, 29, 37). This is perhaps not surprising, since in the absence of HAART, CD4 T cell restoration and preservation during HIV infection are generally less efficient at mucosal sites than in blood (23, 38). Ongoing low levels of HIV replication, the persistence of proviral DNA within infected cells of the mucosa (19, 43, 52, 56), and the poor penetration of drugs through to the mucosa have been implicated in the incomplete reconstitution of mucosal CD4 counts seen in individuals receiving HAART (17, 34).

Many studies have demonstrated that prolonged HAART is associated with a waning of HIV-specific cytotoxic T-lymphocyte (CTL) responses in blood, suggesting that ongoing antigenic stimulation provided by low-level HIV replication is necessary for the persistence of HIV-specific immune responses (1, 22, 31, 42, 60). However, other studies report the opposite findings, where blood HIV-specific responses increase after the initiation of HAART (2, 46). These discrepancies could be attributable to variations in both the time of treatment initiation and the degree to which viral replication is suppressed (44).

The influences of HAART on mucosal anti-HIV responses may be similar to those seen in blood. In the rectal mucosa, for example, the total suppression of HIV replication by HAART significantly reduced HIV-specific CD8 T cell responses at this site (12). This supports the hypothesis that persistent antigenic stimulation may be needed at mucosal surfaces in order to support cytotoxic responses at these sites.

HIV-specific T cell responses have also been identified in the genital tracts of HAART-naïve HIV-infected women (25,

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† Supplemental material for this article may be found at <http://jvi.asm.org/>.

[∇] Published ahead of print on 4 August 2010.

52). The female genital tract has a large proportion of activated CD4 T cells expressing CCR5 that are preferentially infected by HIV (26). Little is known about either fluctuations in genital tract CD4 T cell numbers during HAART or the impact of therapy on the immune function of these cells. We have previously shown significantly reduced CD4/CD8 ratios at the cervixes of chronically HIV infected women compared to uninfected women, suggesting that CD4 T cells are significantly depleted in the female genital tract during HIV infection (41). Previous studies have found near-complete restoration of CD4 T cell numbers in the gut mucosa following HAART (7, 38, 54) and have suggested that the differences observed in post-HAART CD4 T cell numbers may be attributable to differences in pre-HAART CD4 counts, the stage of HIV infection, or the specific gut regions where biopsy specimens were taken (54). The aim of this study was to investigate the impact of HAART on female genital tract CD4 T cell reconstitution, inflammation, local HIV shedding in genital secretions, and HIV-specific T cell immunity in the genital tracts of HAART-compliant women in South Africa.

MATERIALS AND METHODS

Study participants. One hundred and eight women with chronic HIV infections who attended the Nyanga Day Hospital, Cape Town, South Africa, were recruited for this study. Of these women, 45/108 (41.7%) had initiated antiretroviral therapy and 63/108 (58.3%) were therapy naïve (see Fig. S1 in the supplemental material). Women who either were menstruating at the time of sampling, were postmenopausal, had undergone a hysterectomy, had vaginal discharge, or had visible or reported sexually transmitted infections (STIs) were excluded from the study. The study was approved by the Faculty of Health Sciences Human Research Ethics Committee of the University of Cape Town, South Africa. Written informed consent was obtained from all women before initiation of the study.

Collection and processing of cervical specimens. Cervical lymphocytes were collected using a Digene cervical sampler according to a previously described method (25, 41). Briefly, cells were collected by using a single gentle 360° rotation of a cytobrush at the cervical os. The cytobrush was placed in 3 ml transport medium (R10, consisting of RPMI-1640 medium supplemented with 5 mM glutamine, fungin, penicillin-streptomycin, and 10% fetal calf serum [FCS]). Processing was performed within 4 h of collection to maximize recoverable cell yields and to maintain cellular viability. Cervical samples that had visible red blood cell contamination were discarded. Each cytobrush was flushed ~30 times with R10 in the collection tube using a Pasteur pipette. The cell suspension was then transferred to a clean 15-ml tube and centrifuged at 2,300 rpm ($1,000 \times g$) for 10 min. The supernatant was stored at -80°C for analysis of HIV shedding and genital inflammation. The pelleted cells were resuspended in R10 prior to processing for counting and phenotyping using an automated Guava cell counter (41) (Guava Technologies, Hayward, CA).

PBMC isolation. Blood was collected by venipuncture into sterile acid-citrate-dextrose anticoagulated Vacutainer tubes (Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation with Leucosep tubes and were counted using an automated Guava cell counter and Viacount staining (Guava). Plasma was stored at -80°C for viral load determination. Cell concentrations were adjusted to $2 \times 10^6/\text{ml}$, and the cells were rested for 16 h at 37°C under 5% CO_2 . All experiments were carried out on fresh PBMCs.

