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Cycling of Gut Mucosal CD4+ T Cells Decreases after Prolonged Anti-Retroviral Therapy and is Associated with Plasma LPS Levels

Emily J. Ciccone[1], **Sarah W. Read**[2], **Peter J. Mannon**[3], **Michael D. Yao**[4], **Jessica N. Hodge**[1], **Robin Dewar**[5], **Cheryl L. Chairez**[1], **Michael A. Proschan**[6], **Joseph A. Kovacs**[7], and **Irini Sereti**[1,*]

[1] Laboratorie of Immunoregulation, NIH, Bethesda, MD, 20892.

[2] Division of AIDS, NIH, Bethesda, MD, 20892.

[3] Division of Gastroenterology and Hepatology, University of Alabama-Birmingham, Birmingham, AL, 35294.

[4] Laboratorie of Host Defenses, NIH, Bethesda, MD, 20892.

[5] SAIC-Frederick, Inc., Frederick, MD 21702.

[6] Biostatistics Research Branch, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, 20892.

[7] Critical Care Medicine Department, NIH, Bethesda, MD, 20892.

Abstract

The gut mucosa is an important site of HIV immunopathogenesis, with severe depletion of CD4+ T cells occurring during acute infection. The effect of prolonged anti-retroviral therapy (ART) on cycling and restoration of T lymphocytes in the gut remains unclear. Colon and terminal ileal biopsies and peripheral blood samples were collected from viremic, untreated, HIV-infected participants, patients treated with prolonged ART (>5 years), and uninfected controls and analyzed by flow cytometry. In the gut, the proportion of cycling T cells decreased and the number of CD4+ T cells normalized in treated patients in parallel with β7 expression on CD4+ T cells in blood. Cycling of gut T cells in viremic patients was associated with increased plasma LPS levels, but not colonic HIV-RNA. These data suggest that gut T cell activation and microbial translocation may be interconnected while prolonged ART may decrease activation and restore gut CD4+ T cells.

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* Corresponding author. Mailing Address: NIH-NIAID NIH-NIAID, 10 Center Drive, Building 10-11B07A, Bethesda, MD 20892. Phone: (301) 496-5533 Fax: (301) 480-9978 isereti@niaid.nih.gov.

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INTRODUCTION

The gut mucosa is an important site of early SIV and HIV pathogenesis. The majority of CD4+ T cells in the gut express CCR5, the co-receptor for HIV, and exhibit an activated, memory phenotype presumably due to constant exposure to dietary and bacterial antigens (1-3). HIV preferentially infects activated, memory CD4+ T cells; therefore, gut mucosal T lymphocytes provide a large pool of target cells that are susceptible to HIV infection and supportive of active HIV replication (1-7). During acute SIV and HIV infection, gut mucosal CD4+ T cells are rapidly and preferentially depleted, primarily in the lamina propria (LP), before HIV-mediated changes are observed in peripheral blood or other lymphoid tissues such as lymph nodes and the spleen (4, 6, 8-14). Gut CD4+ T cell depletion and direct HIV effects on the gut epithelium (15) can disrupt its integrity allowing for the translocation of microbial products, a phenomenon that has been associated with immune activation in chronically infected patients (16).

The degree to which the CD4+ T cell population in the gut can be reconstituted with antiretroviral therapy is still unclear. There is evidence indicating that, in response to less than four years of antiretroviral therapy (ART), CD4+ T cell restoration in the gut is delayed and incomplete, regardless of the stage of infection during which ART is initiated (4, 10, 17, 18). However, recent studies, some evaluating the effects of long-term (>4 years) ART, have suggested that restoration of gut mucosal CD4+ T cells can occur (19, 20) and may happen more rapidly if treatment is initiated during acute infection (21-23). Importantly, many of these studies have quantified immune reconstitution in response to ART only by relative proportions of T cells. This may result in an underestimation of CD4+ T cell recovery because of significant expansion or accumulation of CD8+ T cells (24, 25). In addition, evidence from the SIV model has suggested that gut CD4+ T cell loss may be reflected in the proportion of peripheral blood lymphocytes expressing high levels of the lymphocyte homing marker, $\alpha 4\beta 7$ (26); this association has yet to be evaluated in human HIV infection in the absence or presence of ART.

Immunophenotype and gene expression profiles of gut mucosa T lymphocytes indicate that both the CD4+ and CD8+ T cell activation observed in HIV infection persist in the gut despite ART (17, 21). However, there are limited data characterizing the effect of therapy on T cell cycling and the relationship between T cell proliferation and reconstitution. It is known that HIV infection causes increased cycling of CD4+ and CD8+ T cells in peripheral blood and lymph nodes that decreases in response to ART (27-29). In the gut mucosa, immunohistochemical analysis has suggested that HIV causes an increase in T cell proliferation that persists despite short-term treatment but may normalize with prolonged (3-7 years) therapy (17).

The primary goal of this study was to measure immune restoration and T cell cycling in the colon and terminal ileum (TI) of HIV-infected patients and to evaluate the potential stimuli of T cell cycling, both in the peripheral blood and at the tissue level in the gut mucosa. In addition, we evaluated potential associations between the proportion of CD4+ T cells expressing $\beta 7$ integrin in peripheral blood and gut CD4+ T cell populations. We observed that significant CD4+ T cell restoration can occur in the gut (that parallels $\beta 7$ expression in

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blood) after prolonged ART. In addition, the proportion of cycling CD4+ T cells decreases or normalizes in the gut after prolonged ART and is associated with plasma LPS levels suggesting that gut immune activation and microbial translocation may be interconnected at the tissue level.

