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Gut epithelial barrier and systemic inflammation during chronic HIV infection

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Abstract

Objective—Microbial translocation and innate immune action characterize HIV infection.

Continued gut mucosal dysfunction during treatment and its relationship to CD4+ T cell recovery has not been well described.

Design—A cross-sectional study was performed of ART-suppressed (immunologic responders with CD4 >500 and immunologic non-responders with CD4 <350), untreated HIV-infected, and seronegative participants consenting to gut biopsies and a blood draw.

Methods—Neutrophil infiltration as a surrogate response to epithelial breach, colorectal epithelial proliferation as a measure of repair, and mucosal apoptosis by immunohistochemistry was determined in gut biopsies. Plasma markers of monocyte activation (sCD14), immune activation (IL-6), and indoleamine 2,3-dioxygenase-1 activity (plasma kynurenine/tryptophan (KT) ratio) were concurrently measured.

Results—Each HIV-infected group had greater neutrophil infiltration than controls. Similarly, untreated HIV-infected participants and ART-suppressed immunologic responders had increased epithelial proliferation compared with controls, but immunologic non-responders had no

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Authorship:

Contribution: PWH, JDE and MS served as the chief investigators, designed the study, and developed the protocol and the statistical analysis plan; PWH and MS identified the patient subgroups and tissue blocks; MS and JMI supervised the mucosal sampling program and JNM and SGD provided patient samples; RMD, RA, and CD performed the laboratory investigations while JMM and JDE coordinated their implementation and interpretation; PWH, JDE, and MS performed the statistical analysis, generated the tables and figures, and interpreted the data; MS wrote the first draft of the manuscript and all authors reviewed, revised, and approved the final manuscript.

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appreciable increase in epithelial proliferation despite elevated neutrophil infiltration. The CD4+ T cell count was positively correlated with epithelial proliferation and was modestly negatively correlated with neutrophil infiltration in ART-suppressed patients. Epithelial proliferation was inversely correlated with mucosal apoptosis, and apoptosis was linked to plasma sCD14 and modestly to KT ratio.

Conclusions—Neutrophil infiltration and mucosal apoptosis remain abnormally high despite ART. Epithelial proliferation increases in HIV, but may be impaired in immunologic non-responders. Whether mucosal apoptosis is a cause or consequence of epithelial proliferative defects is unclear, but appears to be associated with systemic inflammation. The impact of ART and interventions targeting the gut epithelial barrier in treated HIV infection warrant further investigation.

Keywords

HIV; immune activation; microbial translocation; epithelial proliferation; inflammation

BACKGROUND

Despite dramatic improvements in life expectancy in the modern antiretroviral therapy (ART) era, HIV-infected individuals, particularly those who start ART at later stages of disease, still have shorter life expectancy than the general population.[1-4] Abnormally high immune activation and inflammation follow HIV infection, persist during ART, and predict non-AIDS-associated morbidities and death.[5-10]

Increased immune activation has been linked to microbial translocation during both untreated and treated HIV infection.[11-14] Whereas the levels of microbial translocation (e.g., as measured by plasma lipopolysaccharide (LPS)) and innate immune activation decrease during suppressive ART, they remain persistently elevated and may contribute to the inflammatory state.[6, 15] Observations in pathogenic simian immunodeficiency virus (SIV) infection argue that intestinal barrier dysfunction is a source of microbial translocation due to extensive neutrophil infiltration, epithelial proliferative response, and persistent gut mucosal apoptosis.[16-18] Treatment of HIV infection decreases epithelial apoptosis in duodenal tissue, but apoptosis remains elevated compared to uninfected controls.[19, 20] Residual barrier dysfunction as measured by soluble markers predict increased mortality during treated HIV infection,[21] but direct measurements of the gut mucosa across a spectrum of CD4 count has not been reported. Given these observations, we sought to explore if epithelial breach persists in the gut compartment of untreated and treated HIV-infected individuals and their impact on systemic immune activation. Specifically, our objective was to characterize neutrophil infiltration as a surrogate for epithelial breach, epithelial repair by measuring crypt proliferation, and mucosal apoptosis, and how they relate to CD4+ T cell recovery and systemic immune activation.