Intracellular cytokine staining. To investigate the function and frequency of HIV Gag-specific responses by CD8 and CD4 T cells, PBMCs ($1 \times 10^6/\text{ml}$) or cervical cells ($\sim 0.15 \times 10^6$ to 1×10^6 lymphocytes/ml) were split into three BD Falcon tubes (500 $\mu\text{l}/\text{tube}$) and were either (i) stimulated with a single pool of 121 HIV subtype C (Du422) Gag overlapping peptides (each peptide was used at a final concentration of 1 $\mu\text{g}/\text{ml}$; peptides were kindly provided by the NIH AIDS Reagent Repository), (ii) stimulated with phorbol myristate acetate (PMA)-ionomycin (10 $\mu\text{g}/\text{ml}$ each; Sigma-Aldrich) as a positive control, or (iii) left untreated for 4 to 6 h at 37°C under 5% CO_2 . Brefeldin A (10

$\mu\text{g}/\text{ml}$; Sigma, St. Louis, MO) was added after the first hour. In these experiments, cell fluorescence was measured by flow cytometry using a FACSCalibur (for cells from 11/45 HAART⁺ and 22/63 HAART⁻ women) or LSRII (for cells from 34/45 HAART⁺ versus 41/63 HAART⁻ women) flow cytometer (BD Immunocytometry Systems [BDIS]). For experiments using the LSRII flow cytometer, PBMCs and cervical cells were first incubated with the LIVE/DEAD Fixable Violet Dead Cell Stain for 20 min at room temperature (RT) and were then washed twice with phosphate-buffered saline (PBS). This step was not included for samples analyzed using the FACSCalibur flow cytometer. All staining was done in 96-well plates. Extracellular staining was performed for 30 min at RT with phenotypic markers using anti-CD8 conjugated to fluorescein isothiocyanate (FITC) or peridinin chlorophyll protein (PerCP) Cy5.5 (Becton Dickinson, San Jose, CA) and, for LSRII experiments only, anti-CD4 labeled with phycoerythrin (PE) Cy5 or PE Cy7 (BD) (this stain was omitted for FACSCalibur experiments). Cells were washed once by addition of 2 ml of 1% FCS-PBS, centrifuged (5 min, $300 \times g$ [1,300 rpm], RT), fixed, permeabilized using BD CytoFix/CytoPerm for 10 min at room temperature, and washed once with Perm wash (BD). Cells were stained with allophycocyanin (APC)- or APC-H7-labeled anti-CD3, PE-conjugated anti-gamma interferon (anti-IFN- γ), or Alexa Fluor 700-conjugated anti-IFN- γ (both from BD Biosciences) for 30 min on ice. Cells were finally washed with 2 ml of 1% FCS-PBS, centrifuged (5 min, $300 \times g$, 1,300 rpm, room temperature), and fixed with BD Cell Fix. Acquisition was performed using either a FACSCalibur or an LSRII flow cytometer (BD Immunocytometry Systems), with FlowJo (Tree Star, Inc.) for data analysis. Figure S2 in the supplemental material shows the gating strategy used to define IFN- γ production by either CD4 or CD8 T cells in response to Gag.

Cervical cytobrush samples were excluded if the positive control (PMA-ionomycin) failed (samples from 10/45 HAART⁺ versus 18/63 HAART⁻ women) or if the samples contained less than 250 CD3⁺ events by flow cytometry (8/45 HAART⁺ versus 10/63 HAART⁻ samples [see Fig. S1 in the supplemental material]). Of the 62 samples remaining, 27/62 were from HAART⁺ women and 35/62 were from HAART⁻ women. An average (\pm standard deviation [SD]) of 1,247 ($\pm 1,404$) CD3⁺ T cells were acquired per cervical cytobrush sample during intracellular cytokine staining.

Measurement of HIV loads in cervical secretions and plasma. Viral loads were determined in cervical secretions and plasma samples using NucliSens Easyq HIV 1, version 1.2. Cervical secretions were obtained after flushing of the cytobrush 30 times in 3 ml transport medium and removal of cells by centrifugation ($250 \times g$ for 10 min). Plasma was obtained from ACD-anticoagulated whole blood following Ficoll density gradient centrifugation. The detection limit of this assay was 50 HIV RNA copies/ml. Women were considered to be shedding HIV in their genital secretions if they had cervical viral loads of ≥ 300 HIV RNA copies/ml of cervical wash fraction.

