MAJOR ARTICLE

Differences in HIV Burden and Immune Activation within the Gut of HIV-Positive Patients Receiving Suppressive Antiretroviral Therapy

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Background. The gut is a major reservoir for human immunodeficiency virus (HIV) in patients receiving antiretroviral therapy (ART). We hypothesized that distinct immune environments within the gut may support varying levels of HIV.

Methods. In 8 HIV-1-positive adults who were receiving ART and had CD4+ T cell counts of >200 cells/μL and plasma viral loads of <40 copies/mL, levels of HIV and T cell activation were measured in blood samples and endoscopic biopsy specimens from the duodenum, ileum, ascending colon, and rectum.

Results. HIV DNA and RNA levels per CD4+ T cell were higher in all 4 gut sites compared with those in the blood. HIV DNA levels increased from the duodenum to the rectum, whereas the median HIV RNA level peaked in the ileum. HIV DNA levels correlated positively with T cell activation markers in peripheral blood mononuclear cells (PBMCs) but negatively with T cell activation markers in the gut. Multiply spliced RNA was infrequently detected in gut, and ratios of unspliced RNA to DNA were lower in the colon and rectum than in PBMCs, which reflects paradoxically low HIV transcription, given the higher level of T cell activation in the gut.

Conclusions. HIV DNA and RNA are both concentrated in the gut, but the inverse relationship between HIV DNA levels and T cell activation in the gut and the paradoxically low levels of HIV expression in the large bowel suggest that different processes drive HIV persistence in the blood and gut.

Trial registration. ClinicalTrials.gov identifier: NCT00884793 (PLUS1).

The gut plays critical roles in human immunodeficiency virus (HIV) transmission, pathogenesis, and persistence for patients receiving combined antiretroviral therapy (ART). In patients who are receiving ART and whose viral load is undetectable (<40 copies/mL), HIV DNA [1–6], RNA [1, 3, 4, 7–10], and even replication-competent virus [2] have been recovered from the gut. Although most studies have reported detection of HIV DNA in the gut, RNA detection has been more variable

[1, 3, 4, 7–10], and relatively few studies have attempted to quantify the levels of HIV RNA [3, 7, 10] or DNA [3, 5, 6] in ART-suppressed patients. These studies differed in many important respects, including the stage of HIV infection, gut site and method of sampling, tissue processing, and assay for HIV.

A recent study by Chun et al [6], who used colonoscopic-guided biopsy specimens from the terminal il-

eum, showed that HIV DNA levels per 1 million CD4+ cells were, on average, 5 times higher in the gut than in the peripheral blood. It is not known whether there is a similarly disproportionate concentration of HIV RNA in the gut. To our knowledge, no study has sought to systematically measure both HIV DNA and HIV RNA levels throughout the gut of ART-suppressed patients or to compare ratios of HIV RNA to HIV DNA in the gut to those in peripheral blood mononuclear cells (PBMCs).

To address the gap in the understanding of HIV burden across the spectrum of gut tissue, we systematically measured levels of HIV DNA, HIV RNA, and T cell activation throughout the small and large bowel of chronically HIV-infected, ART-suppressed adults. We hypothesized that distinct immune environments in the gut would support varying levels of HIV persistence.

MATERIALS AND METHODS

Study participants. HIV-positive adults who met the entry criteria were recruited from 2 hospital-based HIV clinics. Inclusion criteria included the following: (1) age of 18–65 years, (2) infection with HIV type 1, (3) receiving ART for ⩾12 months, (4) no change in ART regimen for ⩾3 months, (5) CD4+ T cell count of >200 cells/μL, and (6) viral load of <40 copies/mL for >6 months prior to study entry. Exclusion criteria included factors that would increase the risk from sedation, endoscopy, or biopsy. The study was approved by the institutional review board of the University of California, San Francisco.

Study design. Blood was sampled at screening and at study entry. At study entry, after blood samples were collected, all participants underwent esophagogastroduodenoscopy and colonoscopy, with 7–10 biopsy specimens each collected from the duodenum, terminal ileum, ascending colon, and rectum. Duodenal biopsy specimens were collected with a 2.8-mm forceps (Radial Jaw 4; Boston Scientific). Colonoscopic biopsy specimens were collected with a 3.7-mm forceps (Radial Jaw 3; Boston Scientific) or a 2.8-mm forceps (Radial Jaw 4; Boston Scientific).

