MAJOR ARTICLE

The Distribution of HIV DNA and RNA in Cell Subsets Differs in Gut and Blood of HIV-Positive Patients on ART: Implications for Viral Persistence

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(See the editorial commentary by Henrich and Gandhi on pages 1189–93 and the major article by Jain et al 1202–11.)

Even with optimal antiretroviral therapy, human immunodeficiency virus (HIV) persists in plasma, blood cells, and tissues. To develop new therapies, it is essential to know what cell types harbor residual HIV. We measured levels of HIV DNA, RNA, and RNA/DNA ratios in sorted subsets of CD4$^+$ T cells (CCR7$^+$, transitional memory, and effector memory) and non-CD4$^+$ T leukocytes from blood, ileum, and rectum of 8 ART-suppressed HIV-positive subjects. Levels of HIV DNA/million cells in CCR7$^+$ and effector memory cells were higher in the ileum than blood. When normalized by cell frequencies, most HIV DNA and RNA in the blood were found in CCR7$^+$ cells, whereas in both gut sites, most HIV DNA and RNA were found in effector memory cells. HIV DNA and RNA were observed in non-CD4$^+$ T leukocytes at low levels, particularly in gut tissues. Compared to the blood, the ileum had higher levels of HIV DNA and RNA in both CD4$^+$ T cells and non-CD4$^+$ T leukocytes, whereas the rectum had higher HIV DNA levels in both cell types but lower RNA levels in CD4$^+$ T cells. Future studies should determine whether different mechanisms allow HIV to persist in these distinct reservoirs, and the degree to which different therapies can affect each reservoir.

Keywords. HIV; HIV-1; ART; persistence; reservoir; CD4$^+$ T cell; gut; intestine; ileum; rectum.

Most patients infected with human immunodeficiency virus (HIV) achieving virologic suppression with antiretroviral therapy (ART) have low levels of HIV RNA in the plasma [1–4] and cell-associated HIV DNA and RNA in the blood [5–10], lymphoid tissues [5, 11–14], and gut [15–25]. The main obstacle to eradication of HIV is thought to be a population of latently infected resting memory CD4$^+$ T cells [6, 7, 13, 26], though HIV has been observed in many other cell types [27] and on follicular dendritic cells [12, 13, 28]. Within CD4$^+$ T cells, HIV DNA may be disproportionately found in certain subpopulations, including resting memory CD4$^+$ T cells [29], activated CD4$^+$ T cells [21], CCR6$^+$ CD4$^+$ T cells [30, 31], resting CD4$^+$ T_{Reg} cells [32], and central or transitional memory CD4$^+$ T cells [33].

In peripheral CD4$^+$ T cells from ART-suppressed patients, central memory (T_{CM}) and transitional memory (T_{TM}) CD4$^+$ T cells contribute the most to the total pool of HIV DNA in patients with high and low CD4 counts, respectively [33], whereas effector memory (T_{EM}) cells contribute less [33]. However, little is known about the distribution of HIV DNA within CD4$^+$ T memory subsets of the lymphoid tissues and gut, which likely represent the main anatomic reservoirs

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for HIV. Prior studies in ART-suppressed patients have shown that HIV DNA levels per $CD4^+$ T cell are higher in the gut than the blood [21, 25]. Given the expected predominance of T_{EM} cells in the gut and the observation that T_{EM} cells in blood tend to have less HIV DNA [33], it seems paradoxical that HIV DNA would be concentrated in the gut relative to blood. We hypothesized that $CD4^+$ T memory subsets in the gut are infected at different frequencies than the blood, or that HIV may exist in cells other than $CD4^+$ T lymphocytes.

Another unanswered question is whether certain cell populations in either blood or gut, including T_{CM} and T_{TM}, are transcribing more HIV RNA. Most studies of HIV reservoirs have focused on HIV DNA, but the majority of proviral DNA is not transcribed, as revealed by RNA:DNA ratios <0.5 [25, 34]. HIV RNA-positive cells have been found in both blood and gut of ART-suppressed patients [9, 10, 15, 17, 20, 23, 25], but it is unclear whether these cells produce virus, and whether they reflect reactivation from latency, recent infection, or long-lived cells that chronically or intermittently transcribe HIV. These cells are of interest because transcription is a prerequisite for expression of HIV proteins and virions, which could be a large factor driving the persistent immune activation in patients on suppressive ART.

Given these unanswered questions, we sought to measure the levels of HIV DNA, HIV RNA, and RNA/DNA ratio (average transcription per infected cell) in subsets of $CD4^+$ T cells and other leukocytes from blood, terminal ileum, and rectum. We hypothesized that cell frequencies, infection frequencies, and HIV transcription levels may vary by cell type and anatomic location.

