RESEARCH ARTICLE | NOVEMBER 01 2010

Disruption of Intestinal CD4+ T Cell Homeostasis Is a Key Marker of Systemic CD4+ T Cell Activation in HIV-Infected Individuals ⊘

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Disruption of Intestinal CD4$^+$ T Cell Homeostasis Is a Key Marker of Systemic CD4$^+$ T Cell Activation in HIV-Infected Individuals

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HIV infection is associated with depletion of intestinal CD4$^+$ T cells, resulting in mucosal immune dysfunction, microbial translocation, chronic immune activation, and progressive immunodeficiency. In this study, we examined HIV-infected individuals with active virus replication ($n = 15$), treated with antiretroviral therapy ($n = 13$), and healthy controls ($n = 11$) and conducted a comparative analysis of T cells derived from blood and four gastrointestinal (GI) sites (terminal ileum, right colon, left colon, and sigmoid colon). As expected, we found that HIV infection is associated with depletion of total CD4$^+$ T cells as well as CD4$^+$ CCR5$^+$ T cells in all GI sites, with higher levels of these cells found in ART-treated individuals than in those with active virus replication. While the levels of both CD4$^+$ and CD8$^+$ T cell proliferation were higher in the blood of untreated HIV-infected individuals, only CD4$^+$ T cell proliferation was significantly increased in the gut of the same patients. We also noted that the levels of CD4$^+$ T cells and the percentages of CD4$^+$Ki67$^+$ proliferating T cells are inversely correlated in both blood and intestinal tissues, thus suggesting that CD4$^+$ T cell homeostasis is similarly affected by HIV infection in these distinct anatomic compartments. Importantly, the level of intestinal CD4$^+$ T cells (both total and Th17 cells) was inversely correlated with the percentage of circulating CD4$^+$Ki67$^+$ T cells. Collectively, these data confirm that the GI tract is a key player in the immunopathogenesis of HIV infection, and they reveal a strong association between the destruction of intestinal CD4$^+$ T cell homeostasis in the gut and the level of systemic CD4$^+$ T cell activation. *The Journal of Immunology*, 2010, 185: 5169–5179.

P athogenic HIV infection of humans and SIV infection of Asian macaques are consistently associated with a rapid, severe, and largely irreversible loss of mucosal CD4$^+$ T cells, including those resident in the gastrointestinal (GI) tract (1–7). This loss has been attributed to both direct virus infection as well as bystander death of uninfected cells (2, 3) and is in part related to the presence in mucosal tissues of large numbers of memory/activated CD4$^+$ T cells expressing the main HIV/SIV coreceptor CCR5 (8). According to the current paradigm, this depletion of CD4$^+$ T cells will result in an overall loss of mucosal immune function and epithelial integrity that, in the GI tract, will ultimately cause the passage of microbial products from the intestinal lumen to the systemic circulation in a process commonly referred to as "microbial translocation" (9–13). In turn, microbial translocation is thought to be a key factor determining the state of chronic, generalized immune activation that is typically associated with HIV infection and appears to predict the speed of disease progression as well as, if not better than, the level of plasma viremia (14–18). However, the exact relationship between GI tract CD4$^+$ T cell depletion and microbial translocation remains unclear, and in fact depletion of intestinal CD4$^+$ T cells has also been shown in the nonpathogenic models of infection of sooty mangabeys and African green monkeys, in which mucosal immunity is preserved and microbial translocation does not occur (19, 20). More recently, emphasis has been placed on the loss of intestinal CD4$^+$ T cells producing IL-17, henceforth referred to as Th17 cells, which are depleted in HIV/SIV infections of humans and macaques, but are preserved in sooty mangabeys and African green monkeys (21–23). Additionally, other factors, such as mucosal inflammation, cytokine production, and epithelial apoptosis, as well as the dysregulation of other cell types, such as neutrophils, macrophages, NK cells, and CD8$^+$ T cells, may be involved in the HIV-associated mucosal immune dysfunction, although their relative contributions to pathogenesis remain ill-defined (2, 24–29).

Although there is a consensus on the fact that the natural history of HIV infection is associated with this major CD4$^+$ T cell depletion in the GI tract, it remains controversial whether and to what extent this depletion can be reversed when HIV replication is fully suppressed by antiretroviral therapy (ART). According to some studies, CD4$^+$ T cell reconstitution in the GI tract of ART-treated HIV-infected individuals is delayed and incomplete (30–37), with full restoration occurring only if therapy is initiated

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Abbreviations used in this paper: ART, antiretroviral therapy; GI, gastrointestinal; LC, left colon; N/A, not applicable; RC, right colon; SC, sigmoid colon; TI, terminal ileum.

relatively early during the course of infection (30, 34). This lack of mucosal CD4+ T cell reconstitution has been attributed to an incomplete suppression of virus replication at the level of mucosal sites as well as excessive collagen deposition in the same tissues (38, 39). Other studies, in contrast, suggest that prolonged virus suppression by ART is associated with levels of CD4+ T cells in the GI tract that are similar to those observed in healthy uninfected controls (40, 41). Another topic that, to the best of our knowledge, is still understudied is the relationship between CD4+ T cell homeostasis and CD4+ T cell activation at the level of both peripheral blood and the GI tract. In particular, it is unclear how systemic immune activation is related to intestinal immune activation, depletion of GI CD4+ T cells, and loss of Th17 cells.