Processing of blood samples. Sixty-eight milliliters of blood was collected for plasma and PBMCs in acid-citrate-dextrose tubes (BD Bioscience). Plasma was obtained by centrifuging twice at 1000 g for 10 min without braking. The buffy coat from the first spin was used to isolate PBMCs by centrifugation on Ficoll according to the procedure recommended for Dynal Invitrogen Bead Separations. PBMCs were resuspended in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and 2 mmol/L ethylenediaminetetraacetic acid (buffer A), and aliquots were saved for flow cytometry, HIV DNA extraction, and HIV RNA extraction. A Dynabeads Untouched human CD4+ T cell kit (Invitrogen) was used with

6×10^7 PBMCs to isolate CD4+ by negative selection; 5×10^5 CD4+ T cells were saved for flow cytometry, whereas the remainder were frozen as cell pellets for HIV DNA and RNA extraction.

Isolation of gut cells. Six to nine biopsy specimens from each site (initially placed in Roswell Park Memorial Institute 1640 medium with L-glutamine, penicillin, streptomycin, and 15% fetal calf serum) were separated into single cells by use of a modification of a method published elsewhere [11, 12]. Briefly, biopsy specimens were subjected to 3 rounds of collagenase digestion, mechanical disruption (by passing through a blunt 16-gauge needle), clarification (by passing through a 70-μm cell strainer), and washing. The 3 aliquots of strained and washed gut cells were then combined, counted, and resuspended in buffer A. Next, 5×10^5 cells were set aside for flow cytometry, and the remainder were divided and frozen at −80°C for subsequent DNA or RNA extraction.

High-volume plasma HIV RNA. Plasma HIV RNA levels were measured using a modification of the RealTime HIV RNA assay (Abbott) that involves pelleting virions from up to 30 mL of plasma (detection limit, <0.5 copies/mL; S. Yukl et al, unpublished data, 2010). Plasma was layered into 10 mL of 6% iodixanol (OptiPrep Density Gradient Medium [Sigma] diluted 10-fold in PBS) in 50-mL polypropylene tubes (Beckton), and centrifuged at 47,810 g for 3 h at 4°C without braking. Viral pellets were resuspended in a total of 1000 μL of PBS, and the HIV RNA level was measured according to the Abbott protocol. Copy numbers were extrapolated from the cycle threshold (Ct) values of the standards and then adjusted for the concentration factor.

Extraction of cell-associated HIV DNA by real-time polymerase chain reaction (PCR). DNA was extracted using the DNA Blood Mini kit (Qiagen) and measured using a NanoDrop 1000 spectrophotometer. Three replicates of 500 ng of DNA from each sample were then used in a real-time Taqman PCR assay for HIV DNA that uses primers and a locked nucleic acid probe from the Gag region. This assay detected a 10-copy standard 19 of 20 times (detection rate, 95%) with a mean value of 9.5 ± 1.7 copies ([13]). The primers used were G-19–2-F (5′-AGCAGCYATGCAAATGTTA-3′; HXB-2 positions 1374–1392) and G-20-R (5′-AGAGAACCAAGGGGAAGTGA-3′; HXB-2 positions 1474–1493). The probe, 5′-CCATCAATGAGGA-3′ (HXB-2 positions 1400–1412; the locked bases are underlined), was dual-labeled with 6-FAM(5′) and Black quencher BHQ-1(3′). The reaction volume was 50 μL, with primer and probe concentrations of 200 nmol/L, 1× Taqman master mix, and 500 ng of sample DNA. The cycling conditions were 50°C for 2 min, 95°C for 5 min, and then 50 cycles of 95°C for 15 s and 59°C for 1 min. External standards (concentration, 10^5: 1) were prepared from DNA extracted from serial dilutions of known numbers of 8E5 cells (National Institutes of Health

AIDS Reagent Program), each of which contains 1 integrated HIV genome per cell.

HIV DNA copy numbers were extrapolated from the Ct values of the samples and expressed as the number of copies per 10^6 cells (assuming 1 μg of total DNA corresponds to 160,000 cells). To account for variation in the number of CD4+ T cells in different samples, results were also normalized by the percentage of all cells that were CD45+CD3+CD4+ (measured by flow cytometry) and expressed as the number of copies per 10^6 CD4+ T cells. To verify the DNA concentrations and assess for PCR inhibitors, samples from 4 patients were assayed using a separate real-time PCR for β-actin.