METHODS

Subjects and Samples

Eight HIV-positive adults on ART were recruited from the San Francisco Veterans Affairs Medical Center (SFVAMC). Inclusion criteria included $CD4^+$ T-cell counts ≥350 cells/μL and viral load <50 copy/mL for ≥6 months. The study was approved by the local Institutional Review Board of the University of California, San Francisco, and the SFVAMC. All participants gave informed consent. At entry, subjects underwent phlebotomy followed by colonoscopy with 15 biopsies from the terminal ileum (7 subjects) and 15 biopsies from the rectum (7 subjects). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll [25]. Biopsies from each site were dissociated to a single cell suspension by collagenase digestion and mechanical disruption as described elsewhere [25].

Cell Sorting

Small aliquots of PBMCs, ileal cells, and rectal cells were set aside for phenotyping, and the remaining cells were sorted on a BD FACS ARIA II (BD Bioscience). Cells were stained as described elsewhere [25] with LIVE/DEAD violet stain (Invitrogen) and the fluorescently conjugated monoclonal antibodies CD45-Pacific Blue, CD3-Alexa 700, CCR7-APC, CD45RO-FITC (all BD Bioscience), CD4-ECD (Beckman Coulter), CD8-QDot605 (Invitrogen), and CD27-Alexa750 (ebioscience) to differentiate $CD4^+$ T-cell maturation subsets (Supplementary Table 1). Cells from PBMCs, ileum, and rectum were sequentially gated to define singlets, $CD45^+$ leukocytes, and live cells. $CD4^+$ T cells were then defined by coexpression of CD3 and CD4 and lack of CD8. The following populations were sorted: (1) leukocytes other than $CD4^+$ T cells (non-$CD4^+$ T leukocytes; includes $CD3^+CD4^-$,$CD3^-CD4^-$,$CD3\text{-}CD4^+$); (2) $CCR7^+$ "lymphoid homing" $CD4^+$ T cells (T_{R7+}); (3) $CCR7^-CD45RO^+CD27^{High}$ transitional memory $CD4^+$ T cells (T_{TM}); and (4) $CCR7^-CD45RO^+CD27^{Low}$ effector memory $CD4^+$ T cells (T_{EM}). Table 1 shows the gating criteria and the expected cell types in each population. The $CCR7^+$ population includes both naive and T_{CM} $CD4^+$ T cells, which were grouped together because of the extremely low frequency of $CCR7^+$ cells in the gut. The 4 sorted

Table 1. Sorting Scheme

Sorted population	Criteria	Includes
Leukocytes other than $CD4^+$ T cells (non-$CD4^+$ T leukocytes)	$CD45^+$ but excluding $CD3^+$ $CD4^+$ (ie, $CD45^+CD3^-CD4^-$, $CD45^+CD3^-CD4^+$, $CD45^+CD3^+CD4^-$)	$CD8^+$ T cells $CD4^-CD8^-$ T cells B cells Granulocytes Monocytes Macrophages Dendritic cells NK cells Hematopoietic stem cells
$CCR7^+$ "lymphoid homing" $CD4^+$ T cells (T_{R7+})	$CD45^+CD3^+CD4^+CD8^-CCR7^+$	Naive $CD4^+$ T cells Central memory $CD4^+$ T cells "Other memory" $CD4^+$ T cells
Transitional Memory $CD4^+$ T cells (T_{TM})	$CD45^+CD3^+CD4^+CD8^-CD45RO^+CCR7^-CD27^{High}$	Transitional memory $CD4^+$ T cells
Effector memory $CD4^+$ T cells (T_{EM})	$CD45^+CD3^+CD4^+CD8^-CD45RO^+CCR7^-CD27^{Low}$	Effector memory $CD4^+$ T cells

populations from each site were then pelleted and flash frozen for subsequent nucleic acid extraction.

HIV DNA

Total DNA and RNA were extracted from each cell population using Trireagent (Molecular Research Center), with DNA isolated using the alternative protocol with back extraction buffer. The RNA was treated with DNase and further purified on Qiagen RNEasy Mini or Micro Columns. Total DNA (up to 40% of the elution or a maximum of 100 000 cell equivalents) was tested for HIV DNA in duplicate using a modification of a published real-time polymerase chain reaction (PCR) assay that uses primers and a probe from the LTR region and can detect a single copy of HIV in brain tissue [35]. Primers were F522-43 (5′GCCTCAATAAAGCTTGCCTTGA3′; HXB2 522-543) and R626-43 (5′GGGCGCCACTGCTAGAGA3′; 626-643). The probe, 5′CCAGAGTCACACAACAGACGGGCACA3′, was dual-labeled with 6-FAM(5′) and Black Hole Quencher. Reaction volume was 50 µL with 25 µL of 2× Gene Expression Master Mix (Applied Biosystems), 10 pmol of each primer and probe. Cycling conditions were 50C for 2 minutes, 95C for 10 minutes, then cycles of 95C for 15 seconds and 59C for 1 minute. External standards were prepared from DNA extracted from known numbers of 8E5 cells (NIH AIDS Reagent Program), each of which contains one integrated HIV genome per cell.

