Relationship Between Genital Drug Concentrations and Cervical Cellular Immune Activation and Reconstitution in HIV-1-Infected Women on a Raltegravir Versus a Boosted Atazanavir Regimen

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Abstract

Determinants of HIV-infected women's genital tract mucosal immune health are not well understood. Because raltegravir (RAL) achieves relatively higher genital tract concentrations than ritonavir-boosted atazanavir (ATV), we examined whether an RAL-based regimen is associated with improved cervical immune reconstitution and less activation in HIV⁺ women compared to an ATV-based regimen. Peripheral blood, cervical brushings, cervical–vaginal lavage (CVL), and cervical biopsies were collected from HIV⁺ women on tenofovir disoproxil fumarate and emtricitabine (TDF/FTC) and either RAL ($n=14$) or ATV ($n=19$) with CD4⁺ T cells >300 cells/mm³ and HIV RNA <48 copies/ml. HLA-DR⁺CD38⁺ T cells were measured in blood and cervical cells using flow cytometry, CD4⁺ and CD8⁺ T cells were quantified in cervical biopsies by immunofluorescent analysis, and HIV RNA (VL), ATV, and RAL concentrations were measured in CVL. In a linear regression model of log(CVL concentration) versus both log(plasma concentration) and treatment group, the RAL CVL level was 519% (95% CI: 133, 1,525%) higher than for ATV ($p<0.001$). Genital tract VL was undetectable in 90% of subjects and did not differ by regimen. There were no significant differences between groups in terms of cervical %HLA-DR⁺CD38⁺CD4⁺ or CD8⁺ T cells, CD4⁺ or CD8⁺ T cells/mm², or CD4:CD8 ratio. After adjusting for treatment time and group, the CVL:plasma drug ratio was not associated with the cervical CD4:CD8 ratio or immune activation ($p>0.6$). Despite significantly higher genital tract penetration of RAL compared to ATV, there were no significant differences in cervical immune activation or reconstitution between women on these regimens, suggesting both drug regimens achieve adequate genital tract levels to suppress virus replication.

Introduction

ALTHOUGH INTRODUCTION OF POTENT antiretroviral therapy (ART) in the mid-1990s initially led to dramatic improvements in survival of HIV-1 (HIV)-infected individuals,^{1–3} morbidity from complications of HIV infection in the United States continues and has not substantially declined in recent years.⁴ During the ART era, a significant amount of HIV-related morbidity in women is attributable to genital tract disease including vaginal candidiasis, reactivation of herpes simplex virus (HSV), and human papilloma virus (HPV)-related cervical dysplasia and cancer.⁵ Little is known

about the determinants of genital tract CD4⁺ T cell recovery in HIV-infected women receiving ART and whether a specific ART regimen could influence genital tract immune reconstitution and activation.

Declines in genital tract HIV RNA concentration have been well documented after ART initiation.⁶ Nevertheless, HIV RNA is sometimes detected in genital tract samples from women with undetectable plasma viral loads.^{7–10} Low-level viral replication and/or inflammation may impair CD4⁺ T cell reconstitution at mucosal sites resulting in local defects in immunity.⁸ Activated, i.e., HLA-DR⁺CD38⁺(DR⁺38⁺) CD4⁺ T cells in the cervix express high levels of CCR5, suggesting

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they may be uniquely susceptible to HIV.¹¹ In an *ex vivo* cervical model, activated (CD38⁺) CD4⁺ T cells were preferentially infected.¹² Thus, activated genital tract CD4⁺ T cells could contribute to local HIV replication and subsequent cell death, impairing mucosal immune reconstitution in HIV-infected women. It is important to determine if the proportion of activated CD4⁺ T cells could be changed by improved genital antiretroviral drug concentrations. Furthermore, it is not known whether optimized drug levels in the genital tract would enhance reconstitution of CD4⁺ and CD8⁺ T cells.

One study demonstrated higher percentages of CD4⁺ T cells in the cervix of HIV-infected women primarily receiving nonnucleoside analogue reverse transcriptase inhibitor (NNRTI)-based ART (~30%) compared to ART-naïve women (~20%), but this study was confounded by significantly different peripheral blood CD4⁺ T cell counts in the two groups.¹³ Another study used cervical cells collected via cytobrush from HIV-infected women on now outdated ART regimens and HIV-uninfected women and showed no difference in absolute cervical CD4⁺ T cell counts between these two groups.¹⁴ Importantly, the CD4⁺ T cell count alone may not be completely reflective of prognosis.¹⁵ In the genital tract, inflammation may further augment numbers of both CD4⁺ and CD8⁺ T cells,¹⁶ therefore, the CD4:CD8 T cell ratio may be more reflective of immune reconstitution. Cervical immune activation and CD4:CD8 T cell ratio are important measures of genital mucosal immunity and whether a specific ART regimen could affect these measures is an important question.