METHODS

Subject recruitment and sample collection

Since 2006, HIV-infected individuals and controls have been recruited and consented from the SCOPE cohort at UCSF for sigmoidoscopy and collecting relevant GI biopsy samples for research purposes. The SCOPE cohort is an ongoing longitudinal study of over 1500 HIV-infected and uninfected adults based at San Francisco General Hospital. SCOPE was designed to characterize the natural history of both antiretroviral-treated and untreated HIV disease with standardized interviews and specimen collection. Participants come from across the Bay Area. Using this cohort, 73 HIV negative controls, HIV viremic untreated, and individuals suppressed with ART with archived gut biopsies were selected. The ART group included HIV-infected patients maintaining undetectable viral loads (<40 copies/mL) on stable ART for at least one year and were subdivided by the extent of peripheral blood CD4+ T cell recovery: immunologic non-responders (CD4+ T cell count <350 cells/mm³) and immunologic responders (CD4+ T cell count > 500 cells/mm³).

Prior to their procedure, study participants underwent a blood draw and received a Fleet enema, and rectosigmoid biopsies (each ~3 mm in diameter) were then obtained between 10 and 20 cm from the anus using jumbo forceps. Four biopsies were formalin-fixed and paraffin-embedded (FFPE) for immunohistochemistry. The available FFPE colonic tissue blocks and the plasma samples were the basis of this study.

Immunohistochemistry and Quantitative Image Analysis

To minimize bias, investigators quantifying mucosal measurements were blinded to group status. Immunohistochemistry was performed as previously described.[22] Antibodies used in this study were rabbit monoclonal cleaved caspase-3 (Cell Signaling; clone 5A1E), rabbit monoclonal anti-Ki67 (1:100; clone SP6; Labvision/Thermo Scientific) and rabbit polyclonal anti-myeloperoxidase (1:2,000; Dako). All stained slides were scanned at high magnification (x200) using the ScanScope CS System (Aperio Technologies), yielding high-resolution data from the entire tissue section. Representative regions of interest (ROIs; 250 to 500 mm²) were identified and high-resolution images extracted from these whole-tissue scans. The percent area of the lamina propria that stained for cleaved caspase-3 (including colonic epithelial cells) or myeloperoxidase was quantified using Photoshop CS5 and Fovea tools. Colonic crypt epithelial cell proliferation was measured using the Aperio Scanscope ruler tool on coronally aligned colonic crypts (in which the bases, middle portions, and tops were in the same focal plane). From the crypt base, the extent of epithelial cells undergoing proliferation was divided by the entire length of the crypt and the median proportion of crypt length for each patient staining positive for Ki67 is reported. Not all stains were performed on all samples due to the absence of intestinal epithelial crypts aligned coronally or tissue blocks no longer available for sectioning.

Cleaved cytokeratin-18 stain, specific for epithelial apoptosis and used in a non-human primate study,[17] was also assayed in this study. However, several distinct challenges rendered the marker too variable for this study. First, due to the small biopsies and the cleaved cytokeratin-18 stains primarily colonic enterocytes at the crypt surface, tissue

orientation was variably aligned to precluding sufficient visualization of intact crypt structures. Secondly, due the lower extent of enterocyte apoptosis as compared to observations in acute SIV-infected macaques, there was considerable variability in detection of cleaved cytokeratin-18. Finally, because we utilized biopsies from the SCOPE cohort that spanned sample collections over 5 years, we found that the cytokeratin-18 staining was not robust in older archived tissue specimens and variable background noise. Therefore, while tested, we were concerned about interpretability of the cytokeratin-18 marker.