HIV RNA

RNA (up to 36% or 100 000 cell equivalents) was assayed for total processive HIV RNA transcripts in duplicate using primers and probe from the LTR region (as above). Reaction volume was 50 µL with 25 µL of 2× One Step RNA-to-Ct mix (Applied Biosystems), 10 pmol of each primer and probe, and 1.25 µL of 40× RT (Applied Biosystems). Cycling conditions were 48C for 20 minutes, 95C for 5 minutes, then 60 cycles of 95C for 15 seconds and 59C for 1 minute. External standards of genomic HIV RNA were prepared by extracting the RNA from lab stocks of NL4-3 virions and then quantifying the RNA via replicate measurements using the Abbot Real Time assay.

Normalization of HIV Copies to Cell Numbers

HIV levels were normalized to cell equivalents using 2 methods: first, using the cell counts from the sorts and the known elution and PCR input volumes; and second, by assaying the extracted RNA and DNA for the housekeeping genes Glyceraldehyde Phosphate Dehydrogenase (GAPDH) and Telomerase Reverse Transcriptase (TERT), respectively. In total, 2 µL of sample nucleic acid was tested in duplicate for GAPDH or TERT in a reaction with 2.5 µL of 20× primer/probe mix (Applied Biosystems) using the PCR conditions described above and external standards of RNA or DNA extracted from known numbers of peripheral $CD4^+$ T cells. Results were expressed as HIV copies/10^6 cells of each sorted cell type. To calculate an HIV burden that accounts for the frequency of each cell type as well as the HIV levels within each cell type, for each site, we calculate the fractional contribution of each cell type to the total measured HIV DNA or RNA in 10^6 leukocytes.

Statistics

Cell frequencies and HIV levels in different cell populations and anatomic sites were compared across patients using the Wilcoxon signed rank test with 2-tailed *P* values as calculated on GraphPad Prism 5.0.

RESULTS

Clinical Characteristics

The 8 study subjects (Table 2) were all men, with median age of 57. Other median data include HIV duration 22 years, ART

Table 2. Clinical Characteristics

Subject	Age	Sex	Years HIV	CD4 Nadir	Time on ART, yr	Last suppression, yr	CD4/CD4%	Viral load	ART regimen
A648	64	M	26	0	12	3.6	492/27%	<20 detected	3TC/AZT/TDF/r/LPV
A649	50	M	12	218	12	4.7	762/35.5%	<20 ND	3TC/TDF/EFV
A650	56	M	22	155	16	5.0	548/28%	<20 detected	3TC/ABC/r/LPV
A652	58	M	26	1	14	4.5	699/31.5%	<20 ND	FTC/TDF/EFV
A653	51	M	23	230	14	2.0	1032/29%	41	FTC/TDF/r/ATV
A654	57	M	14	430	13	4.0	636/39%	<20 detected	FTC/TDF/r/ATV
A727	44	M	7–16	303	1.33	1.1	547/29%	<20 ND	FTC/TDF/EFV
A728	57	M	22	264	3	2.9	434/30%	<20 ND	FTC/TDF/EFV
Median	56.5		22	224	12.5	3.8	592/29.5%	<20	

Abbreviations: ABC, abacavir; ART, antiretroviral therapy; ATV, atazanavir; AZT, azidothymidine; EFV, efavirenz; FTC, emtricitabine; HIV, human immunodeficiency virus; LPV, lopinavir; M, male; ND, not detected; r, ritonavir (boosting); TDF, tenofovir; 3TC, lamivudine.

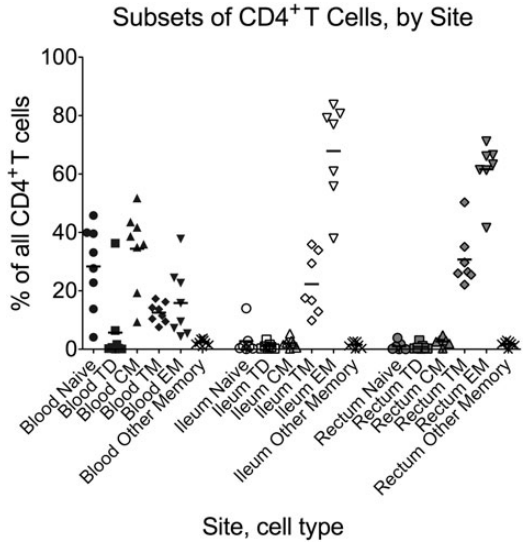


Figure 1. Flow cytometry was used to determine the proportion of all $CD4^+$ T cells from each site (blood, ileum, rectum) that were naive, terminally differentiated effector (Td), central memory (CM), transitional memory (TM), effector memory (EM), or "other memory" cells based on expression of CD3, CD4, CD45RO, CCR7, and CD27 (Table 1). Circles represent naive T cells; squares, terminally differentiated T cells; triangles, central memory T cells; diamonds, transitional memory T cells; inverted triangles, effector memory T cells; X's, all other memory T cells. Black, blood; white, terminal ileum; grey, rectum. Bars represent the means.