Many first-line ART regimens achieve high concentrations of only two medications in the female genital tract secretions. Generally, in the female genital tract, concentrations of NNRTIs and protease inhibitors (PIs) are substantially lower compared to those in plasma, whereas concentrations of nucleos(t)ide analogue reverse transcriptase inhibitors (NRTIs) are similar to or higher than those found in plasma.^{17–19} The commonly used PI atazanavir (ATV), when combined with ritonavir, achieves genital fluid concentrations of 30% or less of those in plasma,^{17,19} although a recent study describes higher genital-to-plasma concentrations.²⁰ Importantly, with the introduction of raltegravir (RAL), which achieves concentrations in the female genital tract fluids higher than those in plasma,^{21,22} ART regimens that deliver robust concentrations of three antiretroviral drugs to the female genital tract became available for the first time. Little is known about immune reconstitution and ongoing inflammation in the female genital tract on these newer ART combinations.

The purpose of this study was to determine if an RAL-based regimen is superior to an ATV-based regimen in terms of female genital tract mucosal immune health. We hypothesized that HIV-infected women receiving an RAL-based regimen would exhibit better cervical immune reconstitution and less immune activation in the cervicovaginal compartment compared to those receiving an ATV-based regimen.

Materials and Methods

Study subjects

HIV-infected women with a CD4⁺ T cells/mm³ ≥ 300 and HIV RNA copies/ml < 48 for a minimum of 6 months, who were receiving tenofovir disoproxil fumarate and emtricitabine (TDF/FTC) combined with either RAL or ATV, were recruited to the study. Women with a history of a hys-

terectomy, active substance abuse, bleeding diathesis, known carcinoma of the cervix, currently using oral glucocorticoids or other immunosuppressive agents, current vaginal symptoms, or current pregnancy were excluded. Two study visits occurred within a 2-week time period, specially avoiding menses in reproductive-age women. Women were asked to abstain from sex for at least 3 days prior to study visits. Whole blood and cervical–vaginal lavage (CVL), cytobrush samples, and samples for wet prep and *Chlamydia trachomatis*/*Neisseria gonorrhea* testing were collected at the first visit. Blood, CVL, cervical biopsies and samples for wet prep, *Chlamydia trachomatis*/*Neisseria gonorrhea*, HSV, and HPV testing were collected at the second visit. Informed consent was obtained in accordance with the Colorado Multiple Institutional Review Board.

Clinical specimens

Peripheral blood CD4⁺ and CD8⁺ T cell counts were determined by flow cytometry using an LSR-II flow cytometer (BD Immunocytometry Systems, San Jose, CA) and plasma HIV-1 RNA concentration was measured by the Roche COBAS TaqMan 96 HIV-1 test (Indianapolis, IN). Genital tract samples were evaluated for *Chlamydia trachomatis* and *Neisseria gonorrhea* using nucleic acid amplification using Roche COBAS Amplicor (Indianapolis, IN); HSV by real-time polymerase chain reaction (PCR) using Roche Mag-NAPur extraction system and amplified using the Roche LightCycler 1.0 real time PCR (Indianapolis, IN); and HPV DNA by the *digene* HC2 HPV DNA Test, which uses signal-amplified nucleic acid hybridization and chemiluminescence for the qualitative detection of 18 types of HPV DNA (Qiagen, Germantown, MD). CVL was collected using 5 ml of sterile saline to lavage the cervical vaginal compartment; fluid was then centrifuged and supernatant was stored at –70°C for later testing for drug concentrations and HIV RNA. In addition, genital samples were evaluated with direct microscopy for clue cells, *Trichomonas*, and *Candida*. The blood was tested for treponemal antibodies to screen for syphilis at both time points. All clinical assays (treponemal antibodies, wet prep, HSV, HPV, chlamydia, and gonorrhea) were performed in the University of Colorado Hospital Clinical or Molecular Correlates Laboratory (Aurora, CO).