Measurement of antiretroviral drug concentrations in plasma. Antiretroviral drug concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS-MS) using the modified methods described by Chi et al. (10) and Mistri et al. (39). Phenomenex Gemini C₁₈ (5 by 2 by 5 μm) and Synergy Fusion C₁₈ RP (50 by 2 by 4 μm) columns were used for NNRTIs/Pis and NRTIs, respectively. The plasma calibration curves of efavirenz, nevirapine, lopinavir, and ritonavir were linear over the range of 0.20 to 15 mg/liter, 0.25 to 10 mg/liter, 0.05 to 20 mg/liter, and 0.025 to 5 mg/liter, respectively. The plasma calibration curves of lamivudine, zidovudine, and stavudine were linear over the range of 0.02 to 6 mg/liter. Details of the lower limit of quantification (LLOQ), accuracy, and intraday and interday precision for each antiretroviral drug are included in Table S1 in the supplemental material.

Measurement of inflammatory cytokine concentrations in genital secretions. The concentrations of interleukin 1 β (IL-1 β), IL-6, and IL-8 in cervical supernatants were determined using Quantikine immunoassay enzyme-linked immunosorbent assay (ELISA) kits (detection limits, 0.070 pg/ml for IL-6, 1 pg/ml for IL-1 β , and 1.5 pg/ml for IL-8) (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The plates were read on a VersaMax ELISA microplate reader, and data were analyzed using SoftMax Pro software (version 4.3.1; Molecular Devices, CA).

Statistical analysis. A Mann-Whitney U test for nonparametric unmatched data was used for independent sample comparisons; Wilcoxon rank tests were used for nonparametric dependent sample comparisons; Chi-square tests were used for comparison of proportions, and Spearman tests were used for correlations, using GraphPad Prism, version 5.0. *P* values of ≤ 0.05 were considered significant.

RESULTS

Of the 108 HIV-infected women enrolled in the study, 27/45 women receiving HAART (HAART⁺) and 35/63 women naïve to HAART (HAART⁻) were eligible for inclusion in this study to investigate the impact of HAART on HIV shedding and T cell immunity in the female genital tract (Table 1; see also Fig. S1 in the supplemental material). The majority of HAART⁺ women were on first-line combination HAART (88.9%) (40), which includes two nucleoside reverse transcriptase inhibitors (NRTIs) and one nonnucleoside reverse transcriptase inhibitor (NNRTI) (Table 2). Of these women, 37% were on lamivudine (3TC)-stavudine (d4T)-efavirenz (EFV) triple-drug therapy and 25.9% were on 3TC-zidovudine (AZT)-EFV (Table 2). Only 11.1% of the women in this study (NY044, NY124, and NY243) were on second-line therapy, which included two NRTIs and two protease inhibitors (Combivir [3TC-AZT] and Kaletra [ritonavir-lopinavir]). The HIV-infected women receiving HAART had been on therapy for a median of 35.5 months (range, 2 to 80 months). The majority of HAART⁺ HIV-infected women (85.1%) were found to have antiretroviral drugs detectable in plasma at the time of study, indicating drug compliance (Table 2).

Impact of HAART on CD4 reconstitution in blood and in the female genital tract. HIV-infected women receiving HAART had significantly higher CD4 counts in blood than HAART-naïve women (Table 1 and Fig. 1A) (*P*, 0.02). We observed a significant positive correlation between time on therapy and blood CD4 cell counts (*P*, 0.05) (data not shown). For the majority of women on HAART, CD4 counts increased over time on therapy at an average rate of 32 CD4 cells/year of therapy (data not shown).

We have previously shown that HIV-infected women have significantly reduced CD4/CD8 ratios at the cervix compared to uninfected women, indicating a skewing toward CD8 T cell dominance in the genital tracts of HIV-infected women (41). To investigate the impact of HAART on CD4 reconstitution in the genital tract, we compared the percentages of CD4 T cells at the cervix of HAART⁻ and HAART⁺ women (Fig. 1B). We found that HAART⁺ women had significantly higher frequencies of CD4 T cells in their genital tracts than HAART⁻ women (*P*, 0.03), and correspondingly higher cervical CD4/CD8 ratios (data not shown), suggesting that HAART was associated with improved CD4 T cell numbers at the genital mucosa.

Impact of HAART on suppression of viremia in blood and at the cervix. In HAART⁻ women, the amount of HIV RNA detected in genital secretions was significantly associated with the amount of HIV RNA circulating in the blood (Rho, 0.7; *P*, 0.003 [data not shown]), indicating that genital tract HIV shedding was related to the level of plasma viremia.