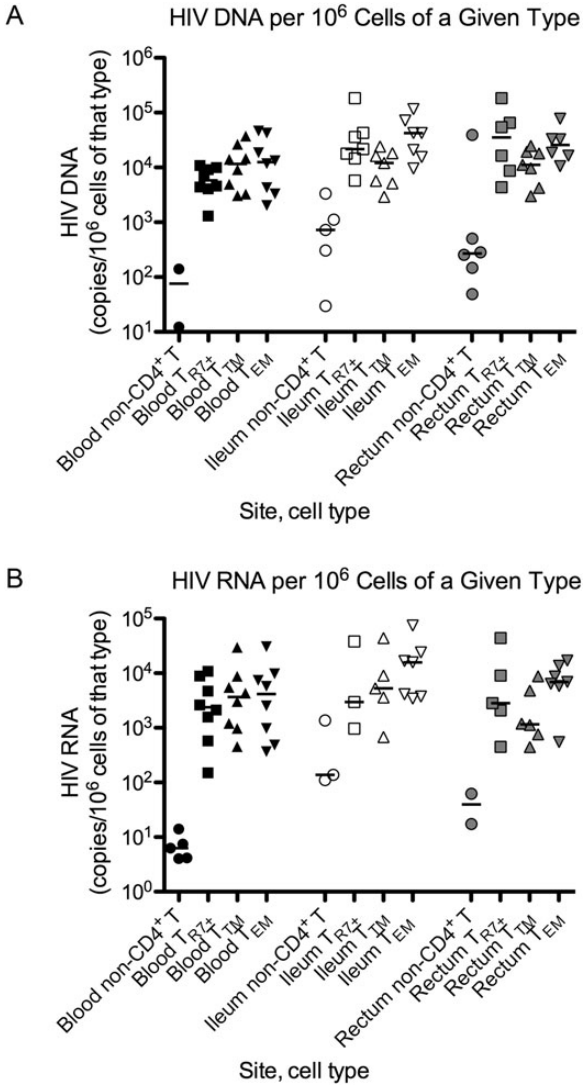


Figure 2. *A* and *B*, HIV DNA (*A*) and HIV RNA (*B*) in sorted cells. Cells from blood (*black*), ileum (*white*), and rectum (*gray*) were sorted into leukocytes other than $CD4^+$ T cells (non-$CD4^+$ T; *circles*) and 3 populations of $CD4^+$ T cells: (1) $CCR7_+$ (T_{R7+} ; *squares*), which includes naive, central memory, and "other memory"; (2) transitional memory (T_{TM}; *triangles*); and (3) effector memory (T_{EM} ; *inverted triangles*). HIV levels are expressed as copies/10^6 cells and graphed on a log scale. Only samples with detectable HIV levels are shown. Bars represent the medians.

duration 12.5 years, CD4 nadir 224, CD4 count 592, and CD4% of 29.5%.

$CD4^+$ T Cell Maturation Subset Frequencies

In the blood of HIV-positive subjects, the largest populations of $CD4^+$ T cells (Figure 1) were T_{CM} ($P = .016$, $P = .023$ for comparison to T_{TM}, terminally differentiated [T_{TD}]) and naive ($P = .023$ for comparison to T_{TM}). In contrast, in both gut sites, most $CD4^+$ T cells were T_{EM} ($P = .016$ for comparison to all other populations, except $P = .031$ for rectum T_{EM} vs T_{TM}) and T_{TM} ($P = .016$ for comparison to naive, T_{CM}, T_{TD}). The percentage of T_{CM} and naive cells was greater in blood compared to either gut site, whereas the percentage of T_{EM} cells was greater in both gut sites compared to blood, and the percentage of T_{TM} cells was higher in rectum than blood ($P = .016$ for all comparisons).

Normalization Using Cell Counts and Housekeeping Genes

There was a strong linear correlation between the HIV levels as measured by cell counts and by housekeeping genes (Supplementary Figure 1*A* and 1*B*), with a slope of close to unity.

Cell-Associated HIV DNA and RNA in Each Cell Type

Despite low yields of sorted T_{R7+} and T_{TM} $CD4^+$ T cells from the gut, HIV DNA was detectable in all T_{EM}, T_{TM}, and T_{R7+} samples except for one rectal T_{R7+} sample with the lowest cell count (Supplementary Table 2). In contrast, despite generally high cell counts and TERT levels from non-$CD4^+$ T leukocytes, HIV DNA was detected in the peripheral non-$CD4^+$ T leukocytes in only 2 of 8 subjects, compared to 5 of 7 for ileal non-$CD4^+$ T leukocytes and 6 of 7 for rectal non-$CD4^+$ T leukocytes.