Plasma inflammatory marker measurements

Cryopreserved plasma was thawed and batched for assessment of biomarkers of gut epithelial barrier function, inflammation, monocyte activation, and indoleamine 2,3-dioxygenase-1 (IDO-1) activity. ELISA using commercially available kits were performed according to the manufacturer's instructions in duplicates: IL-6, sCD14, I-FABP2 (all from R&D Systems, Inc., MN), and D-dimer (Diagnostic Stago, Inc., NJ). Concentrations of kynurenine and tryptophan were quantitated in plasma by liquid chromatography-tandem mass spectrometry, as previously described, as a marker of IDO-1 activity.[23]

Statistical Analysis

Pairwise comparisons between groups in continuous variables were assessed with Wilcoxon ranksum tests. Correlations were examined using Spearman's rank correlation. These analyses were performed using Stata 11.0 (StataCorp LP, College Station, TX) and GraphPad Prism v5.0b (GraphPad Software, Inc., La Jolla, CA). Adjusted differences were compared between groups with multivariable linear regression, transforming variables as appropriate to satisfy model assumptions.

RESULTS

Patient characteristics

A total of 15 viremic untreated, 24 ART-suppressed immunologic non-responders, 15 ART-suppressed immunologic responders, and 19 HIV-uninfected controls were studied (Table 1). The median age was greater than 44 years and was not significantly different between groups. All but three participants were men (2 viremic, 1 immunologic responder). Among viremic subjects, the median viral load was 4.6 log₁₀ copies/mL, and the median CD4⁺ T cell count was 510 cells/mm³, reflecting relatively early stage disease. Among ART-suppressed participants, median CD4⁺ T cell counts reflected our sampling strategy: 628 cells/mm³ in immunologic responders, and 229 cells/mm³ in non-responders.

Neutrophil infiltration persists despite ART

All HIV-infected groups had higher levels of neutrophil infiltration compared to HIV-uninfected controls (P = 0.04 for all groups, Figure 1 and 2A), with the highest levels in immunologic non-responders (median 0.7% vs. 0.3%, P=0.001) (Table 2). Higher neutrophil infiltration tended to associate with lower CD4⁺ T cell counts (rho = -0.31, P=0.06). This suggests that epithelial breach and translocation of microbes persist despite ART and may be linked to the degree of immune restoration.

Limited epithelial proliferation among individuals with incomplete immunologic recovery

Given that increased neutrophil infiltration has been associated with epithelial barrier damage and breach in the SIV macaque model [16], and shown here to be increased with HIV infection, we hypothesized that epithelial proliferation would increase as a host compensatory mechanism to repair gastrointestinal epithelial damage. Indeed, similar to observations in SIV+ rhesus macaques [17], HIV+ viremic participants had higher median levels of epithelial cell proliferation (defined as the proportion of crypt length staining positive for Ki67) than HIV-uninfected controls (55% vs. 40%, $P=0.01$), as did immunologic responders (52%, $P=0.02$, Table 2 & Figure 2B). However, immunologic non-responders – despite having abnormally high neutrophil infiltration – had similar levels of epithelial proliferation to HIV-uninfected controls ($P=0.57$) and significantly lower levels than viremic subjects ($P=0.02$) and immunologic responders ($P=0.04$). Moreover, among ART-suppressed individuals, crypt epithelial proliferation was positively correlated with CD4+ T cell count (ρ 0.43, $P=0.02$, Figure 3A) and modestly correlated with the nadir CD4+ T cell count (ρ 0.32, $P=0.05$, not shown).

Increased mucosal apoptotic activity linked to impaired epithelial proliferation

HIV-infected viremic untreated individuals had higher median levels of mucosal apoptosis (defined as the proportion of tissue area staining positive for caspase-3) than uninfected controls (2.9% vs. 0.1%, $P<0.001$) as did treated subgroups ($P=0.001$, Table 2 and Figure 2C). ART-suppressed participants (pooling responders and non-responders) had lower median mucosal apoptosis levels than did viremic untreated participants ($P=0.004$), but still higher levels than uninfected controls ($P<0.0001$). Among ART-suppressed individuals, a lower level of epithelial proliferation (suggesting diminished reparative activity) was correlated with increased mucosal apoptosis (ρ -0.53 , $P=0.02$, Figure 3B).