RESULTS

Clinical Characteristics of Patient Groups

Clinical characteristics of the three groups are shown in Table 1. Nine of the viremic patients were ART-naïve, while five had a therapy interruption of at least six months duration prior to biopsy. Subgroup analysis of ART-naïve patients of the No ART group yielded similar conclusions for the main statistical comparisons. All treated participants initiated ART during chronic infection and had been on therapy for a median of 8 years with virologic suppression to <50 copies/mL for at least two years at the time of the biopsy. Reportable histopathologic findings were observed in four No ART patients (one case of tubular adenoma, one case of focal acute colitis, and two cases of spirochetosis), four VL<50 patients (two cases of tubular adenoma and two cases of focal acute colitis), and three HIV-uninfected controls (two cases of tubular adenoma and one case of spirochetosis). All participants were clinically stable and asymptomatic at time of biopsy and did not require therapy for any of the above findings. Excluding patients with incidental findings on histopathologic specimens (focal enteritis or spirochetosis) did not alter any of our main reported statistical analyses.

The proportion of gut CD4+ T cells in treated patients was lower than that of uninfected controls despite prolonged ART

At both gastrointestinal (GI) sites, the proportion of CD4+ T cells in the No ART group was significantly lower than that of VL<50 patients (Colon: 5.9% v. 38.3%, TI: 6.6% v. 30.3%; p<0.001 for both comparisons) and controls (Colon: 5.9% v. 54.2%, TI: 6.6% v. 37.6%; p<0.001) (Fig. 1A). A similar pattern was observed in peripheral blood (Fig. 1A). In addition, the percentage of CD4+ T cells in the gut was lower in VL<50 patients as compared to controls (Colon: 38.3% v. 54.2%, p=0.004; TI: 30.3% v. 37.6%, p=0.023; Fig. 1A).

The proportion of CD8+ T cells was higher in viremic patients as compared to controls in both colon and TI (Colon: 67.7% v. 22.0%, TI: 74.9% v. 40.7%; p<0.001) (Fig. 1B). This was also true in comparison to the VL<50 group (Colon: 67.7% v. 42.3%, p<0.001; TI: 74.9% v. 52.8%, p=0.015; Fig. 1B). As with CD4+ T cells, the differences between groups were similar to those seen in peripheral blood (Fig. 1B). The proportion of CD8+ T cells of the VL<50 group was higher than that of controls in the colon (42.3% v. 22.0%, p=0.001), but not in the TI.

The absolute number of gut mucosal CD4+ T cells did not differ between uninfected controls and patients treated with prolonged ART

In contrast to what was observed when comparing proportions of CD4+ T cells, there was no statistically significant difference in the absolute number of CD4+ T cells/ gm of tissue

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between controls and the VL<50 group in either colon or TI (Colon: 3.6×10^6 v. 3.9×10^6, p=0.474; TI: 1.6×10^6 v. 1.0×10^6, p=0.114), mimicking the pattern seen in peripheral blood (Fig. 1A). Fewer CD4+ T cells were observed in both colon and TI of viremic patients as compared to HIV-uninfected controls (Colon: 0.56×10^6 v. 3.6×10^6, p=0.039; TI: 0.29×10^6 v. 1.6×10^6; p=0.007) (Fig. 1A).

At both gut sites, the absolute number of CD8+ T cells in the No ART group was higher than in the HIV-uninfected controls and VL<50 patients, although the differences were more pronounced in the colon (Colon, No ART: 11.8×10^6 v. HIV-: 1.3×10^6, p<0.001 and v. VL<50: 4.6×10^6, p=0.014; TI, No ART: 3.6×10^6 v. HIV-: 1.6×10^6, p=0.019 and v. VL<50: 2.7×10^6, p=0.035) (Fig. 1B).

The proportion and absolute number of CD4+ and CD8+ T cells differed between colon and terminal ileum

In controls and treated patients, the proportion of CD4+ T cells was significantly higher in colon compared to TI (HIV-, Colon: 54.1% vs. TI: 37.6%, p=0.010; VL<50, Colon: 38.3% vs. TI: 30.3%, p=0.006; Supplementary Fig. 1A). Conversely, the proportion of CD8+ T cells was significantly higher in the ileum. (HIV-, Colon: 22% vs. TI: 40.7%, p=0.002; VL<50, Colon: 43.5% vs. TI: 52.8%, p=0.037) (Suppl. Fig. 1B). In both of the HIV-infected groups, the absolute number of CD4+ (Supp. Fig. 1C) and CD8+ (Suppl. Fig. 1D) T cells per gram of tissue was higher in colon as compared to ileum (CD4+ T cells, No ART, Colon: 1.3×10^6 vs. TI: 0.29×10^6, p=0.010; VL<50, Colon: 3.4×10^6 vs. TI: 1.0×10^6, p=0.004; CD8+ T cells, No ART, Colon: 13×10^6 vs. TI: 3.6×10^6, p=0.020; VL<50, Colon: 4.7×10^6 vs. TI: 2.7×10^6, p=0.008). The proportions of CD4+ (Supp. Fig. 1E) and CD8+ (Supp Fig. 1F) T cells in colon and TI were strongly correlated across all three groups (%CD4+: r=0.86, p<0.001; %CD8+: r= 0.75, p<0.001).