In the blood, T_{TM} $CD4^+$ T cells had more HIV DNA/million cells than T_{R7+} cells ($P = .023$; Figure 2*A*). In the ileum, HIV DNA levels were higher in T_{EM} cells than in T_{TM} cells ($P = .016$). HIV DNA levels in ileal T_{R7+} and T_{EM} cells were higher than in the corresponding cell populations in the blood

($P = .016$, $P = .016$) and tended to be higher in rectal T_{R7+} cells compared to T_{R7+} cells in the blood ($P = .063$).

HIV RNA was detected in non-$CD4^+$ T leukocytes from the blood of 5 of 8 subjects, compared to 3 of 7 for the ileum and 2 of 7 for the rectum. In $CD4^+$ T cells, HIV RNA was always detected in blood and in gut T_{EM} cells but was sometimes not detected in gut T_{R7+} or T_{TM} samples with lower cell counts. Differences in levels of HIV RNA/million cells tended to mirror those found for HIV DNA but did not reach statistical significance (Figure 2*B*).

Contribution to the Total Pool of HIV DNA and RNA in Leukocytes (WBC)

Using the HIV levels within each cell type and the frequency of each cell type (percent of live singlet $CD45^+$ cells), we calculated the HIV content in each cell type in 10^6 white blood cells (WBC; Figure 3). Samples with undetectable HIV levels were assigned a value equal to the detection limit of the assay (assigning them a value of zero did not change the conclusions). The HIV levels in T_{R7+}, T_{TM}, and T_{EM} $CD4^+$ T cells were added to yield an estimate of the total HIV level in $CD4^+$ T cells, which was added to the level in non-$CD4^+$ T leukocytes to give an estimate of the total HIV level in WBC.

In both blood and ileum, the $CD4^+$ T-cell fraction had significantly more HIV DNA than the non-$CD4^+$ T leukocyte fraction ($P = .0078$, $P = .047$), whereas no significant difference was observed in the rectum ($P = .44$; Figure 3*A*). Compared to the blood, both the ileum and rectum had higher HIV DNA levels per 10^6 WBC in the non-$CD4^+$ T leukocyte fraction (0.031, 0.016) and the total $CD4^+$ T-cell fraction ($P = .016$, .031), resulting in higher total levels in WBC ($P = .016$, .031).

Similar trends were observed for HIV RNA levels. The $CD4^+$ T-cell fraction had significantly more HIV RNA than the non-$CD4^+$ T leukocyte fraction in both blood and ileum ($P = .0078$, $P = .047$) but not rectum (Figure 3*B*). Compared to blood, the ileum had higher HIV RNA levels in the non-$CD4^+$ T leukocyte fraction, total $CD4^+$ T-cell fraction, and total WBC ($P = .031$, .016, .016), whereas the rectum had higher HIV RNA levels in the non-$CD4^+$ T leukocyte fraction but lower HIV RNA levels in $CD4^+$ T cells and lower total HIV RNA ($P = .016$, .031, .031).

Proportion of HIV in Each Cell Type

At each site, HIV levels in each cell type per 10^6 WBC were divided by the total HIV level in 10^6 WBC to yield the proportion of HIV in that cell type (Figure 4). Samples that had undetectable HIV levels were assigned a value equal to the detection limit of the assay (assigning them a value of zero did not change the conclusions). In the blood, infected T_{R7+} cells comprised a larger proportion of total HIV DNA than did T_{TM} cells ($P = .039$) despite T_{TM} cells having higher HIV DNA/million sorted cells (Figure 4*A*). In the ileum and rectum, T_{EM} cells comprised the largest proportion of HIV DNA ($P = .016$ for comparison of ileal T_{EM} to non-$CD4^+$ T leukocytes, T_{TM}, or T_{R7+}; $P = .031$ for comparison of rectal T_{EM} to T_{TM} or T_{R7+}). The proportion of HIV DNA in T_{R7+} cells was larger in blood than in either gut site ($P = .016$, $P = .031$), whereas the proportion of HIV DNA in T_{EM} cells was larger in both gut sites compared to blood ($P = .016$, $P = .031$). Rectal and ileal non-$CD4^+$