Whereas we found that 73.7% of HAART⁻ and 4.2% of HAART⁺ women had detectable HIV RNA in plasma, 68.2% of HAART⁻ and 4.5% of HAART⁺ women had detectable HIV RNA in their genital secretions (Table 1). HAART⁻ women had significantly higher plasma viral loads than HAART⁺ women (Table 1 and Fig. 1C) (*P*, <0.0001), while only one HAART⁺ woman had detectable HIV RNA in plasma (NY273, with 3,900 RNA copies/ml [Table 2]). Similarly, HAART⁻ women had significantly higher concentrations

TABLE 1. Clinical characteristics of HIV-infected women included in this study

Patient characteristic	Value for group ^a		<i>P</i>
	HAART ⁻	HAART ⁺	
No.	35	27	
Age (yr)	32 (24–44)	34 (23–44)	0.3
CD4 count (cells/μl)	408 (206–919)	630 (294–1,111)	0.03
Plasma viral load (copies/ml)	7,800 (<50–61,000)	<50 (<50–3,900)	0.006
No. with detectable plasma HIV RNA/total no. (%)	14/19 (73.7) ^b	1/24 (4.2) ^c	<0.0001
Genital viral load (copies/ml)	325 (<50–28,000)	<50 (<50–430)	0.002
No. with detectable cervical HIV RNA/total no. (%)	15/22 (68.2) ^b	1/22 (4.5) ^c	<0.0001
Duration on ARV (months)	–	35.5 (2–80)	

^a Except where otherwise indicated, values are medians (ranges).

^b Only 19/35 plasma and 22/35 genital supernatant samples from HAART⁻ women were available for evaluation of plasma and cervical HIV loads, respectively.

^c Only 24/27 plasma and 22/27 genital supernatant samples from HAART⁺ women were available for evaluation.

of HIV detectable in genital secretions than HAART⁺ women (Table 1 and Fig. 1D) (*P*, 0.001), while only one HAART⁺ woman (NY074) had detectable virus in genital secretions (430 RNA copies/ml [Table 2]). It should be noted that the woman with incomplete plasma suppression (NY273) was not shedding HIV in the genital tract, while the woman shedding HIV in the genital tract (NY074) exhibited complete suppression of HIV in plasma and was HAART compliant (Table 2).

Genital tract inflammation and HIV shedding. We have previously shown that HIV-infected women who shed HIV into their genital secretions had significantly higher genital IL-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) concentrations than women who were not shedding HIV (25). Here we found that HAART⁻ women had significantly higher concentrations of IL-8 (but not IL-1β and IL-6) than HAART⁺ women (*P*, 0.02) (Table 3). For HAART⁻ women, concentrations of IL-1β (*P*, 0.005; Rho, 0.61) and IL-6 (*P*, 0.04; Rho, 0.46) in the genital tract were significantly associated with elevated HIV RNA concentrations in genital fluid (Fig. 2). Despite similar concentrations of IL-1β and IL-6 in genital secretions from HAART⁻ and HAART⁺ women, only one HAART⁺ woman was shedding HIV, and this did not appear to be associated with elevated inflammation in the genital tract (data not shown).

Impact of HAART on HIV-specific T cell responses in the genital tract. To evaluate the magnitude of T cell responses to HIV Gag in the genital tracts of HIV-infected women and to determine the impact of HAART on the reconstitution of T cell immunity in this compartment, we evaluated the frequencies of IFN-γ⁺ CD8 and CD4 T cells derived from the cervixes of HAART⁺ and HAART⁻ women (Fig. 3). We found that HAART⁻ women had significantly higher frequencies of HIV-specific CD8 T cell IFN-γ responses detectable in blood than HAART⁺ women (*P*, 0.03) (Fig. 3A and B, top), suggesting that HAART significantly reduces the magnitude of CD8 T cell responses in blood to HIV (21, 29). In contrast, HAART⁻ and HAART⁺ women did not differ in the magnitude of their cervical CD8 T cell responses to Gag (*P*, 0.2) (Fig. 3A and B, bottom), suggesting that genital tract CD8 T cell responses were not diminished by HAART. In addition, we found that Gag-specific CD8 responses in blood were significantly lower than those detected at the cervix for HAART⁻

TABLE 2. Details of the antiretroviral drug regimen and plasma concentrations for HIV-infected women on HAART included in this study