In peripheral blood, β7 expression was higher on central memory as compared to effector memory CD4+ T cells, and was correlated with both the proportion and absolute number of CD4+ T cells in peripheral blood and both intestinal sites

The heterodimeric integrin, α4β7, is a homing receptor that facilitates migration of lymphocytes to the gut lamina propria and has been shown to bind to and signal through gp120 during HIV-1 infection (30, 31). Previous research has demonstrated that the majority of cells expressing high levels of β7 also express α4 (26). Therefore, the expression of β7 integrin, as well as CCR5, the co-receptor for HIV, was quantified on peripheral blood T lymphocytes (gating strategy shown in Supplementary Fig. 2). The proportion of T lymphocytes that were CD4+β7hi was lower in the No ART group than in either controls (0.62% v. 4.2%, p<0.001) or treated individuals (0.62% v. 2.0%, p=0.003); in addition, β7hi expression was lower in the VL<50 group as compared to controls (2.0% v. 4.2%, p=0.019) (Fig. 2A). A similar pattern was observed for CD4+CCR5+ T cells, although there was no statistically significant difference between the HIV-and VL<50 groups (Fig. 2B). The expression of these two surface markers was also measured on central and effector memory subsets (Fig. 2C). In all three groups, β7hi expression (Fig. 2D) was lower and expression of CCR5 (Fig. 2E) was higher in effector memory as compared to central memory CD4+ T cell

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populations. There were no differences across study groups in either β7hi or CCR5 expression on central or effector memory CD4+ T cells.

The proportion of β7hi CD4+ T cells in peripheral blood correlated with both the percent and absolute number of CD4+ T cells in the periphery (data not shown) and at both gut sites (Fig. 3A, D). The strongest correlations were observed when the analysis was restricted to HIV+ groups only. In contrast, among HIV+ individuals, the proportion of CCR5+ CD4+ T cells did not correlate with the percentage or absolute number of CD4+ T cells in the colon or TI (Fig 3B, E). However, the proportion and absolute number of CD4+ T cells in the PB did significantly correlate with the percent and absolute number of gut CD4+ T cells, respectively (Fig. 3C, F).

In colon, the proportion of cycling CD4+ T cells was substantially decreased in response to prolonged ART and was not associated with colonic tissue HIV-RNA

In both colon and ileum, the percentage of CD4+ T cells that expressed Ki67 was significantly higher in untreated patients as compared to HIV-uninfected controls (Colon: 12.0% v. 2.2%, TI: 12.0% v. 1.9%; $p \leq 0.001$) and ART-treated patients (Colon 12.0% v. 3.0%, TI: 12.0% v. 3.9%; $p \leq 0.001$) (Fig. 4A). A similar pattern was observed in peripheral blood (Fig. 4A). In colon and peripheral blood, there was no significant difference in the proportion of cycling CD4+ T cells between uninfected controls and treated patients (PB: 2.0% v. 2.8%, p=0.110; Colon: 2.2% v. 3.0%, p=0.055), however, the difference remained significant in TI (HIV-: 1.9% v. VL<50: 3.9%; p=0.007). The proportion of cycling CD8+ T cells did not differ between uninfected controls and treated patients in either peripheral blood or TI, but was slightly higher in controls (HIV-: 3.3% v. VL<50: 2.7%, p=0.027) in the colon (Fig. 4B). There were no statistically significant differences in the proportions of cycling CD4+ and CD8+ T cells between colon and ileum in any of the three study groups (Fig. 4A-B).

To evaluate the relationship between T cell cycling and immune restoration, we examined associations between the proportion of cycling T cells and both the proportion and absolute number of total CD4+ and CD8+ T cells in the HIV-infected groups. There was a strong inverse correlation between the percentage of CD4+Ki67+ T cells and both the percentage of CD4+ T cells and CD4+ T cell counts in PB (Table 2). In the colon, the percentage of CD4+Ki67+ T cells inversely correlated with the proportion of CD4+ T cells, but not with the number of CD4+ T cells/gm of tissue. In PB, the percentage of CD8+Ki67+ T cells correlated with the proportion of CD8+ T cells, but not CD8+ T cell count, and neither correlation was significant in the colon or TI (Table 2). Finally, β7hi expression on CD4+ T cells in PB inversely correlated with Ki67 expression on CD4+ T cells in the peripheral blood, colon and TI; however, the relationship did not appear to be linear (Supplementary Fig. 3).

Among viremic participants, there was a statistically significant correlation between both the percentage of CD4+Ki67+ and CD8+Ki67+ T cells in PB and plasma HIV-RNA. However, neither the percent of CD4+Ki67+ nor of CD8+Ki67+ in colon was associated with colonic or plasma HIV-RNA (Table 2). Both the percentages of CD4+Ki67+ and CD8+Ki67+ in PB

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correlated with the same subset in colon (r=0.69 and r=0.68; p, ≤0.001). Plasma and colonic HIV-RNA levels did not correlate (r=0.39, p=0.230).