Extraction of cell-associated HIV RNA by real-time quantitative reverse-transcription PCR (qRT-PCR). RNA was extracted using the Rneasy kit (Qiagen) with on-column digestion by use of RNase-free DNase (Qiagen). To maximize the sensitivity of the qRT-PCR assays, which approaches 1 copy per reaction [14, 15], primers and probes were matched to each participant on the basis of the sequence of HIV DNA in peripheral CD4+ T cells. Unspliced HIV RNA was measured using primers from the *pol* region (HXB-2 positions 2536–2562 and 2634–2662). Multiply spliced HIV RNA encoding Tat and Rev was measured using primers from *tat* exon 1 (HXB-2 positions 5956–5979) and *tat/rev* exon 2 (HXB-2 positions 8433–8459). Total multiply spliced HIV RNA was measured using primers from *rev* exon 1 (HXB-2 positions 6012–6045) and *tat/rev* exon 2 (HXB-2 positions 8433–8459). The qRT-PCR was performed under conditions described elsewhere [16]. HIV copy numbers (the mean number of replicate measurements) were extrapolated using the formula $Ct = I + S \log_{10}$ (copy number), where the slope S was derived from an external standard curve and the intercept I was calculated for each patient using the mean Ct value from all sites at which 50% of replicates were detectable, reflecting 1 copy [14, 15, 17].

HIV RNA copy numbers were normalized to cellular input into the PCR, as determined both by total RNA concentration (measured by the NanoDrop 1000), assuming that 1 ng of RNA correspond to 1000 cells [18], and by levels of glyceraldehyde phosphate dehydrogenase RNA, as determined by a separate qRT-PCR. Results (expressed as the number of copies per 10^6 cells) from the 2 different methods of normalization correlated well. To account for variation in the number of CD4+ T cells in different samples, copy numbers were further normalized by the percentage of all cells that were CD45+CD3+CD4+.

Extraction of cell-associated HIV RNA by in situ hybridization (ISH). Gut biopsy specimens were fixed in 4% paraformaldehyde overnight and then transferred to 80% ethanol. Fixed gut biopsy specimens were assayed for cell-associated HIV RNA by ISH with sulfur 35–labeled HIV-1 antisense riboprobe (Lofstrand Labs Limited) and quantitative image analysis, as described elsewhere [19].

Immunophenotyping. CD45, CD3, CD4, CD8, CD38, and HLA-DR levels on blood and gut cells were measured using a flow cytometry method adapted from methods published elsewhere [20]. The following antibodies were used: CD45-allophycocyanin, CD3–Pacific Blue (BD Bioscience), CD4–electron-coupled dye (Beckman Coulter), CD8–Q-Dot605 (Invitrogen), CD38-phycoerythrin (BD Bioscience), and HLA-DR–fluorescein isothiocyanate (BD Bioscience). Cells were washed with PBS with 1% BSA (Wash Buffer), resuspended in Wash Buffer with 1% human γ-globulin, incubated for 15 min at 4°C, stained with 25 μL of antibody mix or controls for 30 min at 4°C, washed twice, fixed with 0.5% formaldehyde, and stored at 4°C overnight.

Data were acquired on a customized LSR II flow cytometer (BD Bioscience) and analyzed using Flowjo software (Treestar).

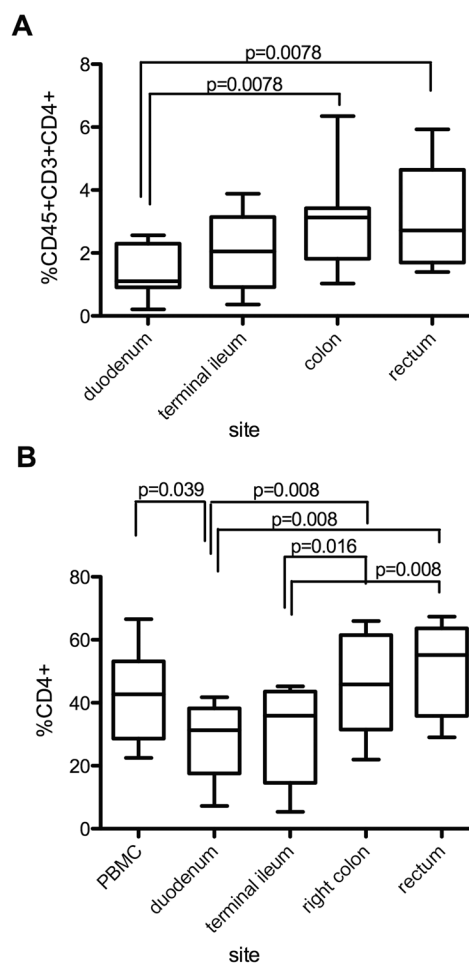**Figure 1.** CD4 contents indicated as the percentage of all cells (*A*) or the percentage of T cells (*B*) that express CD4, as measured by flow cytometry on peripheral blood mononuclear cells (PBMCs) or suspensions of total gut cells. Whiskers represent the maximum and minimum, upper and lower box borders represent the 75th and 25th percentile, respectively, and the horizontal line indicates the median.