Patient ID ^a	Age (yr)	CD4 count (cells/ μ l)	Viral load (copies/ml) in:		HAART regimen ^b	Duration of HAART (mo)	Plasma antiretroviral drug level (μ g/ml) ^c						
			Plasma	Genital tract			0	2	4	6	8	10	12
NY039	26	NA ^d	<50	51	3TC-AZT-EFV	26							
NY044	26	743	<50	<50	Combivir-Kaletra	32							
NY045	33	189	<50	72	3TC-d4T-EFV	39							
NY061	30	317	<50	<50	3TC-d4T-EFV	2							
NY062	42	630	<50	<50	3TC-AZT-EFV	10							
NY074	29	484	<50	430	3TC-AZT-EFV	11							
NY124	29	NA	NA	NA	Combivir-Kaletra	12	*						
NY139	31	416	<50	<50	3TC-d4T-EFV	14							
NY169	23	NA	NA	<50	3TC-d4T-EFV	6							
NY276	30	1,111	<50	<50	3TC-d4T-EFV	61							
NY293	43	666	NA	NA	3TC-d4T-EFV	39							
NY063	34	642	<50	<50	Combivir-EFV	44							
NY076	37	745	<50	<50	Combivir-EFV	80							
NY098	39	370	<50	<50	3TC-d4T-EFV	55							
NY129	32	335	<50	<50	3TC-d4T-EFV	68							
NY273	37	743	3,900	<50	Combivir-EFV	69							
NY166	36	349	<50	<50	Combivir-NVP	15							
NY111	27	910	NA	<50	Combivir-NVP	58							
NY191	38	577	<50	<50	Combivir-NVP	70							
NY149	33	798	<50	<50	AZT-EFV	54							
NY006	32	675	<50	<50	3TC-TDF-NVP	2							
NY029	31	NA	<50	NA	Combivir-NVP	59							
NY294	44	294	120	<50	3TC-d4T-NVP	18							
NY235	NA	NA	<50	<50	Combivir-EFV	NA							
NY243	35	NA	<50	NA	Kaletra-EFV	58							
NY016	NA	366	<50	NA	Combivir-d4T	NA							
NY276	31	879	<50	<50	3TC-d4T-EFV	45							

^a ID, identification number.

^b 3TC, lamivudine; AZT, zidovudine; EFV, efavirenz; d4T, stavudine; NVP, nevirapine; TDF, tenofovir. Combivir is a coformulation of lamivudine and zidovudine in one pill. Kaletra is a coformulation of the HIV protease inhibitors lopinavir (LPV) and ritonavir (RTV).

^c NA, not available.

^d *, no plasma supernatant was available for determination of antiretroviral drug concentrations.

women (P , 0.03; Fig. 3A, top versus bottom), and this comparison approached significance for HAART⁺ women (P , 0.07) (Fig. 3B, top versus bottom).

HIV-specific CD8 responses detected in blood were not predictive of cervical CD8 T cell responses in either HAART⁻ (Rho, 0.16; P , 0.40) or HAART⁺ (Rho, 0.11; P , 0.63) women (data not shown). For HAART⁺ women, we also detected no correlation between the frequency of Gag-specific IFN- γ ⁺ CD8 T cells either in blood or at the cervix and improved CD4 counts in these compartments (data not shown). This indicates that CD4 reconstitution is probably not associated with an altered CD8 HIV-specific response in either compartment.

Previous studies have shown that CD4 T cell immunity in blood is impaired during HIV infection (3, 48) but is partially

restored after the initiation of HAART (2, 4, 53). In this study, few HIV-infected women had detectable CD4 T cell responses to HIV Gag at the cervix or in blood (Fig. 3, open bars), although the magnitude of these responses was significantly higher at the cervix than in blood in HAART⁻ women (P , 0.04) (Fig. 3A, top and bottom). No relationship was observed between the frequencies of IFN- γ ⁺ CD4 T cells specific to Gag at the cervix and in blood, confirming that the compartments were independent (data not shown). In contrast to the differences we observed between Gag-responsive CD8 T cell frequencies between HAART⁻ and HAART⁺ women, there were no obvious differences between these groups with respect to Gag-responsive CD4 T cell frequencies (P , 0.3) (Fig. 3A and B, top, open bars). There was no association between CD4 T cell recon-