Biomarkers of immune activation and gut epithelial barrier integrity in the peripheral blood

We also compared key peripheral biomarkers of inflammation and immune activation between groups. HIV-infected viremic individuals had higher median levels of the soluble monocyte activation marker and LPS receptor, sCD14, than did controls (2.7 vs. 1.8 $\mu\text{g/ml}$, $P=0.01$, Table 2) and this abnormality persisted in both responders and non-responders despite the administration of effective ART. Similarly, plasma IL-6 levels (a measure of inflammation) and KT ratio (a measure of indoleamine 2,3-dioxygenase (IDO-1) activity) were elevated in all HIV-infected groups compared to uninfected controls ($P=0.04$ for all, Table 2). Activation of the coagulation cascade, as measured by D-dimer, was modestly but not significantly elevated in viremic subjects compared to controls ($P=0.09$), and there was no evidence for a difference between controls and ART-suppressed individuals (data not shown).

Mucosal apoptosis associated with systemic immune activation

To examine if local intestinal pathology is reflected systemically, we examined the relationship between mucosal apoptosis and peripheral biomarkers of inflammation and immune activation. In the entire cohort, levels of mucosal apoptosis were associated with plasma I-FABP levels (ρ 0.31, $P=0.05$, Supplement A). Similar patterns were observed

between mucosal apoptosis and IL-6 ($\rho = 0.29$, $P = 0.07$) and D-dimer ($\rho = 0.22$, $P = 0.17$); however, the relationship between apoptosis and these peripheral biomarkers did not persist within subgroups, suggesting potential confounding by HIV and ART status.

A greater level of mucosal apoptosis was positively associated with higher levels of plasma sCD14 levels ($\rho = 0.46$, $P = 0.04$, Figure 3C) and KT ratio ($\rho = 0.42$, $P = 0.08$, Figure 3D) in ART-suppressed individuals. There was no evidence for a relationship between the extent of epithelial proliferation and neutrophil infiltration and systemic markers of immune activation among all subjects or within any subgroup.

DISCUSSION

While the etiology of persistent immune activation during chronic HIV infection is incompletely understood, compromised barrier function and translocation of microbial products from the gut lumen into the systemic circulation have been implicated as one potential cause.[11, 16, 19, 24, 25] In this study, we show that, similar to observations made in non-human primate models of SIV infection and in smaller studies of HIV-infected individuals,[19, 20] treated and untreated chronic HIV infection show increased neutrophil infiltration in the gut compartment, suggesting ongoing epithelial breach. Epithelial proliferation increased in all treated and untreated individuals, except for those with impaired CD4+ T cell recovery. Consistent with the hypothesis that increased levels of mucosal apoptosis represent a response or consequence to injury, the degree of apoptosis was associated with measures of systemic microbial translocation and of innate immune activation (e.g., sCD14, KT ratio). Interestingly, ART-suppressed immunologic non-responders had increased levels of apoptosis and higher levels of neutrophil infiltration, but inappropriately low epithelial proliferation, suggesting that reduced regenerative activity of the epithelium is a pathologic sequelae in these individuals, as appears to also be the case for the T cell lineage of HIV-infected individuals.[26]

Whether intestinal epithelial barrier dysfunction is simply a consequence of HIV infection or a cause of innate immune activation is unknown, and few data to date have linked direct measures of gut epithelial barrier dysfunction to systemic markers of immune activation in this setting.[17, 27] Presumably, translocating microbial products can cause systemic immune activation via activation of innate sensors including Toll-like receptors, activating myeloid lineage cells, and inducing immunoregulatory enzymes like IDO.[28] Furthermore, the kynurenine pathway of tryptophan catabolism, which may be induced both by host IDO and by gut microbes that elaborate kynurenine pathway enzymes may contribute to Th17 and Th22 depletion in the GALT.[29-31] Since Th17 and Th22 cells are important in the maintenance of epithelial barrier function and clearance of translocated microbial products by coordinating the immune response (e.g., recruitment of neutrophils),[32-34] their depletion by kynurenine pathway catabolites in HIV infection may also contribute to gut barrier dysfunction. Indeed, the KT ratio in the peripheral blood – a marker of kynurenine pathway activity - was correlated with mucosal apoptosis in our study.[28, 35]

As with any retrospective cross-sectional study, there are several important limitations. First, the study participants representing the HIV subgroups are subject to selection and survival

bias. In the modern era, ART is initiated earlier and untreated and treated individuals are less likely to experience the extent of CD4+ T cell depletion and clinical progression experienced by the immunologic non-responders. To the best of our ability, we avoided participants who were long-term non-progressors and adjusted for nadir CD4+ T as a measure of advanced HIV disease. Another limitation of the cross-sectional design is that we cannot determine the relative degree to which gut epithelial barrier dysfunction is a cause or consequence of kynurenine pathway activity, but our study supports a relationship between these factors.