The presence of these gaps in our understanding of the overall impact of mucosal CD4+ T cell depletion and mucosal immune dysfunction in AIDS pathogenesis is in part related to the fact that truly informative studies of the GI tract are difficult to perform in HIV-infected humans. For instance, the actual time of HIV infection is typically unknown, and longitudinal investigation of the GI tract as well as sampling of multiple anatomic sites in the GI tract are unpractical and have potential ethical implications. Of note, in most published studies, only one or two anatomic sites in the GI tract were sampled, thus compromising the investigators' ability to obtain a comprehensive analysis of the intestinal immune system, whose cellular composition, phenotype, and function may vary along its length. Additionally, the size of the sample that can be collected is usually small, thus precluding the ability to conduct studies of many immunological, virological, and histological markers in the same location. To overcome these limitations, intense effort has been devoted to studies of the mucosal immune dysfunction following experimental, pathogenic SIV infection of macaques (11, 42). These studies have been very informative, and they provided a number of key advances in terms of our present understanding of the pathogenesis of primate lentiviral infections, with important ramifications for HIV therapy and prevention. However, as with any other animal models of human disease, the SIV macaque system has a number of shortcomings, including the fact that, in this model, viral replication is several orders of magnitude higher than during HIV infection and that the course of disease progression is much more rapid (1–2 y) (43, 44). It is possible, for instance, that the well-characterized effects of SIV infection on the GI tract of macaques may be more severe than those observed during HIV infection, perhaps being due to different pathogenic mechanisms. For these reasons we think that studies of the impact of HIV infection on the mucosal immune systems of humans are invaluable tools that will improve our understanding of AIDS pathogenesis.

In the present study, we performed a cross-sectional immunological study of four GI tract sites (terminal ileum [TI], right colon [RC], left colon [LC], and sigmoid colon [SC]) in HIV-infected individuals (both viremic and with ART-induced suppression of HIV replication) and healthy uninfected controls. We found that HIV infection with active virus replication is associated with depletion of total CD4+ T cells as well as CD4+CCR5+ T cells in all four GI sites, with ART-treated individuals showing higher levels of CD4+ T cells (although lower than healthy controls). Untreated HIV-infected individuals demonstrate increased levels of CD4+ T cell activation and proliferation in both blood and gut, and these levels were directly correlated with the severity of intestinal CD4+ T cell depletion (both total and Th17 cells). We thus concluded that the GI tract is a key player in the immunopathogenesis of HIV infection, with a strong association existing between the extent of disruption of CD4+ T cell homeostasis in the gut and the level of mucosal and systemic CD4+ T cell activation.

Materials and Methods

Patient population

Twenty-eight HIV-infected individuals were recruited for this study, 13 of whom were on antiretroviral therapy for >1 y, as well as 11 uninfected controls. All individuals who participated in this study gave their informed consent in accordance with the relevant protocol that was approved by the Institutional Review Board of the University of Pennsylvania. For details on the clinical, immunological, and virological parameters of the enrolled subjects, please see Table I.

Blood and GI biopsies

Peripheral blood was collected by venipuncture, and biopsies of the gastrointestinal tract were collected using pinch biopsy forceps from the TI, RC (ascending colon) or LC (descending colon), and the SC during a colonoscopy. Pinch biopsies were placed in either 4% paraformaldehyde and used for immunohistochemistry or in RPMI 1640 and processed to isolate lymphocytes. Lamina propria lymphocytes were isolated as previously described (45). Briefly, the intraepithelial layer was removed by incubating biopsies in 5 mM EDTA solution in calcium- and magnesium-free HBSS for 45 min at room temperature. Following the removal of the epithelial layer, the lamina propria lymphocytes were then isolated by digestion with 1 mg/ml collagenase in RPMI 1640 for 1 h at 37°C. The digested cell suspension was then passed through a 70-μm cell strainer to remove residual tissue fragments. Cells were processed and stained the day of sampling.