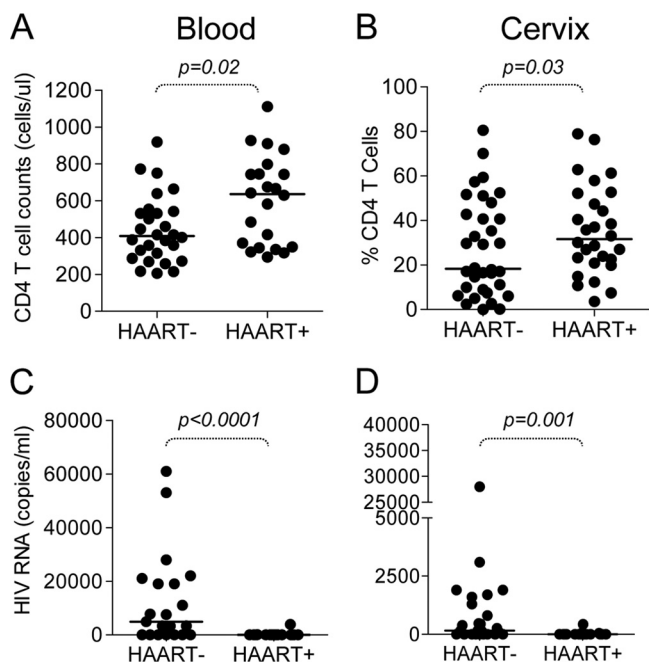


FIG. 1. CD4 T cell reconstitution and HIV suppression in the blood and genital tracts of HIV-infected HAART⁻ and HAART⁺ women. (A) CD4 T cell counts in the blood of HIV-infected HAART⁻ and HAART⁺ women. (B) Percentages of CD4⁺ T cells measured in the genital tracts for HIV-infected HAART⁻ and HAART⁺ women. (C) Plasma HIV loads (RNA copies/ml) for HAART⁻ and HAART⁺ women. (D) HIV loads (RNA copies/ml) in genital secretions from women on and off HAART. Each dot represents an individual woman's CD4 count or viral load; each horizontal line represents the median for the group. Statistical comparisons between HAART⁺ and HAART⁻ women were performed using the Mann-Whitney U test for unmatched nonparametric data. *P* values of <0.05 were considered significant.

stitution in blood or in the genital tract and the frequency of Gag-specific IFN- γ ⁺ CD4 T cells detected in the genital tract or blood (although these were of low magnitude).

Together, these data suggest that reduced plasma viral loads in women receiving HAART are presumably due to decreased antigen loads, associated with significantly reduced Gag-specific CD8 T cell responses in blood. While we found that HAART fully suppressed HIV shedding in the majority of HAART-compliant women in this study, this suppression (which is analogous to a reduction in plasma viral loads) was not associated with a reduction in Gag-specific CD8 T cell responses in the genital tract.

DISCUSSION

Suboptimal bioavailability of antiretroviral drugs at mucosal surfaces has been associated with incomplete immune reconstitution in individuals receiving HAART (17, 34). Several studies speculate that this is a result of low levels of HIV replication within persistently infected cells, in the inflammatory environment associated with mucosal tissue (20, 24, 45, 54, 58). In this study, we show that in one of the regions of greatest HIV endemicity in the world, HAART effectively suppressed both plasma viral loads and genital shedding of viral particles.

TABLE 3. Inflammatory cytokine concentrations in the genital tracts of HIV-infected HAART⁻ and HAART⁺ women

Cytokine	Median cytokine concn (range) for group		<i>P</i>
	HAART ⁻	HAART ⁺	
IL-1 β	50.7 (5.1–535.3)	28.9 (0.07–247.4)	0.5
IL-6	92.1 (6.3–3,442)	63.6 (1.9–316.8)	0.3
IL-8	1,368 (85.0–5,899)	435 (0.6–4756)	0.02

Furthermore, we found that women receiving HAART had significantly better CD4 counts both in blood and at the cervix than women who were not receiving treatment. However, HAART⁻ women had significantly higher frequencies of Gag-specific CD8 T cell responses in blood than HAART⁺ women, a finding that most likely reflects the fact that the latter group experienced reduced antigen loads. Despite showing significantly reduced CD8 T cell responses in blood, we found that cervical CD8 T cell responses to Gag were preserved in women on HAART and were similar in magnitude to those observed in HIV-infected women not on HAART.

We have previously shown that more than 10% of HIV-infected South African women receiving HAART who have undetectable plasma HIV RNA concentrations have >400 HIV RNA copies/ml in their genital secretions (41). In that study, however, HAART compliance was not evaluated. Here we have shown that <5% of HAART-compliant women had any detectable HIV in their genital secretions, an observation that clearly indicates the potential effectiveness of both the national HAART program and treatment adherence in slowing the spread of HIV.

Pharmacokinetics studies have shown that NRTIs penetrate the female genital tract more efficiently than either PIs or NNRTIs (17, 34), achieving higher drug concentrations at the female genital tract than in blood (16, 11, 34). In this study, all of the HAART⁺ women were receiving NRTIs as part of either a first-line treatment regimen (24/27 women), together with NNRTIs, or a second-line regimen (3/27 women), together with PIs. The single HAART⁺ woman with detectable

