Persistence of genital tract T cell responses in HIV-infected women on highly active anti-retroviral therapy (HAART)

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Initiation of highly-active anti-retroviral therapy (HAART) in HIV-infected individuals is associated with control of viraemia, improved CD4 counts and declining systemic HIV-specific immune responses. While HAART effectively reduces plasma viraemia, it remains unclear how effectively anti-retroviral 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 cytobrushes and blood were obtained from 35 HIV-infected women naive to HAART and 27 women on HAART to investigate HIV Gag-specific T cell responses by intracellular IFN-γ staining. IL-1β, IL-6 and IL-8 concentrations were measured by HS-ELISA. We show that in 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 viraemia, HIV-specific CD8 T cell responses in blood were lost while those at the cervix were preserved.

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South Africa currently has the largest number of individuals in the world infected with HIV (5.7 million) with risk of HIV infection in young South African women being almost 6-fold that of young men (28). Highly active anti-retroviral therapy (HAART) suppresses HIV replication and restores peripheral CD4 T cell counts in most HIV-infected individuals (2, 4, 58) 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 anti-retroviral therapy is a blood CD4 count of less than 350 cells/µl or stage IV (AIDS) (60) and the current National recommendations for first line treatment include the nucleoside reverse transcriptase inhibitors (NRTIs) lamivudine and stavudine combined with the non-nucleoside reverse transcriptase inhibitors (NNRTIs), efavirenz 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 viraemia, it is unclear how various anti-retroviral 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 shed fewer HIV virions from mucosal surfaces than women not on HAART (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 is generally less efficient at mucosal sites than it is in blood (23, 38). Ongoing low levels of HIV replication, the persistence of proviral DNA within infected cells of the mucosa (19, 43, 52, 57), and the poor penetration of drugs through to the mucosa have been implicated in 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 CTL responses in blood, suggesting that ongoing antigenic stimulation provided by low level HIV replication is necessary for persistence of HIV-specific immune responses (1, 22, 31, 42, 61). 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 female genital tract of HIV-infected women naïve to HAART (25, 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 cervix 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, 55) and have suggested that variations 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 biopsies were taken (55). 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 female genital tract 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, 45/108 (41.7%) had initiated anti-retroviral therapy and 63/108 (58.3%) were therapy naïve (Supplementary Figure 1). Women who were menstruating at the time of sampling, were post-menopausal, had undergone a hysterectomy, had vaginal discharge, or who had visible or reported 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 3ml transport medium [R10, RPMI-1640 medium supplemented with 5mM glutamine, fungin, penicillin-streptomycin and 10 % fetal calf serum (FCS)]. Processing was performed within 4 hours 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 15ml tube and centrifuged at 2300 rpm (1000xg) for 10 minutes. 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 ACD anti-coagulated vacutainer tubes (Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation using Leucosep tubes and 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 x 10^6 cells/ml and the cells were rested for 16 hours at 37°C and 5% CO₂. 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 (1×10^6 cell/ml) or cervical cells (~0.15-1×10^6 lymphocytes/ml) were split into three BD Falcon tubes (500 µl/tube) and stimulated with either (i) a single pool of 121 HIV subtype C (Du422) Gag overlapping peptides (each peptide was used at a final concentration of 1 µg/ml; peptides were kindly provided by the NIH AIDS Reagent Repository); (ii) PMA/ionomycin (10 µg/ml each; Sigma-Aldrich; positive control); or (iii) untreated for 4-6 hours at 37°C and 5% CO₂. Brefeldin A (10 µg/ml; Sigma, St. Louis, MO) was added after the first hour. In these experiments, cell fluorescence was measured by flow cytometry using FACSCalibur (11/45 HAART+ and 22/63 HAART-) or LSRII (34/45 HAART+ versus 41/63 HAART-) flow cytometers (BD Immunocytometry Systems [BDIS]). For experiments using the LSRII flow cytometer, PBMCs and cervical cells were first incubated with LIVE/DEAD® Fixable Violet Dead Cell Stain for 20 minutes at room temperature (RT), then washed twice with 1% FCS PBS. This step was not included for samples analyzed using the FACS Caliber flow cytometer. All staining was done in 96 well plates. Extracellular staining was performed for 30 minutes at RT with phenotypic markers using anti-CD8 conjugated to FITC or PerCPCy5.5 (Becton-Dickinson, San Jose,
CA), and PECy5 or PECy7 labelled anti-CD4 (BD; only for LSRII experiments; this stain was omitted for FACS Caliber experiments). Cells were washed once by adding 2 ml of 1% FCS PBS, centrifuged (5 min, 300×g, 1300 rpm, RT), fixed and permeabilized using BD CytoFix/CytoPerm for 10 min at room temperature and washed once with Perm wash (BD). Cells were stained with APC or APC-H7 labelled anti-CD3, PE-conjugated anti-IFN-γ or Alexa-fluor 700 conjugated anti-IFN-γ (Both BD Biosciences) for 30 minutes on ice. Cells were finally washed with 2 ml of 1% FCS PBS, centrifuged (5 minutes, 300×g, 1300 rpm, room temperature) and fixed with BD Cell Fix. Acquisition was performed using either a FACS Calibur or LSRII flow cytometer (BD Immunocytometry Systems [BDIS]) with FlowJo (Tree Star, Inc.) being used for data analysis. Supplementary Figure 2 shows the gating strategy used to define IFN-γ production by either CD4 or CD8 T cells in responses to Gag.