Flow cytometry

Multiparameter flow cytometric analysis was performed on whole blood and lymphocytes isolated from intestinal biopsies. Lymphocytes were stained for surface markers at 4°C for 30 min with the following Abs: CD16 Pacific Blue (clone 3G8), CD20 Pacific Blue (clone L27), CD3 Alexa 700 (clone SP34-2), CCR5 allophycocyanin-Cy7 (clone 2D7), and CD45RO PE-Cy7 (clone UCHL1) (all from BD Biosciences, San Jose, CA); CD4 PE-Cy5.5 (clone OKT4; eBioscience, San Diego, CA); CD27 PE-Cy5 (clone 1A4CD27; Beckman Coulter, Fullerton, CA); and CD8 Pacific Orange (clone 3B5; Invitrogen, Carlsbad, CA). Samples assessed for Ki67 were surfaced stained, washed, and permeabilized using BD Perm 2 (BD Biosciences) and then stained intracellularly with Ki67 FITC (clone B56; BD Biosciences) for 30 min at 4°C. At least 50,000 events were collected on an LSRII driven by BD Biosciences Diva software, and analysis was performed using FlowJo (Tree Star, Ashland, OR).

Th17 cells

Mononuclear cells from the peripheral blood or the GI tract were resuspended at 1 million cells/ml in RPMI 1640 supplemented with 10% FBS and stimulated with PMA (5 ng/ml) and ionomycin (1 μg/ml) (Sigma-Aldrich, St. Louis, MO). All stimulations were performed in the presence of monensin (1 μg/ml) (BD Biosciences) for 5 h at 37°C. Stimulated cells were stained for the surface expression of CD3, CD4, CD8, CD45RO, and CD27, permeabilized using BD Perm 2, and stained intracellularly for IL-17 using an anti–IL-17 Alexa 488 Ab (eBioscience). At least 100,000 events were collected on an LSRII driven by BD Biosciences Diva software and analysis was performed using FlowJo (Tree Star).

Immunohistochemistry and immunofluorescence methods

Immunohistochemistry was performed using a biotin-free polymer approach (MACH-3; Biocare Medical, Concord, CA) on 5-μm tissue sections mounted on glass slides, which were dewaxed and rehydrated with double-distilled H$_2$O. Ag retrieval was performed by heating sections in 1× Diva Decloaker reagent (Biocare Medical) in a pressure cooker set at 121°C for 30 s. Slides were stained on an IntelliPATH autostainer (Biocare Medical) with optimal conditions determined empirically that consisted of two blocking steps using Sniper blocking reagent (Biocare Medical) for 10 min and TNB IgG block diluent (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.5% blocking reagent [NEN Life Science Products, Boston, MA]; 2% Sniper blocking reagent and 2% goat serum [Invitrogen]) for an additional 10 min at room temperature. Endogenous peroxidases were blocked with 1.5% (v/v) H$_2$O$_2$ in TBS (pH 7.4). Primary Abs were diluted in TNB IgG block diluent and incubated for 1 h at room temperature. Tissue sections were washed, and mouse/rabbit MACH-3 secondary polymer systems (Biocare Medical) were applied according to the manufacturer's instructions. Sections were developed with 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA), counterstained with hematoxylin, and mounted in Permount (Fisher Scientific, Waltham, MA). All stained slides were scanned

at high magnification (×400) using the ScanScope CS System (Aperio Technologies, Vista, CA) yielding high-resolution data from the entire tissue section. Representative images were acquired from these whole-tissue scans. Primary Abs used for immunohistochemical experiments were mouse anti-human CD4 (clone 4B12; Lab Vision/Thermo Fisher Scientific, Fremont, CA) and rabbit monoclonal anti-human Ki67 (clone SP6; Lab Vision/Thermo Fisher Scientific). Isotype-matched negative control Abs were used in every experiment and uniformly showed no staining (data not shown).

Statistical analysis

Multigroup comparisons were performed using one- and two-way ANOVAs with the Dunn multiple comparison posttest. A Spearman rank correlation was performed on nonparametric data. All graphing and statistical analysis were performed using GraphPad Prism software (GraphPad Software, San Diego, CA).

Results

Study design and patient population

The aim of this study was to conduct a detailed comparative analysis of how HIV infection affects intestinal CD4$^+$ T cell homeostasis, defined as both the level of CD4$^+$ T cell depletion and CD4$^+$ T cell proliferation in mucosal tissues, and to investigate how these events correlate with the level of CD4$^+$ T cell depletion and CD4$^+$ T cell proliferation as measured in peripheral blood. To this end, we conducted a cross-sectional study of a total of 39 individuals, including 15 HIV-infected subjects with active virus replication, 13 HIV-infected subjects treated with ART, and 11 healthy controls (Table I). The study was designed to investigate the immunological changes induced by HIV infection in blood as well as four different anatomic sites within the lower GI tract, that is, the TI, RC, LC, and SC. To the best of our knowledge, this is the first time that this type of detailed comparative analysis has been conducted in a relatively large number of HIV-infected individuals and controls.