A

B
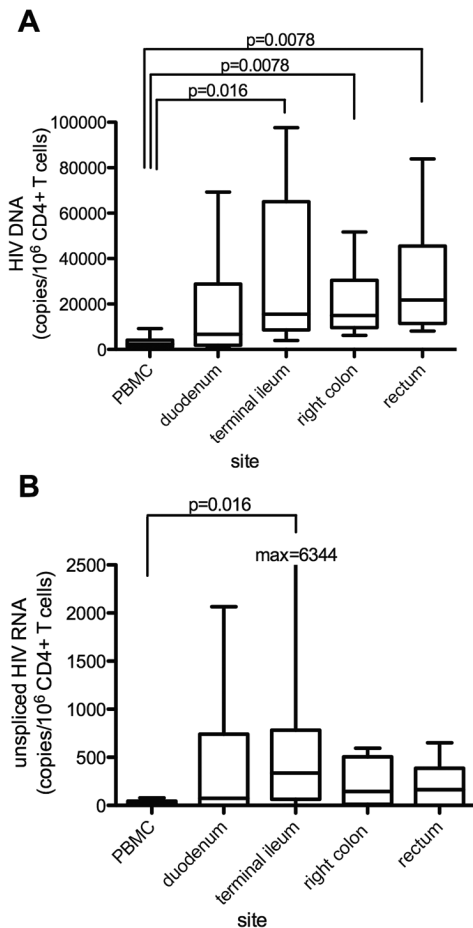
Figure 2. Human immunodeficiency virus (HIV) DNA copy numbers per 10^6 CD4+ T cells (*A*). HIV DNA copy numbers were measured in peripheral blood mononuclear cells (PBMCs) and total gut cells by use of real-time polymerase chain reaction (PCR), normalized to total cell numbers by DNA mass (using a NanoDrop 1000 spectrophotometer) and normalized to CD4+ cells by flow cytometry. Total unspliced HIV RNA levels per 10^6 CD4+ T cells (*B*) were measured using quantitative reverse-transcription PCR, normalized to cell numbers by glyceraldehyde phosphate dehydrogenase and normalized to CD4+ cells by flow cytometry. Whiskers represent the maximum and minimum, upper and lower box borders represent the 75th and 25th percentile, respectively, and the horizontal line indicates the median.

Cells were gated on a scatter plot to remove debris, then sequentially gated for CD45 (to define total leukocytes), CD3 (to define T cells), and CD4 or CD8. CD38 and HLA-DR gates were set using Fluorescent-Minus-One controls for each marker on a PBMC sample then applied to PBMCs and gut samples from the same participant.

Statistical analysis. For each outcome measure, the results from any 2 given sites were compared across all study participants by use of the paired Wilcoxon signed rank test. For each site, pairs of outcome measures were correlated using the Pearson correlation test (for data meeting the D'Agostino and Pearson

son omnibus normality test) or the Spearman correlation test (for all other data). Statistics were calculated using Prism software (version 5.0; GraphPad).

RESULTS

Study population. Of 14 patients who were screened, 13 met the study criteria and 8 consented to enter the study. The 8 participants (all men) had a median age of 51 years (range, 33–63 years), median duration of HIV infection of 15 years (range, 11–22 years), and median CD4+ T cell count nadir of 219 cells/μL (range, 49–469 cells/μL). At study entry, they had maintained viral loads of <40 copies/mL for 2.8–12 years (median, 6.7 years) and had CD4+ T cell counts of 289–1552 cells/μL (median, 478 cells/μL). ART regimens included 2 nucleoside reverse-transcriptase inhibitors (typically emtricitabine and te-
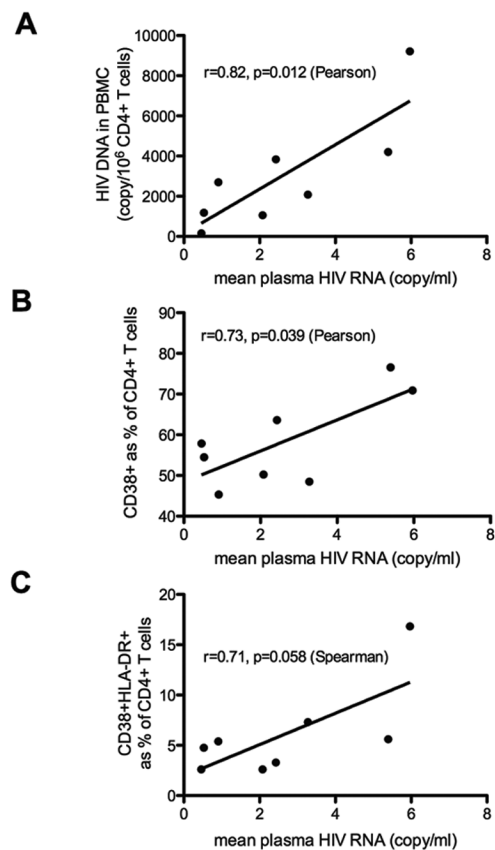