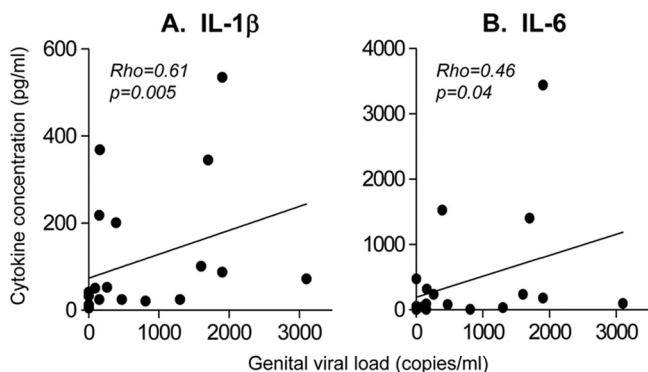


FIG. 2. Relationship between HIV shedding in the female genital tract and inflammatory cytokine concentrations. Concentrations of IL-1 β (A) and IL-6 (B) in genital secretions from HIV-infected HAART⁻ women were compared with the numbers of HIV RNA (copies/ml of cervical supernatant) detected in their genital tracts. Correlations were tested using the Spearman rank test, and *P* values of <0.05 were considered significant.

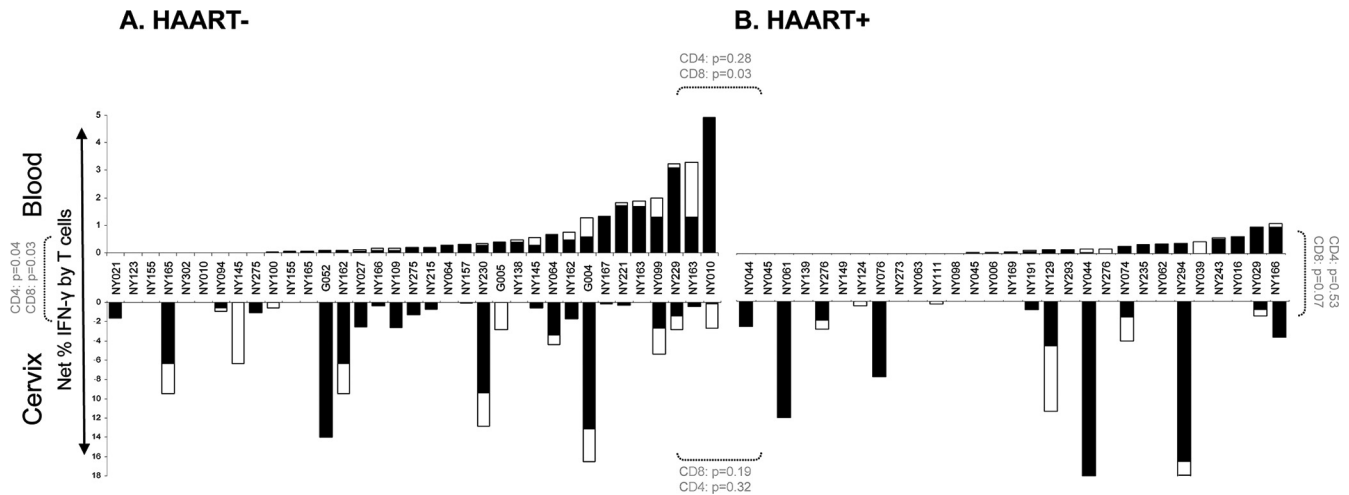


FIG. 3. HIV Gag-specific IFN- γ T cell responses detected in the blood and genital tracts of HIV-infected HAART⁻ (A) or HAART⁺ (B) women. The responses of CD8 (filled stacked bars) and CD4 (open stacked bars) T cells in blood (top) and at the cervix (bottom) to Gag were measured in HAART⁻ (A) and HAART⁺ (B) women. Each bar represents an individual woman's response at the cervix or in blood. Background-corrected net frequencies of IFN- γ ⁺ CD8 T cells are shown. The Mann-Whitney U test was used to compare unmatched samples, while the Wilcoxon rank test was used to compare matched samples. *P* values of ≤ 0.05 were considered significant. *P* values for CD8 and CD4 T cell comparisons are shown separately in panels A and B.

HIV RNA in her genital secretions (430 RNA copies/ml) was taking lamivudine (3TC)-zidovudine (AZT)-efavirenz (EFV) and had clearly detectable levels of all three antiretroviral drugs in her plasma. Although the concentrations of some of the drugs measured in plasma do not provide reliable information on adherence to therapy because of their short half-lives (less than 24 h) (5), it is unlikely that genital HIV shedding in this woman was related to poor adherence. Continued HIV shedding in the presence of fully suppressed plasma viremia in this woman may instead reflect ineffective penetration of drugs to the genital tract. This woman was on a regimen of zidovudine, lamivudine, and efavirenz. While zidovudine and lamivudine have been shown to achieve higher concentrations in the female genital tract than in blood (200% and 400% higher, respectively), efavirenz has comparatively poorer genital exposure (0.6% relative to concentrations in blood) (11).