The proportion of cycling CD4+, but not CD8+, T cells in both PB and colon was strongly associated with plasma LPS levels

Plasma levels of lipopolysaccharide (LPS), a marker of microbial translocation, and soluble CD14 (sCD14), a marker of monocyte activation after stimulation by LPS (16), were quantified. There was no statistically significant difference in plasma or soluble CD14 levels between the three groups, although both markers were higher in viremic patients as compared to controls (LPS, No ART: 46.2 pg/mL v. HIV-: 18.2 pg/mL; sCD14, No ART: 2.07×10^6 pg/mL v. HIV-: 1.74×10^6 pg/mL; p=0.055 for both comparisons) (Fig. 5A). Plasma LPS levels did correlate with the percent of cycling (Ki67+) CD4+ T cells in PB and both gut sites (Fig. 5B) but not with the proportion of cycling CD8+ T cells in either PB or colon (Fig. 5C). These observations were consistent whether the analysis included all three groups or was restricted to HIV-infected individuals. There was no association between sCD14 and cycling CD4+ or CD8+ T cells in either PB or colon.

Multivariate linear regression analysis was performed to further delineate the relationship between LPS and CD4+ T cell cycling in the gut. LPS remained statistically significantly associated with the proportion of CD4+Ki67+ T cells in the colon after controlling for either the proportion (p=0.019) or absolute number (p=0.002) of CD4+ T cells at the same site,

DISCUSSION

In the current study, we found that after prolonged ART, the cycling of CD4+ and CD8+ T cell populations was decreased or normalized and the number of CD4+ T cells was restored in the gut mucosa of HIV-infected patients. The gut CD4+ T cell immune restoration was also reflected in β7 expression on peripheral blood CD4+ T cells. Cycling of gut CD4+ T cell was associated with plasma LPS levels but not with plasma or tissue HIV-RNA levels, indicating that gut T cell activation in the gut may be interconnected with microbial translocation.

In concordance with previous studies (4, 10, 17, 21), we observed that the percentage of CD4+ T cells in both colon and ileum remained significantly lower than that of HIV-uninfected controls despite effective prolonged ART. However, the persistence of a high proportion of CD8+ T cells in HIV-infected patients appeared to result in an underestimation of CD4+ T cell reconstitution. We attempted to address this issue by enumerating the absolute number of CD4+ T cells and observed no difference in absolute CD4+ T cell numbers between HIV-uninfected controls and treated patients at both gut sites. Our findings are in agreement with recent studies using both immunohistochemistry and flow cytometric analyses (19, 20, 32); some of these have suggested that gut CD4+ T cell reconstitution may even exceed what occurs in peripheral blood (20, 32). We noted significant differences in the relative and absolute size of T cell populations in colon and TI, but the proportions of both CD4+ and CD8+ T cells at the two GI locations were strongly correlated across all study groups. This indicates that relative comparisons of CD4+ T cell reconstitution between HIV-infected and HIV-uninfected individuals in colon and TI may

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lead to similar conclusions, but that direct comparisons of studies sampling different GI sites may not be feasible.

It has also been proposed that initiating ART therapy during acute infection may result in more rapid and complete reconstitution of the CD4+ T cell population in the gut (21-23). Three of the four patients in this study who reconstituted their CD4+ T cell counts in the colon to values higher than the median of the HIV-uninfected group had peripheral nadir CD4+ T cell counts of less than 250 cells/μL. This suggests that CD4+ T cell restoration may occur despite substantial disease progression before ART initiation.

The mechanism by which T cell reconstitution in the gut mucosa occurs remains unclear. Microarray analysis of jejunal lymphocytes has demonstrated that the expression of genes related to cell trafficking, but not local proliferation, were increased in patients receiving ART as compared to uninfected controls (10). Our finding that the proportion of cycling T cells is similar in treated patients and uninfected controls also suggests that CD4+ T cell populations in the gut may be sustained by trafficking of T cells to the gut mucosa as opposed to local proliferation after prolonged therapy. However, there may be transient increases in T cell cycling at earlier time points after ART initiation that may contribute to T cell reconstitution, a possibility that could not be tested in this study due to our enrollment of chronically infected individuals treated with prolonged ART. Further longitudinal studies may thus be necessary to determine factors associated with substantial recovery of CD4+ T cells in the gut mucosa and the clinical significance of this immune reconstitution.

This study is the first to examine the relationship between β7hi expression in the blood and gut T cell populations in humans and in the context of ART. Evidence from the rhesus macaque model of SIV infection has suggested that CD4+ T cells expressing the lymphocyte homing marker α4β7 may play a role in gut pathogenesis and immune reconstitution, and therefore may serve as a surrogate marker for intestinal CD4+ T cells (26). Our observation that a strong association exists between β7hi, but not CCR5, expression on blood CD4+ T cells and CD4+ T cell populations at both gut sites is consistent with this hypothesis. Interestingly, in this cross-sectional study we observed similar associations between both the proportion and absolute number of the CD4+ T cell subset in blood and the same populations in colon and ileum, suggesting that measurement of the α4β7 subset may not be necessary to track intestinal CD4+ T cell populations in the context of chronic untreated infection or after prolonged ART. However, given that during acute infection CD4+ T cell depletion occurs more rapidly in the gut as compared to blood (4, 6, 8-14), it will be of great interest to evaluate the association between α4β7 expression in the peripheral blood and gut CD4+ T cell populations during acute HIV infection.