## Phenotype of T cells isolated from the GI tract of healthy individuals

To better assess how HIV affects CD4$^+$ T cell homeostasis in the GI tract, we first examined the frequency and phenotype of intestinal T cells (derived from TI, RC, LC, and SC) in a group of 11 healthy individuals and compared their phenotype with that of T cells isolated from peripheral blood. As shown in Fig. 1A and 1B, the levels of CD4$^+$ and CD8$^+$ T cells (assessed as percentage of total CD3$^+$ T cells) were overall similar in the blood and at each site of the GI tract, although we observed a significantly greater frequency of CD8$^+$ T cells in the TI when compared with both the LC and SC. We next examined the relative distribution of naive (CD27$^+$CD45RO$^-$) and memory (CD45RO$^+$) T cells in the GI tract and blood of the same individuals. Fig. 1C (top) shows representative contour plots of CD45RO and CD27 staining of T cells from the blood and the intestine. As expected, we found that peripheral blood contains a significantly higher percentage of

Table 1. *Characteristics and infection status of study participants*

Patient ID	Gender	Age	Viral Load	HIV Status	ART
MALT1016	Male	55	N/A	Negative	N/A
MALT1017	Male	56	N/A	Negative	N/A
MALT 1019	Male	25	N/A	Negative	N/A
MALT 1040	Male	52	N/A	Negative	N/A
MALT1045	Female	30	N/A	Negative	N/A
MALT 1050	Female	52	N/A	Negative	N/A
MALT 1052	Male	49	N/A	Negative	N/A
MALT 1001	Female	70	N/A	Negative	N/A
MALT1002	Female	48	N/A	Negative	N/A
MALT 1012	Female	29	N/A	Negative	N/A
MALT 1041	Male	49	N/A	Negative	N/A
MALT 1022	Male	61	<75	Positive	Yes
MALT 1023	Male	58	<75	Positive	Yes
MALT 1024	Female	49	<75	Positive	Yes
MALT 1027	Male	55	<75	Positive	Yes
MALT 1028	Male	57	<75	Positive	Yes
MALT 1031	Male	36	<75	Positive	Yes
MALT 1032	Female	50	<75	Positive	Yes
MALT 1004	Male	50	<75	Positive	Yes
MALT 1006	Male	52	86	Positive	Yes
MALT 1008	Male	53	<75	Positive	Yes
MALT 1013	Female	53	<75	Positive	Yes
MALT 1014	Female	48	<75	Positive	Yes
MALT 1039	Male	46	<75	Positive	Yes
MALT 1021	Male	43	101,665	Positive	No
MALT 1026	Male	42	4,884	Positive	No
MALT 1033	Male	45	47,022	Positive	No
MALT 1042	Male	58	100,808	Positive	No
MALT 1047	Male	48	3,298	Positive	No
MALT 1049	Male	50	2,647	Positive	No
MALT 1051	Male	51	8,552	Positive	No
MALT 1055	Male	44	6,006	Positive	No
MALT 1007	Male	50	500,000	Positive	No
MALT 1015	Female	50	3,565	Positive	No
MALT 1030	Female	46	10,257	Positive	No
MALT 1035	Male	46	8,148	Positive	No
MALT 1036	Male	46	66,963	Positive	No
MALT 1053	Female	43	500,000	Positive	No
MALT 1054	Male	44	6,762	Positive	No

N/A, not applicable.