Interestingly, despite high mucosal apoptosis in untreated subjects, myeloperoxidase staining was only modestly increased, supporting the notion that the high levels of kynurenine pathway activity observed in untreated HIV infection may impair recruitment of neutrophils.[28, 32] Further attention to the relationships between mucosal immunity, the elaboration of specific cytokines that enhance epithelial homeostasis (e.g., IL-17, IL-22, and IL-6) or instigate further injury (e.g., TNF α , IFN γ), and their integrated effects on systemic immune activation is warranted.

Increases in epithelial proliferation, as measured by Ki67 staining, during chronic HIV infection suggest ongoing damage and subsequent repair of the epithelial barrier, as has been demonstrated in the SIV rhesus macaque model.[16] Interestingly, the exact opposite association was observed in immunologic non-responders; those with the greatest levels of apoptosis had the weakest epithelial proliferative responses in spite of having the highest level of neutrophil infiltration, which is a tissue marker for GI barrier damage. This suggests an impaired proliferative response in the immunologic non-responders and potentially links failed homeostasis of both epithelial cells and circulating CD4+ T cells. Indeed, lower epithelial Ki67 was associated with lower CD4+ T cell counts among all ART-suppressed participants. Whether dysfunction of epithelial regeneration drives poor CD4+ T cell recovery or vice versa, or whether common immunologic mechanisms drive both of these effects remains unclear.

The alterations in mucosal and epithelial homeostasis described herein may explain why we and others have observed gut dysbiosis, or altered composition of the gut microbial communities, during HIV infection [31, 36-39] along with expansion of the enteric virome, specifically adenovirus infections, that has been linked to epithelial pathology during pathogenic SIV infection [40]. In addition to an anatomical barrier against translocation of immunostimulatory products, the epithelial cells provide innate immune defense by secreting mucins and antimicrobial peptides, which is self-protective and may shape the microbiome but this might possibly impaired during HIV [41-43]. The fact that mucosal measurements did not in and of themselves explain systemic markers of immune activation may suggest that other unmeasured factors contribute to immune activation in this setting including the mucosal microbiome or virome composition, HIV itself, and other chronic co-infections, particularly cytomegalovirus. Alternatively, the relatively small sample size of this study may have limited power to detect relationships between mucosal measurements and systemic biomarkers that have high within-subject variability.

In conclusion, despite the remarkable success of ART, standard therapies do not fully restore health in HIV-infected individuals and persistent inflammation strongly predicts these complications. Our work suggests that elevated apoptosis and residual imbalances in epithelial homeostasis during treated HIV infection is associated with and may contribute to persistent inflammation and poor CD4+ T cell recovery. Earlier treatment may limit the abnormalities observed in the mucosal compartment and enable better CD4+ T cell recovery but this remains to be investigated. Efforts are warranted to define the contribution of the gut epithelial barrier to microbial translocation, immune activation, and shaping of the microbial community.