Unlike in peripheral blood, where the proportion of cycling CD4 + and CD8+ T cells was associated with plasma HIV-RNA, we observed no association between T cell cycling in the colon and viral burden in either tissue or plasma among viremic, untreated patients. This suggests that the factors that most significantly impact T cell proliferation may be different in the two compartments. In peripheral blood, increased T cell cycling in untreated infection is thought to be the result of HIV-induced immune activation as well as a homeostatic response to T cell depletion (29). In our treated group, the median proportion of activated

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CD4+ and CD8+ T cells (as determined by co-expression of HLA-DR and CD38) in blood was significantly lower than that of the untreated group (data not shown). Therefore, it is likely that the prolonged suppression of viral replication with therapy results in a decrease in peripheral immune activation and a subsequent reduction in T cell cycling. In contrast, in the gut mucosa, it is possible that restoration of the CD4+ T cell population itself may result in a decrease in CD4+ T cell cycling by reducing homeostatic signals that trigger local proliferation. In our HIV-infected groups, the proportion of cycling CD4+ T cells in the colon correlated with the proportion of CD4+ gut T cells at the same site, a trend that has been observed in blood both in this study and others (28, 33). However, it was not associated with the number of gut mucosal CD4+ T cells, and all of our treated patients had a relatively small proportion of cycling CD4+ T cells despite demonstrating a wide range of absolute CD4+ T cell counts, arguing against proliferation as a homeostatic response to T cell depletion. Therefore, it is possible that the suppression of viral replication and, in turn, local immune activation (or vice versa), in addition to other unknown mechanisms, may also be contributing to the decrease in T cell cycling observed in the colon after prolonged therapy.

To our knowledge, this is the first analysis of possible associations between plasma levels of LPS and markers of T cell cycling and activation in the gut mucosa. Previous research has examined the relationship between LPS levels and peripheral markers of activation and proliferation. A significant correlation between Ki67+ CD4+ and CD8+ T cells in peripheral blood and plasma LPS levels was observed among treated patients and specifically immunological non-responders (34). While this study is limited by its correlative nature, we found that, among all three groups in our study, the proportion of cycling CD4+ but not CD8+ T cells in colon was associated with plasma levels of LPS, a marker of microbial translocation, suggesting an association between LPS and cellular immune activation in the CD4+ T cell subset both systemically and locally in the gut mucosa. It is unclear whether LPS is a cause (16) or an effect (35, 36) of immune activation. If LPS were the cause of activation-induced cell death through stimulation of the innate immune system, it would be expected to induce increased cycling of both CD4+ and CD8+ T cells. It is possible that microbial translocation may instead be a consequence of HIV-mediated damage to the gut mucosal barrier due to increased local immune activation and CD4+ T cell turnover. In a multivariate analysis, plasma LPS remained significantly associated with colon CD4+ T cell cycling even after controlling for either colon CD4+ T cell proportion or absolute number. Measurements of gut tissue LPS, which has been demonstrated in SIV-infected macaques using immunohistochemistry (Jacob D. Estes, personal communication), would be important for determining the biological mechanism of the interaction between microbial translocation and T cell cycling as well as the clinical significance of this relationship in the context of HIV infection.

In conclusion, we observed that substantial restoration of the gut CD4+ T cell population can occur after therapy and is largely reflected in β7 expression on blood CD4+ T cells. Our findings also suggest that T cell cycling decreases significantly in the gut mucosa in response to prolonged ART and may be regulated by both homeostatic and local stimuli such as those resulting from microbial products.

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MATERIALS AND METHODS

Subjects

Biopsies from colon and terminal ileum as well as peripheral blood samples were collected from 14 viremic HIV-infected individuals (No ART), 12 HIV-infected patients treated with prolonged ART (longer than five years) (VL<50), and 10 HIV-uninfected controls (HIV-). All participants provided informed consent under an IRB-approved NIH Protocol (registered at www.clinicaltrials.gov, NCT# 00001471). Plasma viral loads were determined by ultrasensitive bDNA assay (Versant HIV-1 version 3.0, Siemens Corp., New York City).

Biopsy Processing

To collect colonic and terminal ileum tissue, subjects underwent a routine endoscopy procedure under moderate conscious sedation. Approximately thirty endoscopic biopsies were randomly taken from the gut mucosa at each location with 16 −20 processed for flow cytometric analysis. In groups of five, the biopsies were weighed, placed in 500μL of medium containing RPMI (Mediatech, Herndon, VA) with 10% heat-inactivated fetal bovine serum. Samples were then digested using 1 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO) and 2,000U DNAse I (Invitrogen, Carlsbad, CA) for 30 min at 37°C or 250U benzoase (Novagen, Madison, WI) for 40 min at 37°C before being filtered through a 40 micron screen. After being washed twice with the 10%FBS medium, the resulting cell suspension was counted using a Beckman Coulter Counter to obtain the number of total viable cells. Histopathologic evaluation confirmed sampling of isolated lymphoid follicles in the colon and Peyer's patches/gut-associated lymphoid tissue in the ileum, although the biopsies primarily consisted of lamina propria lymphocytes (data not shown).