FIGURE 1. Frequency and activation status of T cells in HIV uninfected individuals. *A*, The average frequency of CD4+ T cells (pregated on CD3) in the blood (PBMCs) and from four sites along the intestinal tract (TI, RC, LC, and SC) in HIV uninfected individuals. *B*, The average frequency of CD8+ T cells (pregated on CD3) in PBMCs and from the TI, RC, LC, and SC in HIV uninfected individuals. *C* (*top panel*), Representative flow cytometric plots showing the expression of CD45RO and CD27 in cells from blood and the intestines. T cells that express CD45RO and are either CD27+ or CD27− are considered memory cells, while cells that are CD45RO− and CD27+ are naive. The proportions of naive and memory CD4 and CD8 T cells in the blood and various intestinal sites in HIV uninfected individuals are shown (*bottom panel*). *D*, The frequency of CD4+ T cells expressing the nuclear Ag Ki67 in the blood and the intestines. *E*, The frequency of CD8+ T cells expressing the nuclear Ag Ki67 in the blood and the intestines. A one-way ANOVA with a Dunn multiple comparison posttest was used to determine *p* values.
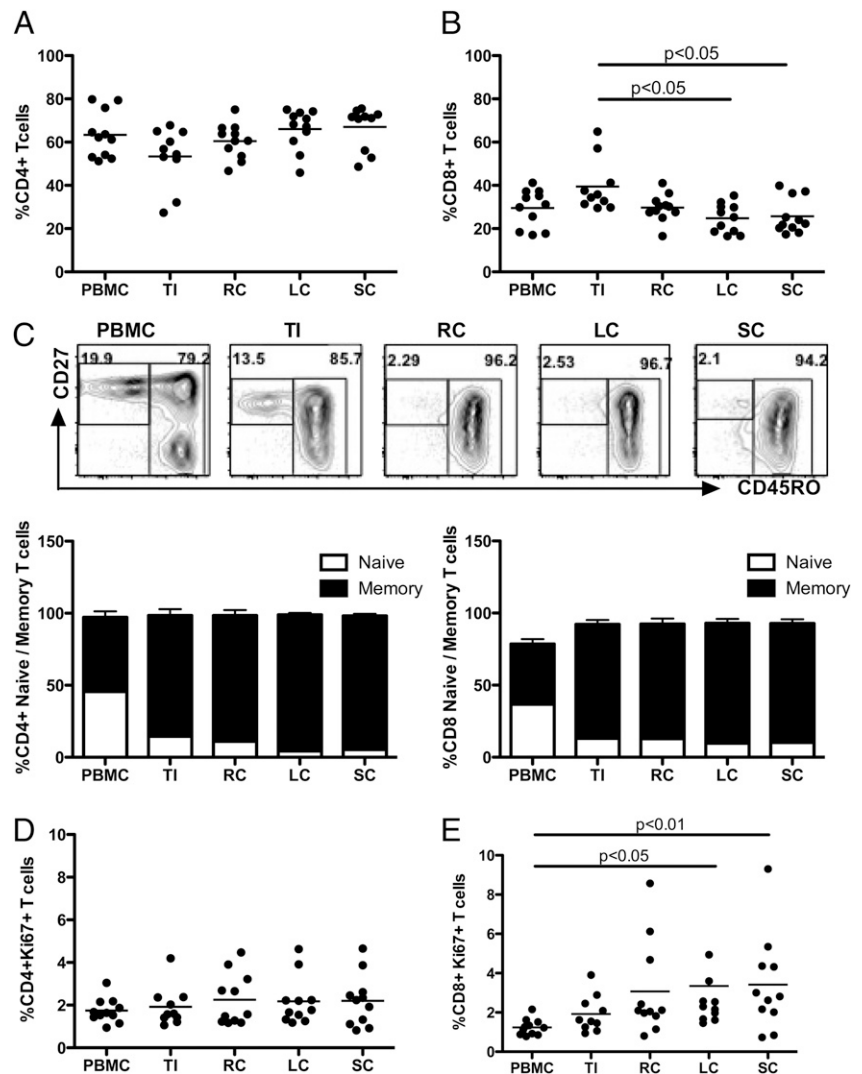
naive cells than do all sites in the GI tract, in which memory cells make up the vast majority (>80%) of CD4 and CD8+ T cells (Fig. 1*C*, *bottom*). Interestingly, the TI has a higher population of naive CD4+ T cells when compared with the LC or SC (Fig. 1*C*, *bottom*), and, in general, we observed a trend toward fewer naive cells when descending from the TI to the SC. We next used the nuclear Ag Ki67 as a marker of activation and proliferation and compared its expression on CD4+ and CD8+ T cells from the blood and GI tract. As shown in Fig. 1*D*, the levels of proliferating CD4+Ki67+ T cells were not significantly different between the blood and the GI tract. In contrast, the fraction of proliferating CD8+Ki67+ T cells tended to be higher in the gut when compared with peripheral blood, with the difference being statistically significant in the LC and SC ($p < 0.01$ and $p < 0.05$, respectively; Fig. 1*E*). Collectively, these data indicate that in healthy individuals the GI tract contains similar proportions of CD4+ and CD8+ T cells than does blood, but it is enriched in memory T cells of both lineages as well as proliferating CD8+ T cells in the LC and SC.