A

B

C

Figure 3. Correlation between the mean plasma human immunodeficiency virus (HIV) RNA level and the HIV DNA content in peripheral blood mononuclear cells (PBMCs) (*A*), the percentage of peripheral CD4+ T cells that are CD38+ (*B*), and the percentage of peripheral CD4+ T cells that are CD38+HLA-DR+ (*C*). Plasma RNA levels indicate the mean of 2 measurements (taken 2 weeks apart) that were made by concentrating virions from up to 30 mL of plasma by use of high-speed centrifugation on a 10% iodixanol density cushion and then assaying the resuspended pellet by use of the Abbott M2000 assay. CD38 and HLA-DR were measured by flow cytometry.

Table 1. Patients with Detectable HIV in Blood Samples or 6–9 Pooled Gut Biopsy Specimens by Site

HIV DNA or RNA	No. (%) of patients with HIV detected					
	PBMCs ($n = 8$)	Blood CD4+ T cells ($n = 8$)	Duodenum ($n = 8$)	Terminal ileum ($n = 8$)	Ascending colon ($n = 8$)	Rectum ($n = 8$)
DNA	8 (100)	8 (100)	5 (62.5)	8 (100)	7 (87.5)	8 (100)
Unspliced RNA	8 (100)	8 (100)	5 (62.5)	7 (87.5)	5 (83.3)[a]	4 (66.7)[a]
Multiply spliced RNA encoding Tat and Rev	2 (25)	5 (62.5)	0 (0)	0 (0)	0 (0)[a]	0 (0)[a]
Total multiply spliced RNA	4 (50)	7 (87.5)	0 (0)	0 (0)	1 (16.7)[a]	1 (16.7)[a]

NOTE. HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell.

[a] Only 6 patients had specimens from the colon and rectum with sufficient cells for RNA detection.

nofovir); plus either efavirenz (3 participants); atazanavir with or without ritonavir (3 participants); nevirapine and lopinavir with ritonavir (1 participant); or nevirapine, atazanavir with ritonavir, and maraviroc (1 participant).

Plasma HIV RNA levels and CD4+ T cell counts. Plasma HIV RNA levels were undetectable in all study participants as measured with the standard Abbott assay, but viral loads were detectable in all participants with our modified high-volume assay. The mean plasma viral loads were 0.5–6 copies/mL (median, 2.3 copies/mL).

CD4 content was measured by flow cytometry in PBMCs and cell suspensions from the 4 gut sites. The percentage of all cells that were CD4+ T cells was lower in all 4 gut sites (range, 0.2%–6.4%) (Figure 1*A*) compared with that in PBMCs (range, 14.3%–44.4%; data not shown). As a percentage of all cells and of T cells, CD4 content increased from the small bowel to the large bowel (Figures 1*A* and 1*B*).

HIV DNA. HIV DNA levels were measured in PBMCs, peripheral CD4+ T cells, and cell suspensions from the 4 gut sites. To account for significant differences between the PBMCs and gut sites in the percentage of all cells that were CD4+ T cells, the HIV DNA levels were normalized to CD4+ T cell content, as measured by flow cytometry. HIV DNA levels per 10^6 CD4+ T cells (Figure 2*A*) were higher in all 4 gut sites compared with those in blood ($P = .016$ for the ileum, $P = .008$ for the colon, and $P = .008$ for the rectum; the P value for the duodenum was not significant). The median HIV DNA level per 10^6 CD4+ T cells in the duodenum was 2.8 times higher than that in the blood; the fold difference was 6.5 for the ileum, 6.3 for the colon, and 9.1 for the rectum. HIV DNA levels per 10^6 CD4+ T cells in the blood correlated positively with the mean plasma HIV RNA level ($r = 0.82$ [Pearson test]; $P = .012$) (Figure 3*A*).

Cell-associated HIV RNA. HIV RNA levels were undetectable by ISH in any of the gut sites at 14 d of film exposure. In contrast, unspliced HIV RNA levels were detectable by qRT-PCR in samples from PBMCs, peripheral CD4+ T cells, and total cells isolated from the 4 gut sites. For participants A185 and A186, the yield of cells from the colon and rectum was

insufficient for testing for HIV RNA levels. Unspliced HIV RNA was usually detectable in the gut, whereas multiply spliced HIV RNA was rarely detectable (Table 1).