HIV-1 Viral RNA Extraction from Gut Tissue

Two biopsies from colon and terminal ileum were snap frozen on dry ice for measurement of tissue HIV-RNA. The samples were weighed and then homogenized in AVL buffer (QIAamp Viral mini kit Cat No. 52,904) using a mini mortar and pestle. Extraction was completed per kit instructions. The Siemens Quantiplex HIV-1 3.0 assay was used to measure HIV-1 RNA copy number. Results are expressed as copies/mg of tissue.

Immunophenotyping

Immunophenotypic analysis was performed on whole blood, cryopreserved peripheral blood mononuclear cells (PBMCs) (in the case of the β7 and CCR5 staining only), and cells extracted from the gut biopsies as described elsewhere (37). Comparison of b7 and CCR5 staining between fresh PBMCs, cryopreserved PBMC stained immediately after thawing, and cryopreserved PBMCs rested for three hours before staining showed that a three-hour rest maintained β7 and CCR5 expression that is often downregulated with freezing (Supplementary Fig. 4). The following antibodies were used for immunophenotyping of whole blood samples: anti-CD3 FITC (clone SK7), anti-CD4 PerCP or APC (clone SK3), and anti-CD8 PerCP (clone SK1), (BD Biosciences, San Jose, CA), and anti-Ki67 PE (clone B56) (BD Pharmingen, San Jose, CA). The following additional antibodies were used for staining of frozen peripheral blood mononuclear cells (PMBC): anti-CD3 APC-Cy7 (clone

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SK7), anti-β7 integrin PE-Cy5 (clone FIB504), anti-CCR5 PE (clone 2D7), CD27 FITC (clone M-T271) (BD Pharmingen, San Jose, CA), CD45RO APC (clone UCHL1) (BD Biosciences, San Jose, CA) and anti-CD4 Qdot 605 (clone S3.5) (Invitrogen, Carlsbad, CA). Samples were acquired using a FACSCalibur or an LSRII flow cytometer (BD Pharmingen, San Jose, CA). The data were analyzed using FlowJo software version 8 (Tree Star, Inc., San Carlos, CA).

Calculation of Absolute Numbers of Gut T cell Subsets

Absolute numbers of CD4+ and CD8+ T cell subsets per gram of gut tissue were calculated by multiplying the total viable cell count by percentages obtained from flow cytometric analysis. The total cell count per gram of tissue was calculated by dividing the viable cell count as determined by Coulter Counter (Beckman Coulter Inc., Fullerton, CA) by the tissue weight. This proportion was then multiplied by the percent of cells in the live lymphocytes gate and that number was subsequently multiplied by the percent of CD3+ lymphocytes. The absolute number of gut CD3+ T cells was then used in conjunction with the subset percentages to determine the absolute number of each T cell subset per gram of biopsy tissue.

Measurement of Lipopolysaccharide (LPS) and Soluble CD14 (sCD14) levels

LPS levels were quantified in duplicate by dilution of plasma samples collected in EDTA tubes to 10% with endotoxin-free water and subsequent heat inactivation of plasma proteins for 15 minutes at 80°C using a Limulus Amebocyte Assay (Lonza Group Ltd., Switzerland). Background was then subtracted and LPS levels were calculated by first setting the y-intercept for the standard regression line at zero and then by the manufacturer's recommended protocol. Soluble CD14 (sCD14) plasma levels were measured in duplicate using a commercially available ELISA assay (R&D Systems, Minneapolis, MN), and analyzed according to manufacturer's recommended procedure.

Statistical Analyses

Values are expressed as medians with interquartile ranges. Kruskal Wallis and Wilcoxon matched pairs tests were used for between and within group comparisons, respectively. Spearman's Rank tests were used to calculate correlation coefficients. Due to the exploratory nature of the work, all p-values of ≤ 0.05 are shown; however, to account for multiple comparisons, $p<0.01$ was considered statistically significant by the authors. Analyses were performed using Prism v5.0 (GraphPad Software, Inc., La Jolla, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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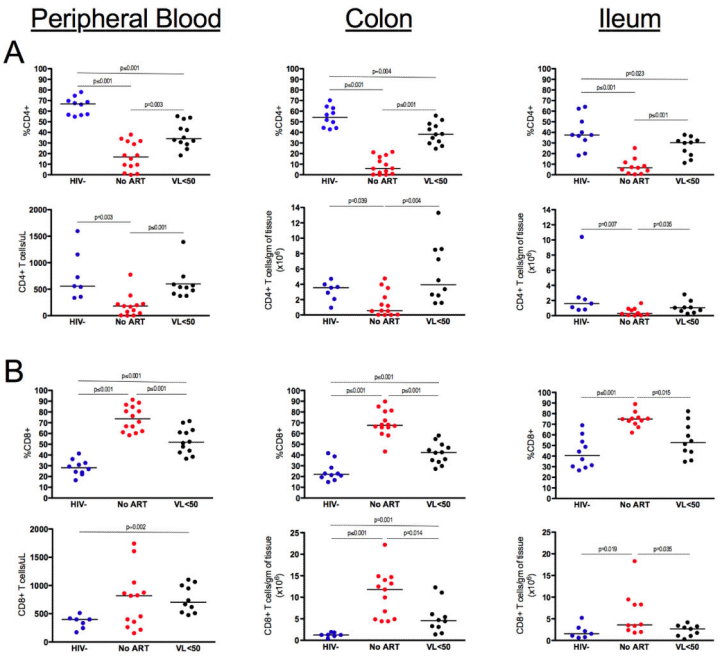

Figure 1.