Cervical cytobrush samples were excluded if the positive control (PMA/ionomycin) failed (10/45 HAART+ versus 18/63 HAART- samples) or if the samples contained less than 250 CD3+ events by Flow cytometry (8/45 HAART+ vs 10/63 HAART-, Supplementary Figure 1). Of the 62 samples remaining, 27/62 were from HAART+ women and 35/62 were from HAART- women. An average of 1247 (± 1404 SD) CD3+ T cells were acquired per cervical cytobrush sample during ICS.

Measurement of HIV viral load 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 the cytobrush 30 times in 3ml transport medium and removal of cells by centrifugation (250 g for 10 min). Plasma was obtained from ACD anti-coagulated 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 ≥300 HIV RNA copies/ml of cervical wash fraction.

**Measurement of anti-retroviral drug concentrations in plasma.** Anti-retroviral 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). The column used for NNRTIs/PIs and NRTIs were Phenomenex Gemini C18 (5x2x5 um) and Synergy Fusion C18 RP (50x2x4 um) columns, respectively. The plasma calibration curves of efavirenz, nevirapine, lopinavir and ritonavir were linear over the range of 0.20-15 mg/L, 0.25-10 mg/L, 0.05-20 mg/L and 0.025-5 mg/L, respectively. The plasma calibration curves of lamivudine, zidovudine and stavudine were linear over the range of 0.02-6 mg/L. Details of the lower limit of quantification (LLOQ), accuracy and intra-day and interday precision for each anti-retroviral drug is included in Supplementary Table 1.

**Measurement of inflammatory cytokine concentrations in genital secretions.** The concentrations of IL-1β, IL-6, IL-8 in cervical supernatants were determined using Quantikine Immunoassay ELISA kits (IL-6: detection limit 0.70pg/ml; IL-1β: detection limit (1 pg/ml); IL-8: detection limit (1.5 pg/ml) (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The plates were read on a VersaMax® ELISA microplate reader and data analysed using SoftMax Pro Ver. 4.3.1 Software (Molecular Devices, CA).

**Statistical analysis.** A Mann-Whitney U Tests for non-parametric unmatched data were used for independent sample comparisons; Wilcoxon rank tests were used for non-parametric dependent sample comparisons; Chi-squared tests were used for comparison of proportions,
and Spearman tests were used for correlations, using GraphPad Prism version 5.0. P values of $p \leq 0.05$ were considered significant.
RESULTS