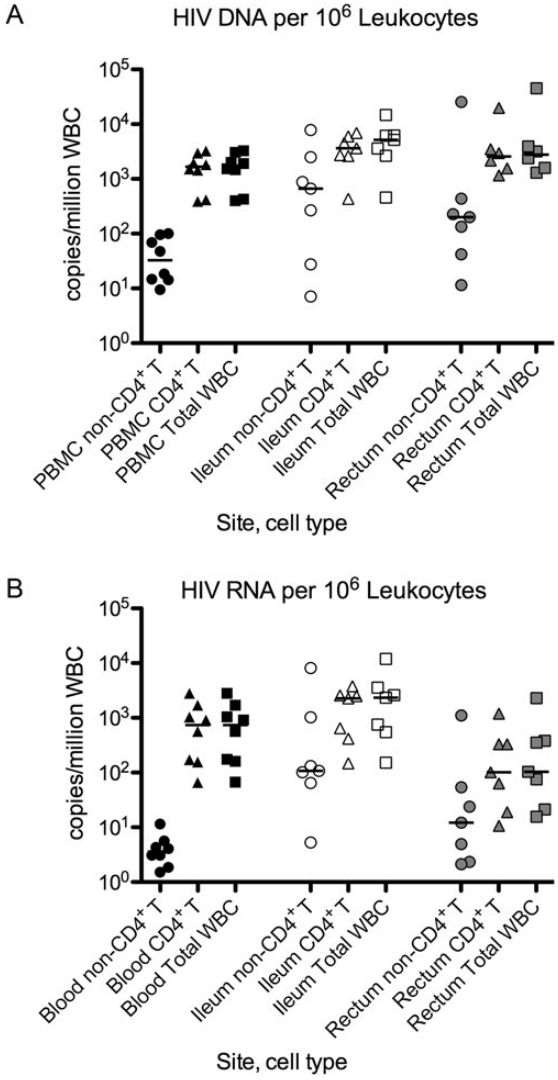
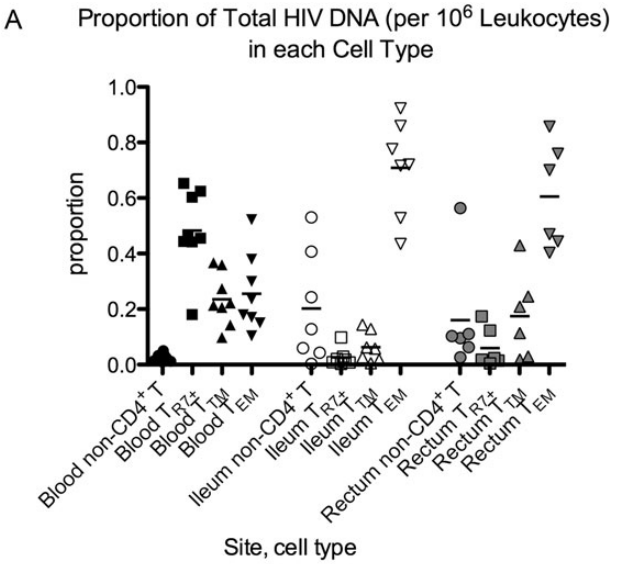


Figure 3. *A* and *B*, HIV DNA (*A*) and RNA (*B*) with normalization to cell frequency (in 10^6 WBCs). For each site (blood, ileum, rectum), we used the HIV levels within each cell type (Figure 2) and the frequency of each cell type (% of live singlet $CD45^+$ cells, as determined by flow cytometry) to calculated the HIV content within each cell type in 10^6 WBCs. Samples that had undetectable HIV levels were assigned a value equal to the detection limit of the assay. The HIV levels in T_{R7+}, T_{TM}, and T_{EM} $CD4^+$ T cells were added to yield an estimate of the total HIV level in $CD4^+$ T cells (*triangles*), which was added to the level in non-$CD4^+$ T leukocytes (*circles*) to give an estimate of the total HIV level in WBCs (*squares*). Data are shown on a log scale. Bars represent the medians. Abbreviations: HIV, human immunodeficiency virus; WBC, white blood cell.



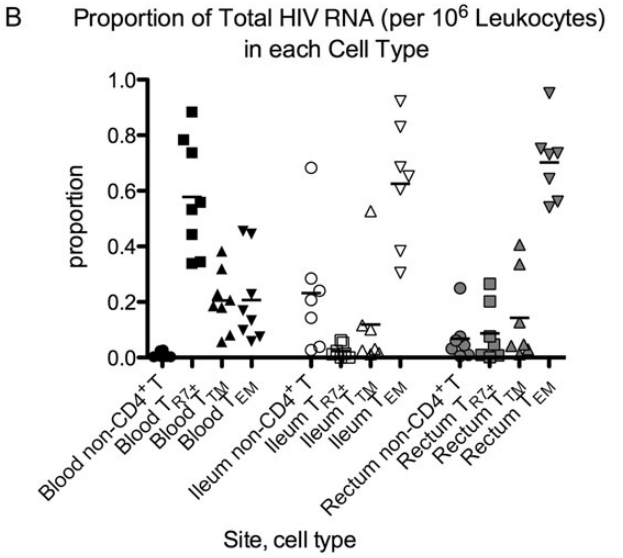


Figure 4. *A* and *B*, Proportion of HIV DNA (*A*) and RNA (*B*) contributed by each cell type. For each cell type, the HIV levels in 10^6 WBCs were divided by the total HIV level in 10^6 WBCs (the sum of the HIV levels in the 4 cell types at that site) to yield the proportion of HIV in that cell type. Samples that had undetectable HIV levels were assigned a maximum value equal to the detection limit of the assay. Symbols are used as in Figure 2. Bars represent the means. Abbreviations: HIV, human immunodeficiency virus; WBC, white blood cell.