HIV infection is associated with a significant CD4+ T cell depletion in all examined regions of the GI tract, which is incompletely restored by ART

Several studies have shown that HIV infection is associated with depletion of intestinal CD4+ T cells (4, 5, 30, 32, 41); however, the extent of this depletion and the ability of ART to restore normal

levels of intestinal CD4+ T cells remains controversial. To examine the impact of HIV infection on the homeostasis of CD4+ T cells in the blood and GI tract, we performed our comparative immunophenotypic analysis in 28 HIV-infected individuals, including 15 untreated subjects with active replication and 13 subjects with viremia fully suppressed by ART for at least 1 y. Fig. 2*A* shows representative pseudo-color plots depicting the fraction of CD4+ and CD8+ T cells (pregated on CD3+ T cells) in the blood and at each sampled site of the gut (TI, RC, LC, and SC) in three representative subjects, that is, a healthy HIV uninfected individual (*top*), an HIV-infected individual on ART (*middle*), and a therapy naive HIV-infected patient (*bottom*). As shown in Fig. 2*B*, and consistent with other studies (4, 5, 30, 32, 41), we observed that HIV infection is associated with a significant depletion of CD4+ T cells from the blood and all sampled intestinal sites. In patients with active replication, the magnitude of the CD4+ T cell depletion was similar in all sampled sites of the GI tract and was consistently more severe in the gut than in peripheral blood (Fig. 2*B*). Of note, in HIV-infected individuals with viremia suppressed by ART, the percentage of CD3+CD4+ T cells was similar in all sampled intestinal sites and in peripheral blood, thus suggesting that ART had induced a significant, albeit incomplete, repopulation of gut CD4+ T cells (Fig. 2*B*). As shown in Fig. 2*C*, the HIV-associated depletion of gut CD4+ T cells (and its attenuation by ART) was confirmed by immunohistochemical staining for CD4
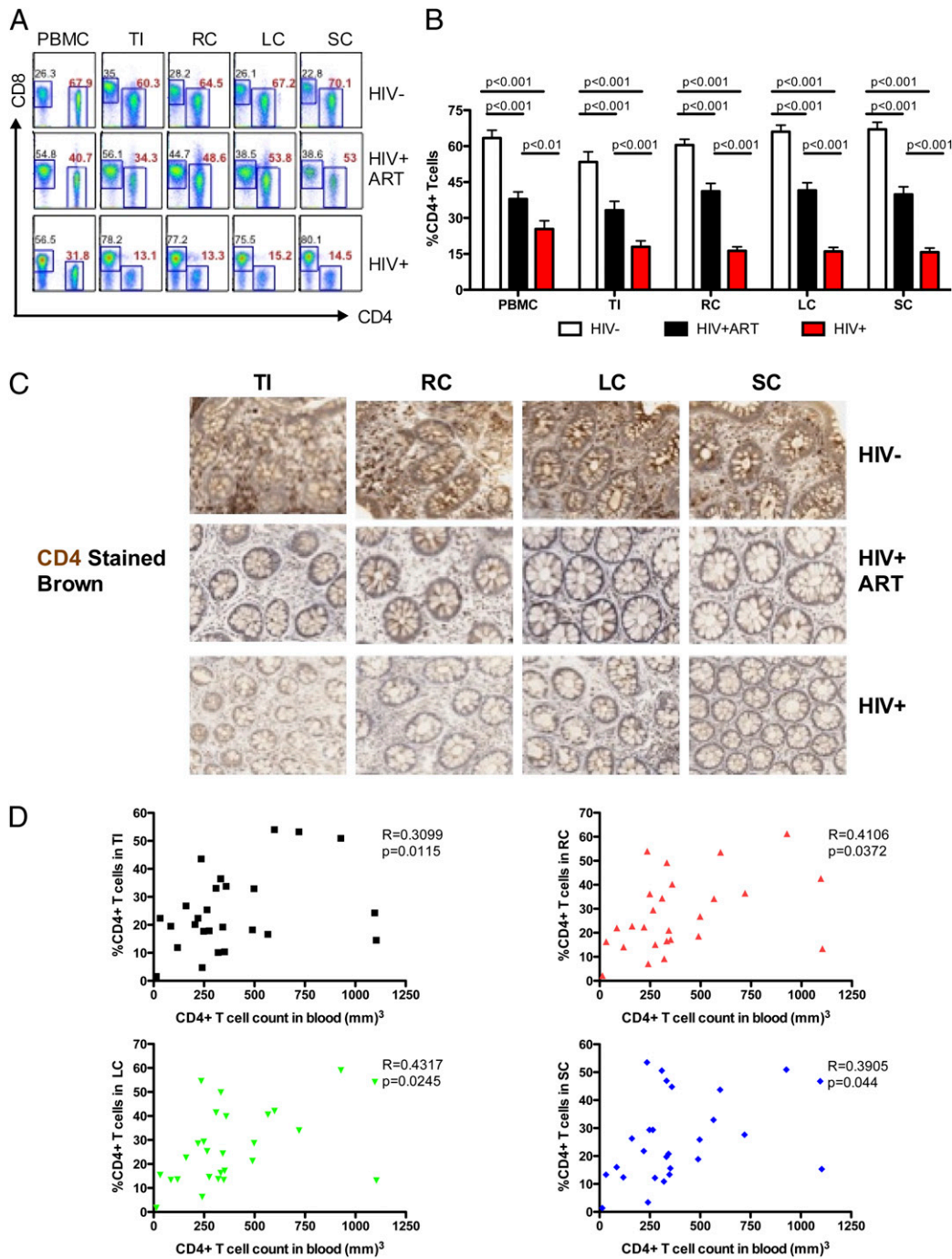
FIGURE 2. HIV infection causes a depletion of CD4$^+$ T cells. *A*, Representative flow plots showing the frequency of CD4$^+$ and CD8$^+$ T cells (pregated on CD3) in the blood (PBMCs) and from four sites along the intestinal tract (TI, RC, LC, and SC) in one HIV uninfected individual and two HIV-infected individuals, one of whom is on ART. *B*, Average frequency of CD4$^+$ T cells from the blood and the intestines in HIV uninfected individuals (white), HIV infected individuals on ART (black), and HIV infected individuals that are not on therapy (red). The *p* values were determined by a two-way ANOVA with a Dunn multiple comparison posttest. *C*, Representative immunohistochemical staining for CD4 (brown, original magnification ×20) in the intestines of one HIV uninfected individual and two HIV infected individuals, one of whom is on ART. *D*, The frequency of CD4$^+$ T cells at each intestinal site studied (TI, RC, LC and SC) is significantly correlated to the number of CD4$^+$ T cells in the blood. A Spearman rank correlation was used to determine the *R* and *p* values.