Of the 108 HIV-infected women enrolled in the study, 27/45 HIV-infected women receiving HAART (HAART+) and 35/63 HIV-infected women naïve to HAART (HAART-) were eligible to be included in this study to investigate the impact of HAART on HIV shedding and T cell immunity in the female genital tract (Table 1 and Supplementary Figure 1). The majority of HAART+ women were on first-line combination HAART (88.9%) (40), which includes two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside 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 Lamivudine (3TC)-Zidovudine (AZT)-Efavirenz (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 2-80 months). The majority of HAART+ HIV-infected women (85.1%) were found to have anti-retroviral 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 Figure 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 ratio at the cervix compared to uninfected women, indicating a skewing towards CD8 T cell dominance in the genital tract of HIV-infected women (41). To investigate the impact of HAART on CD4 reconstitution in the genital tract, we compared the percentage of CD4 T cells at the cervix of HAART- and HAART+ women (Figure 1B). We found that HAART+ women had significantly higher frequencies of CD4 T cells in the genital tract 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 viraemia 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 blood (Rho=0.7; p=0.003; data not shown), indicating that genital tract HIV shedding was related to the level of plasma viraemia.

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 Figure 1C; p<0.0001) with only one HAART+ woman having detectable HIV RNA in plasma (Table 2; NY273 with 3900 RNA copies/ml). Similarly, HAART- women had significantly higher concentrations of HIV detectable in genital secretions than HAART+ women (Table 1 and Figure 1D; p=0.001) with only one HAART+ women (NY074) having detectable virus in genital secretions (430 RNA copies/ml; Table 2). It is important to note 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 have significantly higher genital concentrations of IL-1β, IL-6, IL-8 and TNF-α than women who are 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). In HAART- women, genital tract concentrations of IL-1β (p=0.005; Rho=0.61) and IL-6 (p=0.04; Rho=0.46) were significantly associated with elevated HIV RNA concentrations in genital fluid (Figure 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 female 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 frequency of IFN-γ+ CD8 and CD4 T cells derived from the cervices of HAART+ and HAART- women (Figure 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; Figure 3A and B top panels) 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; Figure 3A and B bottom panels) 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- women (p=0.03; Figure 3A top versus bottom panels) and this comparison approached significance for HAART+ women (p=0.07; Figure 3B top versus
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+ women (Rho=0.11; p=0.63). In HAART+ women, we also detected no correlation between the frequency of Gag-specific IFNγ+ CD8 T cells in either 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 and in blood (Figure 3 clear bars) although the magnitude of these responses was significantly higher at the cervix than in blood in HAART- women (p=0.04; Figure 3A top and bottom panels). No relationship was observed between the frequency of IFN-γ+ CD4 T cells 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 cells 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; Figure 3A and B; top panels clear bars). There was no association between CD4 T cell reconstitution in blood or in the genital tract and the frequency of Gag-specific IFNγ+ CD4 T cells detected in the genital tract or blood (although these were of low magnitude).

Together, this data suggests that reduced plasma viral loads in women receiving HAART is presumably due to decreased antigen load, associated with significantly reduced Gag-specific CD8 T cell responses in blood. While we found that HAART fully suppresses 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 anti-retroviral drugs at mucosal surfaces has been associated with incomplete immune reconstitution in individuals receiving HAART (17, 34). Several studies speculate that this is as a result of low levels of HIV replication within persistently infected cells, in the inflammatory environment associated with mucosal tissue (20, 24, 45, 54, 59). In this study, we show in one of the most HIV endemic regions in the world that HAART effectively suppressed both plasma viral loads and genital shedding of viral particles. Furthermore, we found that women receiving HAART had significantly better CD4 counts in both 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, which most likely reflects the fact that the latter group experience 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 this other 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 compared to blood (16, 11, 34). In this study, all of the HAART+ women were receiving NRTIs as part of either a first line treatment regime (24/27 women) together with NNRTIs or a second line regime (3/27 women) together with PIs. The single HAART+ woman with detectable 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 anti-retroviral drugs in her plasma. Although some of the drug concentrations measured in plasma do not reliably inform on adherence to therapy because of their short half life (less than 24 hours) (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 viraemia 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 compared to blood (200% and 400% higher than blood, respectively), Efavirenz has comparatively poorer genital exposure (0.6% relative to 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 (58, 36) although the impact of therapy on CD4 reconstitution at other mucosa 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 time on therapy. In the female genital tract, we also found a significant increase in the proportion of CD4 T cells in HAART+ women compared to 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 viral load in blood diminishes the magnitude of HIV-specific T cell responses after therapy (1, 22, 31, 43, 61), certain other studies have not found this effect (2, 46). We found that HAART+ women had significantly reduced HIV-specific CD8 T cell responses in blood relative to HAART- women. It has been suggested that low levels of ongoing viral replication (antigenic stimulation) is necessary for the maintenance of immune responses in HIV-infected individuals (1).