T leukocytes tended to comprise a larger proportion of HIV DNA than corresponding cells in the blood ($P = .031$, $P = .078$).

Similar patterns were observed for the distribution of HIV RNA (Figure 4*B*). In blood, T_{R7+} cells comprised a larger proportion of HIV RNA than T_{TM} ($P = .0078$) or T_{EM} cells ($P = .039$) and non-$CD4^+$ T leukocytes comprised the smallest proportion of HIV RNA ($P = .0078$ for all comparisons). In the ileum and rectum, the proportion of HIV RNA was highest in

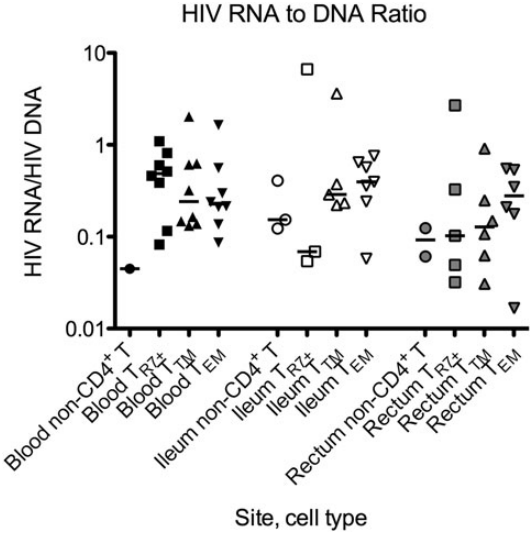


Figure 5. Average transcription per infected cell. For all cell samples in which both HIV RNA and DNA were detectable, the HIV RNA per 10^6 cells (Fig. 2*B*) was divided by the HIV DNA per 10^6 cells (Fig. 2*A*) to determine the RNA/DNA ratio (average transcription per infected cell). Results are graphed on a log scale. Symbols are used as in Figure 2. Bars represent the medians. Abbreviation: HIV, human immunodeficiency virus.

the T_{EM} cells ($P = .016$, .031, .016 for comparison of ileal T_{EM} to T_{R7+}, T_{TM}, and non-$CD4^+$ T; $P = .016$ for comparison or rectal T_{EM} to T_{R7+}, T_{TM}, or non-$CD4^+$ T leukocytes). The proportion of HIV RNA in T_{R7+} $CD4^+$ T cells was higher in blood than in either gut site ($P = .016$, .016), whereas the proportion of HIV RNA in T_{EM} was higher in both gut sites compared to blood ($P = .016$, .016). Ileal and rectal non-$CD4^+$ T leukocytes tended to comprise a higher proportion of HIV RNA than corresponding cells in the blood ($P = .016$, $P = .078$).

RNA/DNA Ratios in Each Cell Type

The RNA/DNA ratio (average transcription per infected cell) was calculated for all cell samples in which both HIV RNA and DNA were detectable. In the blood, the median RNA/DNA ratio was highest in T_{R7+} cells, whereas in both gut sites, the median ratio was highest in T_{EM} cells (Figure 5). However, owing in part to the small numbers of calculable ratios, these differences did not reach statistical significance.

DISCUSSION

In this study of ART-treated HIV-positive patients, HIV infection frequencies and HIV transcription levels differed by cell type and anatomic location. Most $CD4^+$ T cells in the blood were central memory and naive $CD4^+$ T cells, in agreement with prior reports [33]. In contrast, in the ileum and rectum, the predominant $CD4^+$ T-cell types were T_{EM} and, to a lesser degree, T_{TM}. Levels of HIV DNA/million cells were higher in

T_{R7+} and T_{EM} cells in the ileum than in the blood, suggesting some compartmentalization between these 2 sites. When normalized by cell frequencies, most HIV DNA and RNA in the blood were found in T_{R7+} cells, whereas in both gut sites, most HIV DNA and RNA were found in T_{EM} cells. These data indicate that insights provided by blood-based studies may not be indicative of what is happening in the tissues, and that the local host environment in the tissues may have dramatic effects in shaping the size and distribution of the reservoir during ART.

The observation that most HIV in the gut was in T_{EM} cells was surprising and suggests that the factors contributing to HIV persistence in tissues differ from those that contribute to the distribution of HIV DNA in circulating cells in blood. One possibility is that tissue-resident T_{EM} cells persist for long periods. In HIV-uninfected subjects, the half-life of circulating $CD4^+$ T_{EM} cells is shorter than that of T_{CM} cells [36], but it is unclear whether these half-lives account for migration of T_{EM} cells to the tissues, and whether T_{EM} cells survive for longer in the tissues. $CD4^+$ and $CD8^+$ T_{EM} cells persist for years after antigen exposure [37–39], suggesting a longer half-life or continuous renewal, either by homeostatic proliferation of T_{EM} cells or by maturation from another cell type, such as T_{CM} cells [39–41]. The enrichment of HIV in gut T_{EM} cells could be due to maturation and proliferative expansion, or it could reflect an increased susceptibility to de novo infection.