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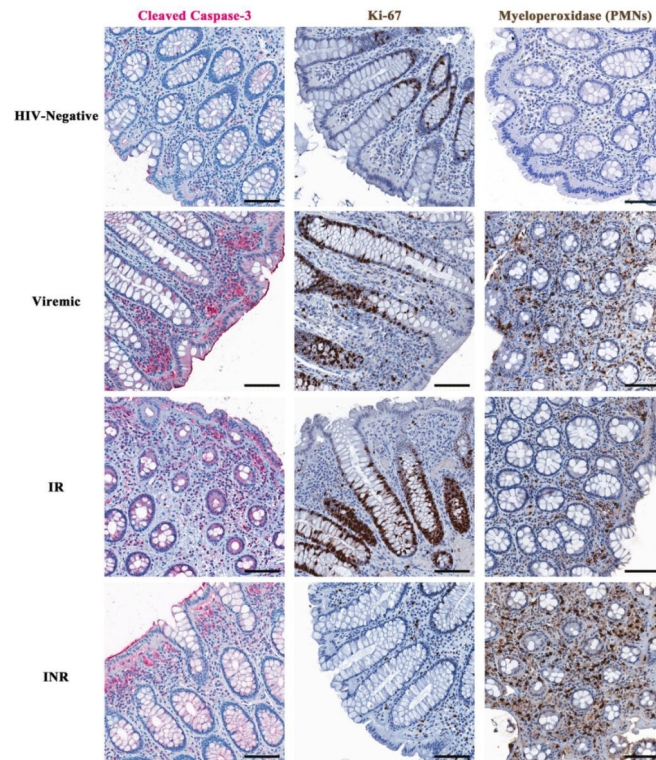


Figure 1. Mucosal apoptosis, crypt epithelial proliferation, and neutrophil infiltration on colorectal biopsies by immunohistochemistry. Mucosal biopsies demonstrate little (HIV-negative) and increased (other groups) cleaved caspase-3 staining (in red). Limited (HIV-negative, INR) and extensive (viremic, IR) colonic epithelial proliferation along the crypt base is demonstrated by Ki67 staining (in brown). Minimal (HIV-negative) versus increased neutrophil infiltration (other groups) was quantified by myeloperoxidase staining (in brown).

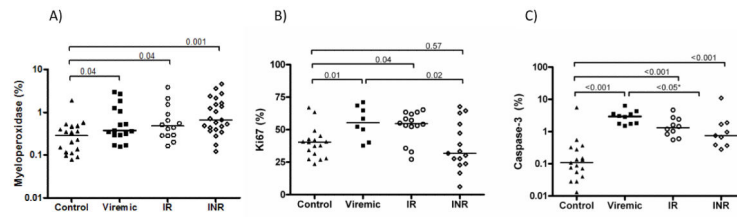


Figure 2.

Comparisons of neutrophil infiltration, epithelial proliferation, and mucosal apoptosis in colorectal biopsies from HIV-infected subgroups and HIV-uninfected controls. A) Neutrophil infiltration was measured by the percent of myeloperoxidase positive staining in rectosigmoid tissue. B) Intestinal epithelial cell proliferation as measured by extent of Ki67 staining along colonic crypt. C) Mucosal apoptosis was measured by percent of cleaved caspase-3 staining in rectosigmoid tissue.

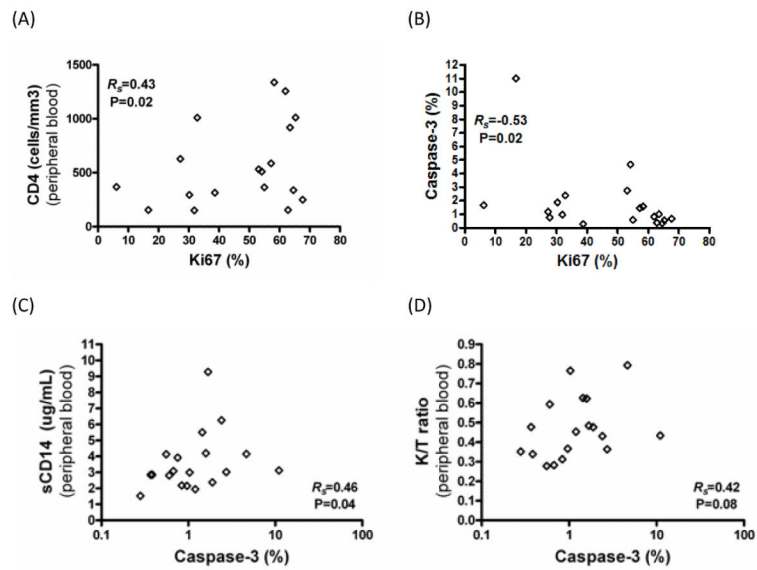


Figure 3.