Quantification of the CD4⁺:CD8⁺ T cell ratio in cervical biopsies

Two punch biopsies were collected from the ectocervix at approximately the 2–3 o'clock and 7–8 o'clock positions. Tischler biopsy forceps were used to obtain the biopsies that measured approximately 5 mm by 2 mm by 2 mm in size. Biopsies were snap frozen in OCT and stored at –70°C. One of the two biopsies was randomly selected to provide absolute quantitation (vs. relative quantitation with flow cytometry) of cervical CD4⁺ and CD8⁺ T cells. Snap frozen cervical biopsies were cut into 4-μm sections and on average eight sections, each approximately 120 μm apart, were evaluated for each subject. Sections were immunostained with fluorescent antibodies to CD3, CD4, and CD8, and visualized by immunofluorescent microscopy. Numbers of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells per mm² of tissue were determined using visual inspection and quantitative image analysis. Cell counts and areas of all sections from each subject were

summed. The total count was divided by the total area to determine cells/mm². The CD4:CD8 ratio was calculated by dividing CD3⁺CD4⁺ cells/mm² by CD3⁺CD8⁺ cells/mm².

Flow cytometry analysis

Cervical cells were collected from the endocervical canal using a cytobrush, as previously described.¹¹ Fresh peripheral blood and cervical cells were stained in real time with antibodies to CD3-PEcy5 (BD Biosciences, San Jose, CA), CD4-APC-H7, CD8-Alexa Fluor 405, CD38-FITC (Invitrogen Life Science), and HLA-DR-APC (BD Biosciences) as previously described.^{11,23,24}

Quantification of raltegravir and atazanavir

RAL and ATV plasma trough concentrations were quantified using a validated LC-MS-MS and HPLC-UV assay, respectively, as previously described.^{25,26} The lower limit of quantification for RAL was 5 ng/ml and that for ATV was 50 ng/ml. The CVL samples were extracted by protein precipitation using methanol and isotopically labeled raltegravir-d₃ and ritonavir-d₆ were used as an internal standards. LC-MS/MS analysis was performed using gradient elution on a Phenomenex Polar-RP (50 × 2.0 mm, 4 μm) HPLC column with water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). Method analytes were detected on an AB Sciex API-5000 triple quadrupole mass spectrometer operated in positive ion mode. Samples were quantified over calibration ranges of 0.2–1,000 ng/ml (ATV) and 1–1,000 ng/ml (RAL) with acceptability criteria of ± 15%.

HIV RNA detection in CVL

A reverse transcription-polymerase chain reaction assay (Abbott RealTime HIV assay, Des Plaines, IL) was used for quantitation of HIV RNA in 1 μl samples of cell-free CVL for amplification with a lower limit of quantification of 40 copies/ml. Virus from the VQA laboratory of the AIDS Clinical Trial Group and WHO 1st International Standard for HIV RNA were used as standards. HIV RNA signals two standard deviations above background, but below the limit of quantification, were recorded as detectable but not quantifiable.

Statistical analysis

The sample size for this study was based on a power analysis using cervical CD4:CD8 T cell ratios from Nkwananya *et al.*¹⁴ in which there was a 6-fold difference in mean CD4:CD8 ratio of cervical T cells between HIV-1-seropositive (0.4) and HIV-1-seronegative (2.7) groups. Enrolling at least 18 subjects in each group was calculated to provide 80% power to detect a 2.3-fold difference in cervical CD4:CD8 ratio between RAL- and ATV-based regimens under the null hypothesis that the group ratio equals 1.0, assuming a coefficient of variation of 1.03 from IQR-based estimated SD of 2.8,¹⁴ and $\alpha = 0.05$.

Hypothesis tests were assumed to be two-sided with a significance level of 0.05. Continuous outcomes were log transformed for normality, as appropriate. Between group demographic and clinical characteristics were compared using Fisher's exact test and two-sample *t*-tests for categorical and continuous outcomes, respectively. Continuous study outcomes were analyzed using linear regression models. For log trans-

formed outcomes, linear regression and *t*-tests report percent change and geometric means, respectively. For analyses of cervical CD4⁺ and CD8⁺ T cell counts, count and area data from multiple sections were summed to calculate cells/mm². R version 2.13.2 software (R Foundation for Statistical Computing, Vienna, Austria, www.R-project.org/) was utilized.