The proportion and absolute number of (A) CD4+ and (B) CD8+ T cells in peripheral blood and gut compartments. Percentages are proportions of CD3+ T lymphocytes. Absolute numbers from gut are the number of cells per gram of biopsy tissue. Black bars indicate median values. Ileum values for the T cell percentages were not available for one VL<50 participant. Absolute number estimates were available for 7 HIV-, 13 No ART (10 for ileum), and 10 VL<50 (9 for ileum) patients.

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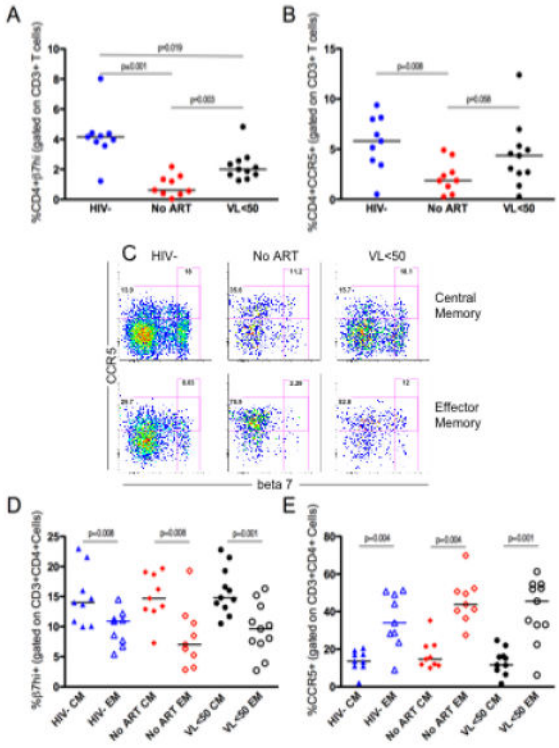

Figure 2.

The proportion of peripheral blood CD4+ T cells expressing (A) β7hi and (B) CCR5 in 9 HIV-, 9 No ART, and 11 VL<50 participants. The gating strategy in Part (C) was used to quantify expression of (D) β7hi and (E) CCR5 on effector (CD27-CD45RO+) and central (CD27+CD45RO+) memory CD4+ T cells. Black bars indicate median values.

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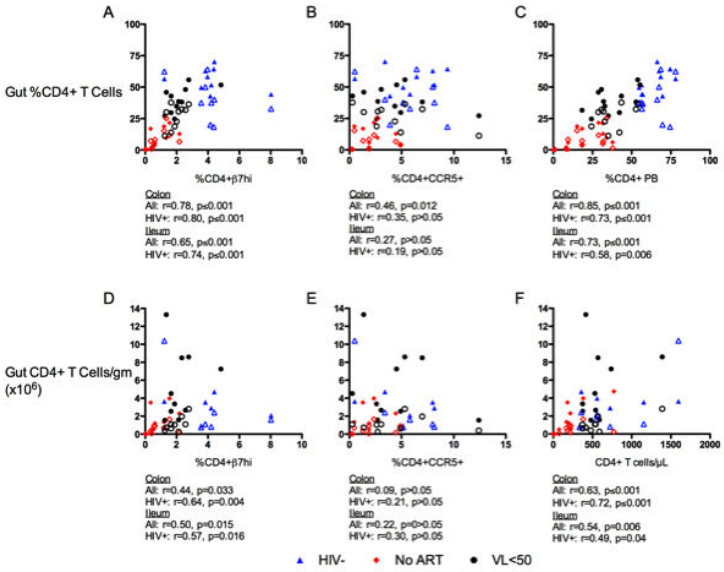

Figure 3.

Comparison of the associations between peripheral blood (A, D) CD4+β7hi, (B, E) CD4+CCR5+, and (C, F) CD4+ T cells and the (A-C) percentage and (D-F) absolute number of CD4+ T cells in colon and terminal ileum. Filled symbols represent colon values, and open symbols reflect terminal ileal values. Spearman rank correlations for comparisons of all three groups and HIV+ groups only are displayed below each graph.

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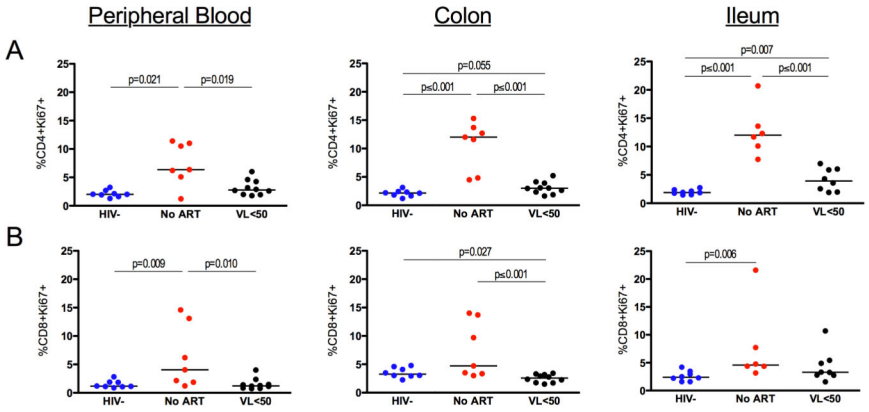

Figure 4.