Intestinal epithelial proliferation is (A) positively correlated with the peripheral blood CD4 count in ART suppressed individuals and (B) negatively correlated with the level of mucosal apoptosis in colorectal biopsies. Mucosal apoptosis is then linked to (C) monocyte activation as measured by soluble CD14 and (D) modestly with induction of indoleamine 2,3-dioxygenase activity as measured by the ratio of kynurenine to tryptophan levels in the peripheral blood.

Table 1

Baseline Characteristics of the Study Subjects

	HIV Negative median (IQR) n=19	HIV+ Untreated median (IQR) n=15	ART+ CD4>500 median (IQR) n=15	ART+ CD4<350 median (IQR) n=24
Age	47 (37-55)	44 (34-51)	49 (47-55)	47 (43-52)
Male, n (%)	16 (100)	13 (86)	14 (93)	24 (100)
CD4+ T cell count (cells/mm ³)	--	510 (347-714)	628 (532-1011)	229 (155-279)
CD4+ T cell nadir (cells/mm ³)	--	400 (320-500)	105 (49-311)	21 (7-56)
Duration of ART, (year)	--	--	9.8 (4.4-10.7)	11.3 (7.8-12)
Viral load (log ₁₀ copies/mL)	Not performed	4.60 (4.32-4.94)	Not detected	Not detected

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Table 2

Measures of intestinal injury, repair, and systemic immune activation.

Measure	Control median (IQR), n=19	VU median (IQR), n=15	P	IR median (IQR), n=15	P*	INR median (IQR), n=24	P*
<i>Intestine</i>							
Caspase-3 (%)	0.10 (0.05-0.21)	2.94 (1.76-3.56)	<0.001	1.32 (0.84-2.41)	<0.001 0.02	0.75 (0.38-1.68)	0.001 0.01
Ki67 (%)	40.4 (31.4-42.8)	55.4 (45.2-66.6)	0.01	54.6 (52.4-62.0)	0.02 0.63	31.8 (23.8-54.3)	0.57 0.02
MPO (%)	0.28 (0.12-0.47)	0.38 (0.30-1.28)	0.04	0.44 (0.29-0.90)	0.04 0.73	0.66 (0.39-1.61)	0.001 0.32
<i>Peripheral blood</i>							
sCD14 (µg/ml)	1.77 (1.54-2.34)	2.71 (2.28-4.06)	0.01	3.33 (2.33-4.19)	0.0002 0.37	3.08 (2.76-4.88)	0.0003 0.33
IL-6 (pg/ml)	1.04 (0.81-1.58)	1.59 (1.37-1.91)	0.04	1.50 (0.94-2.03)	0.10 0.84	1.54 (1.32-2.36)	0.004 0.89
K:T (nM/µM)	35 (33-37)	65 (58-79)	0.0001	51 (36-60)	0.01 0.04	43 (34-49)	0.08 0.001
D-dimer (ng/ml)	246 (207-278)	396 (243-552)	0.09	336 (220-382)	0.16 0.31	241 (198-391)	0.95 0.18
I-FABP (pg/ml)	180 (140-276)	204 (142-250)	1.0	299 (209-407)	0.02 0.08	440 (225-658)	0.0004 0.004

Abbreviations: VU, viremic untreated; ART, antiretroviral therapy; IR, immunologic responder; INR, immunologic non-responder; MPO, myeloperoxidase; K:T, kynurenine:tryptophan; I-FABP, intestinal fatty acid binding protein.

Ki67: controls (n=17); VU (n=8); IR (n=14); INR (n=15)

Casp-3: controls (n=16); VU (n=10); IR (n=10); INR (n=9)

MPO: controls (n=18); VU (n=12); IR (n=15); INR (n=23)

sCD14/IL-6/D-dimer/I-FABP: controls (n=17); VU (n=8); IR (n=14); INR (n=15)

* P-values are reported for comparisons to HIV-negative controls (first row) and to VU (second row).