Results

Demographic and clinical characteristics

Fourteen women taking an RAL-based and 19 women taking an ATV-based regimen were evaluated and their clinical and demographic characteristics are shown in Table 1. There was no significant difference in mean age between the two treatment groups and the majority of women in both groups were nonblack. Women on an ATV-based regimen knew about their HIV diagnosis, on average, 5 years more and took the current drug combination 3 years more than the RAL group. There were no significant differences in current peripheral blood CD4⁺ T cell count or peripheral blood CD4:CD8 ratio [RAL, 1.00 (95% CI: 0.84, 1.18) versus ATV 1.03 (95% CI: 0.78, 1.37), *p* = 0.8] between treatment groups.

All subjects had negative gonorrhea, chlamydia, syphilis, and urine β HCG at both time points and negative HSV PCR at visit two. There was no difference between the numbers of women with positive genital tract HPV DNA between treatment groups. Eight subjects had clue cells present, but no subjects had symptoms of bacterial vaginosis. Women were asked to abstain from sex for at least 3 days prior to study visits, but a large percentage in each treatment group reported no sex for at least 6 months. Hormone therapy was uncommon; one postmenopausal woman on RAL was taking hormone replacement therapy, two women on ATV were using a levonorgestrel-releasing intrauterine system, and one woman on ATV was receiving medroxyprogesterone intramuscular injections.

Antiretroviral drug levels in the plasma and genital tract

The geometric mean plasma trough concentrations (ng/ml) for RAL were 121 (95% CI: 44, 335) and for ATV were 656

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS (MEAN, 95% CI)

	RAL (n = 14)	ATV (n = 19)	p-value
Age, years	44 (38, 50)	43 (38, 48)	0.70
Race, no. (%)			0.72
Black	4 (29%)	7 (37%)	
Nonblack	10 (71%)	12 (63%)	
Years on current antiretroviral regimen	2 (1, 4)	5 (3, 6)	0.02
Years since HIV diagnosis	8 (5, 11)	13 (9, 16)	0.04
Historic nadir CD4 ⁺ T cells/mm ³ ^a	275 (192, 357)	340 (246, 435)	0.27
CD4 ⁺ T cells/mm ³ , geometric mean	663 (495, 890)	758 (652, 882)	0.40
HPV positive, no. (%)	2 (14%)	4 (21%)	1.0
Clue cells positive, no. (%)	2 (14%)	6 (32%)	0.42
Abstinent ≥ 6 months	7 (50%)	8 (42%)	0.70

^aATV, n = 17.

RAL, raltegravir; ATV, atazanavir; HPV, human papilloma virus.

(95% CI: 445, 966). In CVL obtained concurrently, concentrations were 9.5 (95% CI: 4, 22.6) and 4.3 (95% CI: 2.4, 8), respectively. In a linear regression model of log CVL concentration versus both log plasma concentration and treatment group, the relationship between plasma and CVL drug levels was not significantly different between the two treatment groups ($p=0.41$). In the final model, CVL concentration increased in both groups by 84.2% (95% CI, 34.4, 152.3%) for every 1 log increase in plasma concentration ($p<0.001$) (Fig. 1). Furthermore, the RAL CVL concentration was 516% (95% CI, 133, 1525%) higher than the ATV CVL concentration ($p<0.001$) (Fig. 1). Similarly, the CVL to plasma drug concentration ratio was also higher for RAL at 0.078 (95% CI: 0.034, 0.178, $n=14$) compared to ATV at 0.007 (95% CI, 0.004, 0.012, $n=18$, $p=0.001$).

Cervicovaginal HIV RNA detection

HIV-RNA was measured in CVL and in 90% of subjects was undetectable at both time points ($n=30$). Two subjects had detectable CVL HIV RNA at a single time point, but it was below the level of quantitation. Specifically, CVL HIV RNA was <40 copies/ml in one RAL subject and <160 copies/ml in one ATV subject. In one RAL subject, CVL viral load could not be measured at one time point because of inhibition of the PCR assay.