When normalized for CD4+ T cell content (Figure 2*B*), the mean and median unspliced HIV RNA levels were highest in the ileum and were greater in all 4 gut sites compared with levels in the blood (median fold difference for the duodenum, ileum, colon, and rectum, 1.8, 10.2, 1.6, and 2.4, respectively; mean fold difference for the duodenum, ileum, colon, and rectum, 5.7, 12.5, 4.6, and 3.8, respectively), although results were significant only for comparison of levels in the ileum to those in the blood ($P = .016$).
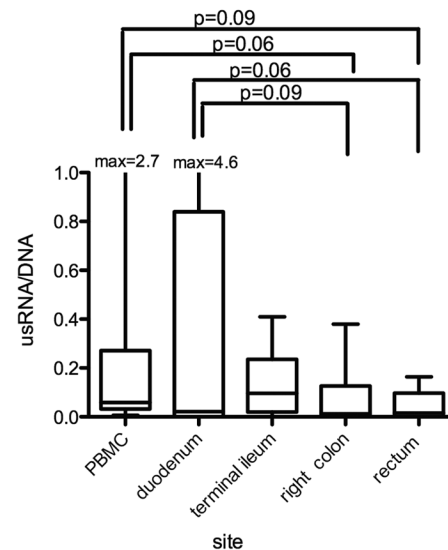

Figure 4. Ratio of unspliced human immunodeficiency virus (HIV) RNA to HIV DNA (UsRNA/DNA). To approximate the transcriptional activity per infected cell, we calculated the ratio of the level of unspliced HIV RNA (expressed as the number of copies per 10^6 cells) to that of HIV DNA (expressed as the number of copies per 10^6 cells; both measures were normalized by nucleic acid mass) in peripheral blood mononuclear cells (PBMCs) and cells from the 4 gut sites. Whiskers represent the maximum and minimum, upper and lower box borders represent the 75th and 25th percentile, respectively, and the horizontal line indicates the median.

A

B

Figure 5. T cell activation indicated by the percentage of CD4+ T cells (*A*) or CD8+ T cells (*B*) that express both CD38 and HLA-DR, as measured by flow cytometry. Whiskers represent the maximum and minimum, upper and lower box borders represent the 75th and 25th percentile, respectively, and the horizontal line indicates the median. PBMC, peripheral blood mononuclear cell.
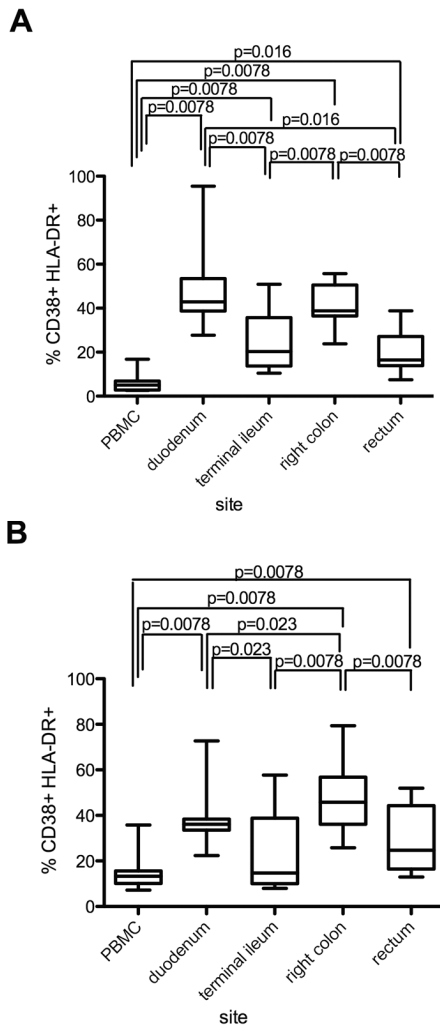
To approximate the transcriptional activity per HIV-infected cell, we divided the unspliced HIV RNA level per 10^6 cells (normalized by RNA mass) by the HIV DNA level per 10^6 cells (normalized by DNA mass) to determine the ratio of unspliced RNA to DNA. Within the gut, the median ratio of HIV RNA to HIV DNA was highest in the terminal ileum (Figure 4). There was a trend of a higher ratio of RNA to DNA in PBMCs compared with that in either site of the large bowel ($P = .062$ for comparison of PBMCs with ascending colon; $P = .094$ for comparison of PBMCs with rectum) and a trend of a higher ratio of RNA to DNA in the duodenum compared with that in either site of large bowel ($P = .094$ for comparison of duodenum to ascending colon; $P = .062$ for comparison of duodenum to rectum).

T cell activation and correlations. Although immune activation markers have not been clearly defined for the gut, we measured CD38 and HLA-DR because these markers have been used to distinguish activated T cells in the peripheral blood and have been extensively correlated with poor prognosis [21–32]. T cell activation levels were higher in all 4 gut sites compared with those in the blood (Figure 5).