While we found significantly reduced blood CD8 T cell responses to HIV Gag in HAART+ women compared to HAART- women, Gag-specific CD8 T cell responses in the genital tract were detected in more than a third of HAART+ women and were not significantly different in magnitude compared to 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 is important to stress, however, that these other studies evaluated fewer participants than were evaluated here and they 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 non-progressors (35, 47) and HIV exposed but persistently sero-negative (HEPS) women (32-33). The maintenance of these responses
could therefore potentially block any breakthrough HIV shedding at the cervix 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 memory 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 (56). 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 level 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 viraemia, 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 female genital tracts of women receiving HAART. We propose that sub-optimal anti-retroviral 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 induce HIV-specific T cell responses.
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### TABLE 1. Clinical characteristics of HIV-infected women included in this study

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>HAART- Median (Range)</th>
<th>HAART+ Median (Range)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>35</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>32 (24-44)</td>
<td>34 (23-44)</td>
<td>0.3</td>
</tr>
<tr>
<td>CD4 Count (Cells/µl)</td>
<td>408 (206-919)</td>
<td>630 (294-1111)</td>
<td>0.03</td>
</tr>
<tr>
<td>Plasma Viral load (copies/ml)</td>
<td>7800 (&lt;50-61000)</td>
<td>&lt;50 (&lt;50-3900)</td>
<td>0.006</td>
</tr>
</tbody>
</table>
| Number of women with detectable plasma HIV RNA [N/total; %] | 14/19 (73.7%)
\(^\text{a}\) | 1/24 (4.2%)
\(^\text{b}\) | <0.0001 |
| Genital Viral load (copies/ml) | 325 (<50-28000) | <50 (<50-430)            | 0.002   |
| Number of women with detectable cervical HIV RNA [N/total; %] | 15/22 (68.2%)
\(^\text{a}\) | 1/22 (4.5%)
\(^\text{b}\) | <0.0001 |
| Duration on ARV (months) | -                     | 35.5 (2-80)            |         |

\(^\text{a}\) Only 19/35 plasma and 22/27 genital supernatant samples from HAART- women were available for evaluation of plasma and cervical HIV load, respectively.

\(^\text{b}\) Only 24/27 plasma and 22/27 genital supernatant samples from HAART+ women.
TABLE 2. Details of the anti-retroviral drug regimen and plasma concentrations in HIV-infected women on HAART included in this study

<table>
<thead>
<tr>
<th>PID</th>
<th>Age (Years)</th>
<th>CD4 count (cells/µl)</th>
<th>Plasma viral load (copies/ml)</th>
<th>Genital tract viral load (copies/ml)</th>
<th>HAART regimen</th>
<th>Duration of HAART (months)</th>
<th>Plasma anti-retroviral drug levels (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NY039</td>
<td>26</td>
<td>na</td>
<td>&lt;50</td>
<td>51</td>
<td>Lamivudine (3TC)-Zidovudine (AZT)-Efavirenz (EFV)</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>NY044</td>
<td>26</td>
<td>743</td>
<td>&lt;50</td>
<td>&gt;50</td>
<td>Lamivudine (3TC)-Zidovudine (AZT)-Efavirenz (EFV)</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>NY045</td>
<td>33</td>
<td>109</td>
<td>&lt;50</td>
<td>72</td>
<td>Lamivudine (3TC)-Stavudine (d4T)-Efavirenz (EFV)</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>NY061</td>
<td>30</td>
<td>317</td>
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*Plasma supernatant was not available for anti-retroviral drug concentration determination