Cellular immune activation

Percentages of HLA-DR⁺CD38⁺CD4⁺ and CD8⁺ T cells were determined in fresh cervical cells collected via cytobrush and in whole blood. There were no significant differ-

ences in the proportion of activated CD4⁺ T cells in the RAL compared to ATV treatment groups on cervical cells [geometric mean, 18.8% (95% CI, 12.6, 26.8) vs. 16.9% (95% CI, 12.3, 23.1), $p=0.35$] or whole blood [geometric mean, 1.7% (95% CI, 1.1, 2.4) vs. 1.5% (95% CI, 1.1, 1.9), $p=0.87$]. In addition, percentages of HLA-DR⁺CD38⁺CD8⁺ T cells did not differ by treatment group in the cervix [geometric mean, RAL 20.1% (95% CI, 13.8, 27.5) vs. ATV 22.6% (95% CI, 16.7, 30.7), $p=0.68$] or whole blood [geometric mean, RAL 4.2% (95% CI, 3.1, 5.7) vs. ATV 4.7% (95% CI, 3.7, 5.0), $p=0.10$]. After adjusting for treatment group, cervical cells had a higher proportion of HLA-DR⁺CD38⁺CD4⁺ and CD8⁺ T cells than whole blood cells ($p<0.001$). The log(CVL:plasma drug ratio) was not a significant predictor of percent cervical HLA-DR⁺CD38⁺CD4⁺ T cells ($p=0.7$) nor percent cervical HLA-DR⁺CD38⁺CD8⁺ T cells ($p=0.6$), after adjusting for treatment group and time on antiretroviral therapy. Specifically, for every one-log unit increase in the CVL:plasma drug ratio there was a 4% (95% CI: -18%, 32%) increase in percent cervical HLA-DR⁺CD38⁺CD4⁺ T cells and a 5% (95% CI: -20%, 13%) decrease in percent cervical HLA-DR⁺CD38⁺CD8⁺ T cells.

Cervical cellular immune reconstitution

Frequencies of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells were determined in cervical biopsies (Fig. 2) as measures of immune reconstitution. After controlling for time on treatment, there were no significant differences between treatment groups in absolute CD4⁺ or CD8⁺ T counts in cervical biopsies (Fig. 3A and B). The peripheral blood CD4:CD8 ratio predicted the cervical CD4:CD8 ratio. Specifically, for every one-log unit increase in peripheral blood CD4:CD8 ratio, there was a 92% (95% CI: 4%, 254%) increase in the cervical tissue ratio ($p=0.038$) (Fig. 3C).

To determine if cervical immune reconstitution was different between the two treatment groups taking into account both CD4 and CD8 T cell counts, cervical tissue CD4:CD8 ratios were compared between the two treatment groups. The cervical tissue CD4:CD8 T cell ratio (geometric mean) in the RAL group, 0.46 (95% CI: 0.33, 0.63), was not significantly different than that in the ATV group, 0.52 (95% CI: 0.36, 0.75, $p=0.6$) (Fig. 3D). After adjusting for time on treatment, the CD4:CD8 cervical T cell ratio was not significantly different in the RAL compared to the ATV group (19% lower; 95% CI: -53%, 40%, $p=0.44$), such that the fold differences that are consistent with our data are 0.47 to 1.40. The CD4:CD8 T cell ratio was significantly higher in whole blood than cervical tissue in both groups ($p<0.002$). Furthermore, after adjusting for treatment group and time on antiretroviral therapy, the log(CVL:plasma drug ratio) was not a significant predictor of the cervical CD4:CD8 ratio ($p=0.4$). Specifically, for every one-log unit increase in the CVL:plasma drug ratio there was a 9.5% (95% CI: -27.3%, 12%) decrease in the CD4:CD8⁺ T cell count.

Discussion

This study evaluated whether two antiretroviral drug regimens that differ substantially in terms of female genital tract penetration affect immune recovery in the cervix. We confirmed that the CVL-to-plasma concentration ratios of RAL were 11-fold higher than those for ATV. Nevertheless, there

FIG. 1. Linear regression model of log cervical-vaginal lavage (CVL) concentration versus both log plasma concentration and treatment group showed that plasma drug concentrations predict genital drug concentrations ($p<0.001$). Raltegravir log CVL levels (dashed line) were significantly higher than atazanavir log CVL levels (solid line) ($p<0.001$). Women taking raltegravir are indicated with closed circles and women taking atazanavir are indicated with open circles.

FIG. 2. Representative images of CD3 (red), CD4 (green), CD8 (blue), and composite immunofluorescent staining in a cervical biopsy specimen (40 $\times$).

was no significant difference in immune activation or cervical CD4:CD8 ratio between women taking RAL and ATV. Furthermore, genital tract viral loads were undetectable in the majority of study subjects, and there were no differences between treatment groups. Finally, genital tract drug levels did not correlate with either measures of immune activation or

reconstitution. These findings suggest that on the background of TDF/FTC therapy, adequate levels of both RAL and ATV are achieved in the female genital tract to suppress HIV replication and enable comparable levels of immune reconstitution.