The mean plasma HIV RNA level correlated positively with the percentage of peripheral CD4+ T cells that were CD38+ ($r = 0.73$ [Pearson test]; $P = .039$) (Figure 3*B*) and tended to correlate with the percentage that were CD38+HLA-DR+ ($r = 0.71$ [Spearman test]; $P = .058$) (Figure 3*C*). For the PBMCs, comparison of HIV DNA levels (per total number of cells or number of CD4+ T cells) to any measure of immune activation (CD38+HLA-DR+, total CD38+, or total HLA-DR+ on CD4+ or CD8+ T cells) yielded a correlation coefficient (*r*) that was invariably positive (Table 2), although *P* values were not always significant. In contrast, for all gut sites, comparison of either measure of HIV DNA level to any measure of immune activation yielded an *r* value that was always negative or neutral (Table 2).

DISCUSSION

In HIV-infected adults with a history of sustained viral suppression who are receiving ART, we measured levels of HIV RNA, levels of HIV DNA, ratios of HIV RNA to HIV DNA, CD4+ T cell counts, and levels of T cell activation in blood and 4 different regions of gut. HIV DNA levels per CD4+ T cell were on average 5–10-fold higher in the gut compared with those in the peripheral blood, in agreement with the findings of Chun et al [6]. If the gut CD4+ T cells have on average 5 times more HIV DNA than CD4+ T cells in the blood (and possibly the rest of the body), and if the gut contains 50%–80% of total body CD4+ T cells, then the gut harbors 83%–95% or more of all infected cells in the body. On the basis of the average (across sites and participants) HIV DNA level of 20,000 DNA copies per 10^6 CD4+ T cells, an estimate of 1.2×10^{11} total body CD4+ T cells, and an estimate of 50% of total body CD4+ T cells residing in the gut, the gut contains 1.2×10^9 infected CD4+ T cells after a median of 7 years of suppressive ART. If 1 in 100 infected cells carries replication-competent HIV, then the gut alone contains 1.2×10^7 latently infected HIV genomes. This estimate exceeds by an order of magnitude earlier estimates of the latent reservoir size in PBMCs and central lymphoid tissues [33, 34].

It is unclear why the gut harbors such a disproportionate concentration of infected CD4+ T cells. Possible explanations include greater rates of initial infection (especially in the rectum, which may be a site of transmission), differences in the content of memory CD4+ T cell subpopulations (such as transitional memory cells) that may harbor more HIV DNA [35],

Table 2. Correlation between HIV DNA Level and Markers of T Cell Activation by Site

Correlation of HIV DNA level	Correlation coefficient				
	PBMCs	Duodenum	Ileum	Colon	Rectum
HIV DNA level per 10^6 total cells					
With percentage of CD4$^+$ T cells					
That are CD38$^+$HLA-DR$^+$	0.36	−0.63	−0.42	−0.39	−0.50
That are CD38$^+$	0.77[a]	0.06	−0.19	−0.29	−0.17
That are HLA-DR$^+$	0.43	−0.63	−0.43	−0.34	−0.26
With percentage of CD8$^+$ T cells					
That are CD38$^+$HLA-DR$^+$	0.26	−0.42	−0.47	−0.47	−0.60
That are CD38$^+$	0.53	−0.36	−0.50	−0.75[b]	−0.52
That are HLA-DR$^+$	0.50	−0.32	−0.45	−0.45	−0.31
HIV DNA level per 10^6 CD4$^+$ T cells					
With percentage of CD4$^+$ T cells					
That are CD38$^+$HLA-DR$^+$	0.74[c]	−0.43	−0.29	−0.31	−0.83[d]
That are CD38$^+$	0.52	−0.05	0.06	−0.23	−0.55
That are HLA-DR$^+$	0.60	−0.43	−0.21	−0.27	−0.63
With percentage of CD8$^+$ T cells					
That are CD38$^+$HLA-DR$^+$	0.36	−0.50	0.00	−0.51	−0.47
That are CD38$^+$	0.29	−0.19	0.00	−0.46	−0.14
That are HLA-DR$^+$	0.55	−0.38	0.02	−0.48	−0.41

NOTE. Correlations were not statistically significant, unless otherwise indicated. HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell.

[a] $P = .024$.
[b] $P = .032$.
[c] $P = .046$.
[d] $P = .015$.

higher rates of replication of integrated HIV DNA by cell division, increased establishment of latent infection, reduced reactivation from latency, slower clearance, trafficking from the blood, or ongoing replication (the latter was suggested by Chun et al [6]).