1 Combivir: A co-formulation of Lamivudine and Zidovudine in one pill
2 Kaletra: A co-formulation of HIV protease inhibitors Lopinavir (LPV) and Ritonavir (RTV)
3* Plasma supernatant was not available for anti-retroviral drug concentration determination
4 Not available; plasma and cervical supernatants for these participants were not available for evaluation
TABLE 3. Genital tract inflammatory cytokine concentrations in HAART- and HAART+ HIV-infected women

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>HAART- Median (Range)</th>
<th>HAART+ Median (Range)</th>
<th>p-value</th>
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<td>IL-1β</td>
<td>50.7 (5.1-535.3)</td>
<td>28.9 (0.07-247.4)</td>
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<td>IL-8</td>
<td>1368 (85.0-58999)</td>
<td>435 (0.6-4756)</td>
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FIGURE LEGENDS

FIG. 1. CD4 T cell restoration and suppression of HIV in HIV-infected women on HAART. (A) Left panel; CD4 T cells were measured from the blood of HIV-infected women (HAART+) and therapy naïve (HAART-) HIV-infected women. (A) Right panel; Association between CD4 counts in blood and time on HAART in HIV-infected women on therapy. (B) Left panel; Percentage of CD4 T cells measured in the female genital tract from HAART+ and HAART- HIV-infected women. (B) Right panel; Association between percentage of genital CD4 T cells and time on HAART in women undergoing treatment. (C) Comparison of HIV shedding in genital secretions (left) and plasma viral load (right) detected in women on and off HAART. Each dot represents an individual woman CD4 count or viral load. Statistical comparisons between HAART+ and HAART- women were performed using the Mann-Whitney U Test for unmatched non-parametric data and associations were tested using the Spearman Ranks test. P values of \( p<0.05 \) were considered significant.

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

FIG. 3. HIV Gag-specific IFN-\( \gamma \) T cell responses detected in blood and in the genital tract of (A) HAART- or (B) HAART+ HIV-infected women. CD8 (solid stacked bars) and CD4 (clear stacked bars) T cell responses in blood (top panel) and at the cervix (bottom panel) to Gag were measured in (A) HAART- and (B) HAART+ women. Each bar represents an individual woman’s response at the cervix or in blood. Background-corrected net frequencies of IFN-\( \gamma \) CD8 T cells are shown. Mann-Whitney U test was used to compare unmatched samples while Wilcoxon Ranks test was used to compare matched samples. \( P \)-values \( \leq 0.05 \) were considered significant. \( P \)-values for CD8 and CD4 T cell comparisons are shown separately on (A) and (B).
FIG. 1. CD4 T cell reconstitution and HIV suppression in blood and in the female genital tract of HAART- and HAART+ HIV-infected women. (A) CD4 T cell counts in blood of HAART- and HAART+ HIV-infected women. (B) Proportion of CD4+ T cells measured in the female genital tract from HAART- and HAART+ HIV-infected women. (C) Plasma HIV load (RNA copies/ml) from HAART- and HAART+ women. (D) HIV load (RNA copies/ml) in genital secretions from women on and off HAART. Each dot represents an individual woman’s CD4 counts or viral load. The line represents the median for each group. Statistical comparisons between HAART+ and HAART- women were performed using the Mann-Whitney U Test for unmatched non-parametric data. P values of p<0.05 were considered significant.
FIG. 2. Relationship between HIV shedding in the female genital tract and inflammatory cytokine concentrations. Concentrations of (A) IL-1β and (B) IL-6 in genital secretions from HAART- HIV-infected women were compared with the amount of HIV RNA (copies/ml of cervical supernatant) detected in the genital tract. Correlations were tested using the Spearman rank test and p values of $p < 0.05$ were considered significant.
FIG. 3. HIV Gag-specific IFN-γ T cell responses detected in blood and in the genital tract of (A) HAART- or (B) HAART+ HIV-infected women. CD8 (solid stacked bars) and CD4 (clear stacked bars) T cell responses in blood (top panel) and at the cervix (bottom panel) to Gag were measured in (A) HAART- and (B) HAART+ 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. Mann-Whitney U test was used to compare unmatched samples while Wilcoxon Ranks test was used to compare matched samples. P-values ≤0.05 were considered significant. P-values for CD8 and CD4 T cell comparisons are shown separately on (A) and (B).