Several studies have reported that CD4 T cells residing at the mucosa of the gastrointestinal tract (8, 23), lung (6), and male genital tract (48) are massively and persistently depleted during HIV infection. Furthermore, HAART has been shown to be ineffective at reconstituting CD4 T cell counts in the gut mucosa (36, 57), although the impact of therapy on CD4 reconstitution at other mucosal sites is unclear. We confirm in this study that women on HAART had significantly higher CD4 T cell counts in blood than HAART⁻ women, and the extent of CD4 reconstitution was associated with the time on therapy. In the female genital tract, we also found a significantly higher proportion of CD4 T cells for HAART⁺ women than for HAART⁻ women, indicating that HAART was also associated with the restoration of CD4 T cell proportions at the genital tract.

Although a number of studies have shown that the suppression by HAART of HIV loads in blood diminishes the magnitude of HIV-specific T cell responses after therapy (1, 22, 31, 43, 60), certain other studies have not found this effect (2, 46). We found that HAART⁺ women had significantly lower HIV-

specific CD8 T cell responses in blood than HAART⁻ women. It has been suggested that low levels of ongoing viral replication (antigenic stimulation) are necessary for the maintenance of immune responses in HIV-infected individuals (1).

While we found significantly lower blood CD8 T cell responses to HIV Gag in HAART⁺ women than in HAART⁻ women, Gag-specific CD8 T cell responses in the genital tract were detected in more than one-third of HAART⁺ women and were not significantly different in magnitude from the responses detected in HAART⁻ women. These Gag-specific CD8 T cell responses at the cervix were found to persist despite almost-complete suppression of genital HIV shedding in HAART⁺ women. In women receiving HAART, genital tract CD8 T cell responses against Gag were significantly higher in magnitude at the cervix than in blood. This finding is in contrast to those of other studies evaluating HIV-specific T cell responses at the cervix (52) and in the gut (12), which reported that mucosal T cell immunity to HIV is diminished in HAART⁺ individuals compared to those not on HAART, with only 1/9 (52) and 3/13 (12) HAART⁺ individuals having detectable HIV-specific IFN- γ T cell responses at the cervix and gut, respectively. It should be stressed, however, that these other studies evaluated fewer participants than were evaluated here and included participants on HAART regimens that differed from those of the women included in our study.

HIV-specific CTLs are known to occur in the genital tracts of HIV-infected women and have been shown to be protective in long-term nonprogressors (35, 47) and HIV-exposed but persistently seronegative (HEPS) women (32, 33). The maintenance of these responses could therefore potentially block any breakthrough HIV shedding at the cervixes of HIV-infected women undergoing therapy. We have previously shown that cervical cytobrush-derived T cells are dominated by effector memory subsets, with central memory and transitional memory being present at lower frequencies (41). While effector memory cells are short-lived, central and transitional mem-

ory cells are more persistent (51). Previous studies have shown that resting memory CD4 cells can support low levels of intracellular HIV replication and can therefore act as “reservoirs” of HIV infection (55). It is therefore plausible that the presence of HIV-specific CD8 T cells in the female genital tract, even during HAART, is the result of ongoing low levels of virus replicating within cells that are HIV reservoirs. Future studies should aim to evaluate HIV reservoirs in the genital tract of HIV-infected women on and off HAART.

In conclusion, we have shown that by effectively reducing blood and cervical viremia, HAART reconstitutes CD4 T cell counts within these compartments. Whereas we confirm previous reports of decreased blood CD8 T cell responses to HIV Gag following HAART, induced by decreases in viral loads, HIV-specific CD8 T cell responses were preserved in the genital tracts of women receiving HAART. We propose that sub-optimal antiretroviral drug concentrations in an environment rich in inflammatory signals may allow for the persistence within this compartment of latent HIV reservoirs within which intermittent local HIV replication persistently induces HIV-specific T cell responses.

ACKNOWLEDGMENTS

We thank the women from the Nyanga Day Hospital who kindly participated in the study, Janine Jones for collecting the specimens, and Darren Martin for reviewing this article and offering constructive comments to improve it.

This study was supported by grants from the Wellcome Trust and the Medical Research Council of South Africa. N.N.M. and J.S.P. received training in the United States as part of the Columbia University-Southern African Fogarty AITRP Program. N.N.M. is funded by the National Research Foundation (South Africa) African Scholarships Program and is a L’Oréal-UNESCO sub-Saharan regional fellow. J.S.P. is a Wellcome Trust Intermediate Fellow in Infectious Diseases.

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