In addition to the HIV in $CD4^+$ T cells, we detected HIV DNA and some RNA in non-$CD4^+$ T leukocytes, which may constitute a sizeable portion of the HIV in the gut but much less in blood. It is possible that the sorted non-$CD4^+$ T leukocyte fraction was contaminated with low levels of $CD4^+$ T cells, including $CD4^{Low}$ T cells. However, the average fold difference in per cell levels of HIV DNA in T_{EM} cells and $CD45^+$ non-$CD4^+$ T leukocytes was 20-fold in the ileum and 4.66-fold in the rectum, which would require that the non-$CD4^+$ T leukocyte fraction were contaminated with >5% T_{EM} cells in the ileum and >20% in the rectum, whereas the predicted contamination with $CD4^+$ T cells (based on similar sorts in other subjects) was <1%. The non-$CD4^+$ T leukocyte population could include $CD8^+$ T cells [29, 42], macrophages, dendritic cells, mast cells and progenitors, and natural killer (NK) cells, all of which have been shown to be reservoirs for HIV [27]. However, the PCR signals could also come from virions trapped on the cell surface or phagocytosis of virions, infected cells, or cellular debris. Future studies will be necessary to confirm whether HIV can infect these cell populations in the gut. The existence of HIV reservoirs in non-$CD4^+$ T leukocytes would require additional consideration in developing viral eradication strategies.

In agreement with prior studies, HIV DNA levels were higher in the rectum and ileum compared to blood [25]. This difference reflects higher HIV DNA levels in both the $CD4^+$ T-cell fraction and the non-$CD4^+$ T leukocyte fraction. The greater HIV DNA in the $CD4^+$ T-cell fraction likely reflects differences in the types of $CD4^+$ T cells in the gut (more T_{EM} cells), as well as differences in the frequency of infection of each cell type. The disproportionate frequency of infected ileal T_{R7+} and T_{EM} could reflect preferential homing of infected cells from the blood, higher rates of de novo infection in the ileum, or increased survival or proliferation of infected cells in the ileum. The ileum also had higher HIV RNA levels than the blood, reflecting higher levels in both non-$CD4^+$ T leukocytes and $CD4^+$ T cells, whereas the rectum had lower total HIV RNA levels than the blood, reflecting lower HIV levels in $CD4^+$ T cells. Compared to the rectum, the ileum is likely to have higher concentrations of inductive sites, with a higher density of $CD4^+$ T cells. The ileal environment may be more likely to cause T-cell activation (resulting in more HIV RNA production) and more likely to support active replication (given that cells are in closer proximity and more likely to be activated).

In both blood and gut, HIV RNA/DNA ratios were typically <0.5, in agreement with prior reports [25, 34]. Assuming that most of the HIV DNA is integrated, and that there is roughly one copy per infected cell, at least half of the infected cells are not transcribing any HIV RNA. Most $CD4^+$ T cells in the gut display markers associated with T-cell activation [25], which may argue against a lack of host transcription factors associated with the resting state, and may suggest another mechanism of transcriptional control. At the same time, at least a fraction of the HIV DNA-positive cells in both blood and gut are spontaneously transcribing HIV RNA, and HIV RNA levels are higher in the ileum than blood. It is possible that this HIV RNA is translated into protein with the capacity to induce immune activation, whereas some HIV RNA could be packaged as virions, which could also contribute to limited, localized replication in the ileum [43] or to residual viremia.

Limitations of our study include the small number of subjects and the low yield of sorted gut T_{R7+} and T_{TM} cells. Both factors may reduce the power to detect differences between cell types or anatomic sites, particularly for RNA and RNA/DNA ratios. As noted previously, CCR7 expression was rare in the gut, making it difficult to sort out pure T_{CM} cells, and CD27 expression in the gut was less discrete than in the blood, blurring the distinction between gut T_{EM} and T_{TM} cells. Finally, normalization of HIV levels to cell numbers can be challenging, particularly for RNA from the gut, although we found a fairly good correlation between GAPDH and the cell counts from the sorts.

Despite these limitations, we found significant differences in the distribution of HIV-infected cell types in the blood, ileum, and rectum. Further study is needed to determine whether different mechanisms allow HIV to persist in the different cell types and sites, and whether levels of replication-competent virus and latently infected cells differ between gut and blood. Given the magnitude of the GI reservoir, new therapies must consider these fundamental differences between cellular reservoirs in the gut and blood.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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