in the lamina propria of the intestinal mucosa at all sampled sites. Interestingly, we observed that, when we examined the entire population of HIV-infected individuals (i.e., both untreated and ART-treated), the level of CD4$^+$ T cell depletion in the blood was significantly directly correlated with the level of CD4$^+$ T cell depletion in the TI (*p* = 0.011), in the RC (*p* = 0.037), in the LC (*p* = 0.024), and in the SC (*p* = 0.044) (Fig. 2*D*).

HIV infection is associated with selective depletion of total and memory CD4$^+$CCR5$^+$ T cells in the GI tract

We next assessed, in our group of HIV-infected individuals and controls, the impact of HIV infection on the level of CD4$^+$ T cells expressing the HIV coreceptor CCR5 in blood as well as in four GI tract sites. As shown in Fig. 3*A*, which depicts a representative flow cytometric contour plot from an HIV uninfected control, and
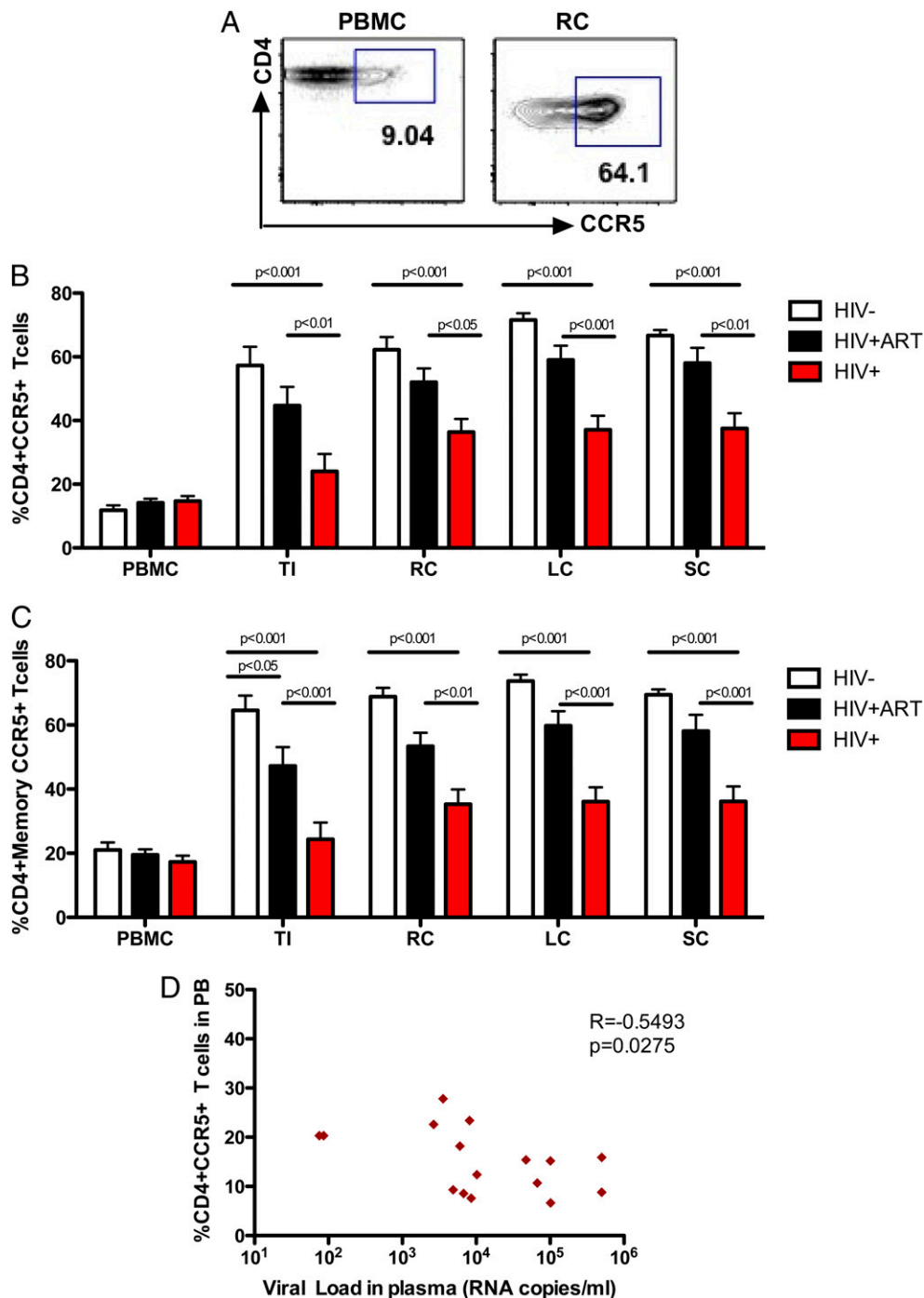
FIGURE 3.   CD4+ T cells expressing the coreceptor for HIV, CCR5, are significantly depleted from all intestinal sites. *A*, Representative flow cytometric plots showing CCR5 staining on CD4+ T cells in the blood and the RC. *B*, Average frequency of CD4+ T cells expressing CCR5 in the blood and from four sites along the intestinal tract (TI, RC, LC, and SC). HIV uninfected patients are shown in white, HIV-infected patients on ART are shown in black, and those not on therapy are shown in red. *C*, The frequency of memory CD4+ T cells expressing CCR5 in the blood and the intestines. *D*, The frequency of CD4+ T cells expressing CCR5 in the blood is inversely related to the viral load in the plasma. A Spearman rank correlation was used to determine *R* and *p* values.