Plasma RAL and ATV concentrations described here are within the range of prior reports.^{22,25,27} Specifically, the

FIG. 3. Cervical tissue (A) $\log_e(\text{CD4}^+ \text{ T cells/mm}^2)$ and (B) $\log_e(\text{CD8}^+ \text{ T cells/mm}^2)$ were not significantly different between the two treatment groups after controlling for time on treatment. Lines indicate means (on the $\log_e$ scale). (C) There was a significant association between the peripheral blood CD4:CD8 ratio and cervical CD4:CD8 ratio using combined results from all women. (D) The cervical tissue CD4:CD8 ratio was not significantly different between the two treatment groups. Lines indicate geometric means. Women taking raltegravir are indicated with closed circles and women taking atazanavir are indicated with open circles.

geometric mean plasma trough concentrations in this study (121 ng/ml for RAL and 656 ng/ml for ATV) were similar to, or above, troughs reported in previous studies (72 ng/ml for RAL and 636 ng/ml for ATV).^{28,29} Cervicovaginal drug concentrations reported here are lower than prior reports,^{20–22} likely because of dilution from the sample collection method (CVL vs. direct aspiration). Cervical vaginal fluid trough concentrations arising from direct aspiration range from approximately 235 to 447 ng/ml for RAL and 100 to 1,440 ng/ml for ATV.^{20–22,30} These concentrations are generally above the trough plasma concentrations recommended for patients with susceptible HIV, which are 72 ng/ml for RAL and 150 ng/ml for ATV, supporting the conclusion that adequate drug levels were achieved in the female genital tract.²⁹

Genital sampling for drug concentrations introduces more variability as compared to plasma drug levels. Specific aspects of genital sampling that introduce variability include sampling technique (lavage versus direct aspirate), volume and consistency of fluid, and sampling frequency. For example, a recent study that sampled the female genital tract using TearFlo wicks²⁰ showed higher genital ATV concentrations than previously reported,¹⁹ although the authors considered the levels within the known variability of measurement in genital samples. Importantly, we found a significant association between plasma and CVL drug concentrations, internally validating our results, and genital:plasma drug ratios were significantly higher in RAL compared to ATV, consistent with prior reports.^{17,19}

Elevated immune activation has been associated with worse outcomes in HIV-infected individuals.^{31–33} Although there was no difference in immune activation between the two treatment groups, we did observe elevated percentages of cervical HLA-DR⁺CD38⁺CD4⁺ T cells in the women described here (geometric mean, 18%, 95% CI: 14, 23%, $n = 33$) compared to a cohort of seronegative women (geometric mean, 7%, 95% CI: 5, 11%, $n = 19$, $p < 0.001$) we previously evaluated using the same methods.¹¹ There was also higher whole blood immune activation in the treated, virally suppressed HIV-seropositive women described here (geometric mean, 1.6%, 95% CI 1.4, 1.8%, $n = 33$) compared to the prior seronegative cohort¹¹ (geometric mean, 1.2%, CI 95%, 1.1, 1.4%, $n = 46$, $p = 0.02$). Thus, elevated levels of cervical immune activation were present in this cohort of HIV-infected women despite the fact that the majority of the cohort had been on suppressive treatment for 2 or more years.

We observed higher percentages of HLA-DR⁺CD38⁺CD4⁺ and CD8⁺ T cells in the cervix compared to whole blood, similar to what we and others have previously reported in both HIV-seronegative women and in HIV-infected women with detectable plasma HIV RNA.^{34–36} Elevated immune activation in the cervix may be due to ongoing antigenic stimulation. Coinfection with other sexually transmitted infections can contribute to inflammation. Nevertheless, all subjects tested negative for *Chlamydia*, gonorrhea, *Trichomonas*, and herpes simplex virus shedding and only a minority of subjects tested positive for HPV or had clue cells present. A limitation to this testing is that more sensitive nucleic acid amplification technology was not used to test for bacterial vaginosis and *Trichomonas*,³⁷ and therefore some infections may have been missed. Another potential source of antigenic stimulation could be imbalances in the vaginal microbiome, as HIV may be

associated with changes in vaginal bacterial diversity.³⁸ Regardless of the underlying cause of elevated immune activation in the cervix of HIV-seropositive women, it may inhibit immune reconstitution at this site, as has been reported in peripheral blood.³³ Future research should be directed at evaluating factors that contribute to ongoing cervicovaginal inflammation and the development of products that could reduce this inflammation and potentially enhance immune recovery.