Although ISH results for HIV RNA levels were negative in all gut sites, unspliced HIV RNA levels were detectable in the majority of gut samples by use of qRT-PCR. ISH can detect as few as 2–5 genomic HIV equivalents per cell but has a detection limit of 10^4 copies per gram of tissue for dispersed HIV RNA, whereas PCR provides very sensitive detection of HIV RNA pooled from many cells but cannot discriminate RNA produced from few or many cells. As measured by qRT-PCR, unspliced HIV RNA levels per CD4$^+$ T cell were higher in all 4 gut sites compared with those in the blood. It is unclear whether this RNA represents reactivation of latently infected cells, stable chronically infected cells, or cells newly infected as a result of ongoing replication. On the basis of the absence of HIV RNA–positive cells detected by ISH, we suspect that productively infected cells in the gut, if present, must be very infrequent or exhibit very attenuated production, and that the HIV RNA detected by qRT-PCR represents modest viral transcription distributed across many HIV DNA–positive cells. This assumption is in agreement with the observation that multiply spliced HIV RNA, which may be found in latently infected cells but is ex-

pressed at high level in productive infection [14], was rarely detected in the gut.

The ratio of HIV RNA to HIV DNA peaked in the ileum, where the median ratio tended to be higher than that of the PBMCs, which suggests that this site may have a greater ratio of productive infection to latent infection and should be sampled in studies aimed at detecting ongoing replication. In contrast, in the large bowel, the median ratio of HIV RNA to HIV DNA tended to be lower than in the PBMCs, which suggests that more of these cells behave as if they are latently infected. However, since most gut lymphocytes display markers of T cell activation, latent infection of these cells may differ from the classic latent infection in the blood, which was originally described in resting CD4$^+$ T cells. Differences between gut sites could reflect differences in T cell activation, memory CD4$^+$ T cell subsets, or the proportion of lymphocytes from lymphoid aggregates.

The infrequent detection of multiply spliced HIV RNA and the trend of a lower ratio of HIV RNA to HIV DNA in most gut sites suggest lower levels of HIV transcription. Given the high degree of T cell activation in the gut, it is very surprising that gut cells have such low levels of HIV transcription, which suggests that they are hyporesponsive to activating stimuli or that T cell activation has different consequences (or activation markers have different meanings) in the gut compared with

the blood. Gut lymphocytes may have a reduced ability to respond to antigens and may resemble more immunotolerant or anergic T cells. Previous studies have shown that CD4$^+$ T cell tolerance and anergy can be caused by epigenetic modification [36–38]. Given that epigenetic modification of the long terminal repeat has also been implicated as a feature of latent infection with HIV [39–46], it is tempting to hypothesize that the unique environment of the gut favors both induction of CD4$^+$ T cell tolerance and HIV latency through epigenetic modification. It is not clear whether these cells would respond to therapies that may reduce latently infected cells in the blood.

Whereas HIV DNA levels in PBMCs tended to correlate positively with markers of T cell activation, in the gut, we found a surprising trend of a negative correlation between HIV DNA levels and T cell activation. Immune activation could have divergent effects on HIV infection. Systemic immune activation may increase the susceptibility of CD4$^+$ T cells to infection, cause replication of proviral DNA by cell division, or serve as a marker for the spread of infection, thus explaining the positive correlation between activation and HIV DNA levels seen in the blood. On the other hand, activation of HIV-specific T cells can lead to death of virally infected cells, and HIV-nonspecific activation can reduce the number of susceptible target cells (by apoptosis) or lead to reactivation and clearance of latently infected cells, thus explaining the negative correlation seen in the gut. If verified, the opposing directions for the correlations seen in the gut and the peripheral blood further suggest that activation may have different consequences for HIV persistence in these 2 sites.

Potential limitations of the study should be noted. First, the number of participants was relatively small, thus limiting generalizability and the power to detect small differences. Second, even though multiple biopsy specimens were collected, there remains the possibility of insufficient sampling. Previous studies have shown that HIV DNA and RNA can be reproducibly quantified from a single endoscopic biopsy specimen [5, 47], and we pooled 6–9 biopsy specimens from each site. Although in situ studies confirmed the presence of lymphoid aggregates (in 50% of ileal biopsy specimens), additional sampling error may have been introduced by the tissue digestion, which resulted in some cell loss and death (arylamine reactive dye staining showed that 75%–80% of gut cells were viable). Third, the normalization per CD4$^+$ cell assumes that all of the HIV is in CD4$^+$ T cells. Finally, the PCR detection methods, while sensitive, do not overcome confounding effects of sampling that occur when target nucleic acids are present at low copy numbers, so that Poissonian effects have a greater influence on results.

Nevertheless, the findings here confirm and extend the important role played by the gut as a reservoir for HIV in patients receiving suppressive ART. Additional studies are needed to better define and distinguish the modes of viral persistence in the blood and different regions of gut and to investigate whether site-specific differences result in different responses to therapies designed to reactivate HIV from latently infected cells.

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