The percentage of cycling (A) CD4+ and (B) CD8+ T cells (Ki67+) in peripheral blood and gut compartments. Included in the analysis are participants who had Ki67 subset information for both PB and colon (8 HIV-, 7 No ART and 10 VL<50). Of these individuals, ileum measurements were not available for one No ART and two VL<50 participants. Black bars indicate median values.

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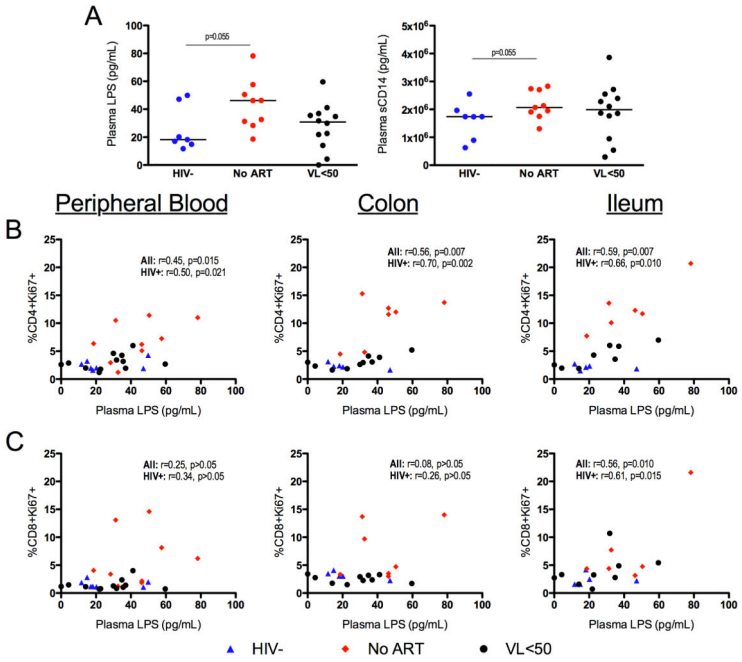

Figure 5.

(A) Plasma levels of LPS and sCD14 in 7 HIV- (blue triangles), 9 No ART (red diamonds), and 12 VL<50 (black circles) individuals. Black bars indicate median values. (B) Correlation between LPS levels and the percentage of CD4+Ki67+ and CD8+Ki67+ T cells in peripheral blood (left column), colon (middle column), and ileum (right column). Colon values were not available for an additional two participants from each group. Spearman rank tests were used to determine correlation and p-values.

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Table 1

Clinical characteristics of study participants.

	HIV- (n=10)	No ART (n=14)	VL<50 (n=12)
Age	46 (40-50)	47 (35-49)	48 (41-50)
CD4+ T cells/μL	773 (496-1,359)	175 (37-263)	535 (384-608)
% CD4+ T cells	45 (38-49)	12 (6-26)	32 (23-38)
CD8+ T cells/μL	399 (313-443)	755 (334-919)	703 (520-988)
% CD8+ T cells	20 (19-25)	60 (53-68)	42 (36-53)
CD4:CD8 ratio	2.13 (1.59-2.58)	0.19 (0.07-0.50)	0.72 (0.45-1.04)
Plasma HIV-RNA (copies/mL)	N/A[B]	31,199 (6,505-216,531)	<50
Colon Tissue HIV-RNA [C] (copies/mg)	N/A	180[D] (49-2,645)	<50
Years on ART	N/A	N/A	8 (5-10)
Nadir CD4+ T cells/μL	N/A	N/A	187 (36-270)

[A] Median values with IQR in parentheses.

[B] N/A: Not applicable

[C] Tissue HIV-RNA only available for eleven of the No ART participants.

[D] Participants with HIV-RNA levels below the limit of detection were assigned a value of 49 copies/mg.

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Table 2

Results of Spearman rank tests comparing the percentage of cycling CD4+ and CD8+ T cells in PB and GI sites with other cellular and viral measurements among HIV-infected individuals. Correlation analyses of plasma and tissue HIV-RNA only included No ART patients.

	%CD4+ PB	**CD4+ T cells/μL (PB)**	**%CD4+ colon**	**CD4+ T cells/gm of tissue (colon)**	**%CD4+ ileum**	**CD4 T cells/gm of tissue (ileum)**
%CD4+Ki67+[A] (at matching site)	−0.59 **0.004**	−0.73 **<0.001**	−0.62 **0.009**	−0.43 0.114	−0.51 0.061	−0.39 0.184

	%CD8+ PB	**CD8+ T cells/μL (PB)**	**%CD8+ colon**	**CD8+ T cells/gm of tissue (colon)**	**%CD8+ colon**	**CD8 T cells/gm of tissue (ileum)**
%CD8+Ki67+[A] (at matching site)	0.55 **0.008**	−0.14 0.539	0.43 0.086	0.36 0.182	0.14 0.621	0.01 0.970

	Plasma HIV-RNA (copies/mL)	**Colonic HIV-RNA (copies/mg)**
%CD4+Ki67+ PB	0.70 **0.025**	0.41 0.327
%CD4+Ki67+ colon	0.39 0.383	0.41 0.419
%CD8+Ki67+ PB	0.78 **0.008**	0.54 0.171
%CD8+Ki67+ colon	0.07 0.880	0.49 0.356

[a] Analysis of HIV+ groups only.