Low-level viral replication leading to inflammation may impair CD4⁺ T cell reconstitution at mucosal sites resulting in local defects in immunity. Multiple studies have shown that HIV is detected in the female genital tract despite viral suppression in the peripheral blood in individuals on two NRTIs and either an NNRTI or a PI.^{8,9,18} In the study described here, 90% of subjects had undetectable genital concentrations of HIV RNA (<40 copies/ml). Our finding that genital tract HIV shedding is uncommon in HIV-infected women on suppressive ART is supported by a recent, more intensive study that sampled genital fluid twice weekly for 3 weeks.²⁰ The lack of HIV RNA in the genital tract of most women suggests that both regimens provide sufficient concentrations of antiretroviral drugs to suppress viral replication.

This is the first study to evaluate cervical immune reconstitution in HIV-infected women by quantifying CD4⁺ and CD8⁺ T cells within cervical biopsies. This technique may provide improved sampling of cervical CD4⁺ T cells, which are primarily located in the lamina propria.³⁹ This layer, which lies deep to the epithelium, may not be as readily sampled using cytobrush sampling. Counter to our hypothesis, we did not find a significant difference in immune reconstitution between the two treatment groups using CD4:CD8 ratios from cervical biopsies. The mean CD4:CD8 ratio was 0.46 and 0.52 in the RAL and ATV groups, respectively, and adjusting for time on treatment did not change these results. Furthermore, converting the CD4:CD8 ratio to a fold-difference in RAL vs. ATV groups (95% CI, 0.47 to 1.40) makes our hypothesized value of a 2.3-fold difference assumed in sample size calculations inconsistent with these data. There are limited data on normal ratios of CD4⁺:CD8⁺ T cells in cervical tissues from HIV-seronegative women; one study reported a ratio of 2.1 in a study using immunofluorescent staining of cervical biopsies⁴⁰ and another reported a ratio of 2.7 using flow cytometry on cervical brush specimens.¹⁴ Both of these values are substantially higher than those observed in the present study, suggesting impaired reconstitution in the HIV-seropositive women in our study. Whether remaining on ART for longer periods of time would improve immune reconstitution is not clear. A relatively low cervical tissue CD4:CD8 ratio in HIV-infected women on ART for multiple years may contribute to ongoing morbidity in the genital tract.

This study has several limitations. First, women on ATV were on the regimen for 3 years more than those on RAL, a not unexpected finding since ATV has been FDA approved for approximately 4 more years than RAL. Another limitation of the study is the small sample size, including a small subset of women on hormonal therapies, which may or may not affect cervical immune reconstitution. Nonetheless, based on the narrow confidence intervals derived from the fold difference of cervical CD4:CD8 ratios between treatment

groups, recruiting four more women on an RAL-based regimen or exclusion of the small number of women on hormonal therapies would not likely change the results of this study. Furthermore, exclusion of women on hormonal therapies would have limited recruitment of this single site study. Second, this study measured trough drug levels in plasma and vaginal secretions at only one time point, which does not represent drug concentrations during the years that a woman has taken ART. In addition, neither unbound, intracellular, or tissue drug concentrations were measured in this study, which might better reflect the concentration at the site of action. For example, a recent study showed that ~10% of RAL trough values were below the IC₉₅ in cervical tissue.²⁷ Another limitation is the dilution effect of the measurement of genital compartment fluids using CVL. Dilution could at least partially account for the lack of detection of HIV RNA in genital secretions compared to other studies.^{7,9,10} Nevertheless, this finding may be a true reflection of viral suppression in the genital tract of these study subjects due to selection of a cohort based on known viral suppression in the plasma.

In summary, no statistically significant differences in cellular immune recovery or activation in the cervix were detected between the RAL and ATV groups despite different genital drug concentrations. Furthermore, persistent cervical immune activation and incomplete cervical cellular immune reconstitution were observed compared to historical HIV-seronegative controls. Future research should focus on understanding the mechanisms that underlie these observations and developing methods for diminishing mucosal inflammation and optimizing immune reconstitution in the female genital tract. Enhancing mucosal immunity in the genital tract and reducing coinfections and malignancies remain a priority, because it is a major site of HIV-related morbidity in women on ART.⁵

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