in Fig. 3*B*, the level of CCR5 expression on CD4+ T cells is significantly higher in all intestinal sites as compared with peripheral blood. In the event of HIV infection, the fraction of circulating CD4+ T cells expressing CCR5 remained similar to that of uninfected controls (Fig. 3*B*). In contrast, and similar to what others have reported (4, 5, 32), we observed a significant depletion of CD4+CCR5+ T cells in all GI sites of HIV-infected individuals. Of note, the most severe depletion was found in the TI, and an

intermediate level of CD4+CCR5+ T cells was observed in patients treated with ART for at least a year (Fig. 3*B*). As shown in Fig. 3*C*, a similar trend was observed for memory CD4+ T cells expressing CCR5. Perhaps surprisingly, however, in our hands the extent of the depletion of intestinal CD4+CCR5+ T cells appeared to be less severe than what has been reported by others during HIV infection (4). Additionally, we found that HIV-infected individuals treated with ART consistently manifest a 15–20% higher fraction

of CD4$^+$CCR5$^+$ T cells (i.e., equivalent to a 30–50% increase) as compared with patients with active virus replication, thus suggesting a major impact of ART in replenishing the pool of intestinal CD4$^+$CCR5$^+$ T cells. Intriguingly, while we found an inverse correlation between plasma viremia and the fraction of CD4$^+$CCR5$^+$ T cells in blood (Fig. 3D), no correlation was observed between viremia and the fraction of CD4$^+$CCR5$^+$ T cells in any of the examined GI tract sites (data not shown). This latter finding suggests that a complex dynamic exists between the depletion of CD4$^+$CCR5$^+$ T cells in the GI tract and the level of HIV replication, which seems unlikely to be the only key factor involved in determining the level of this cell population.

Increased levels of T cell proliferation in the GI tract of HIV-infected individuals

In HIV-infected individuals, disease progression is associated with high virus replication and the presence of a chronic, generalized immune activation (14–18). To determine the relationship between CD4$^+$ T cell depletion and immune activation at the level of the intestinal mucosa, we measured the fraction of proliferating (i.e., Ki67$^+$) T cells in the blood as well as the four GI sites (i.e., TI, RC, LC, and SC) of all subjects included in this study. To normalize for the observed differences in the number of naive cells (which express very low levels of Ki67) in different tissues, we measured the frequency of proliferating cells within the memory CD4$^+$ and CD8$^+$ T populations. As shown in Fig. 4A, we found that HIV infection is associated with a significantly increased fraction of proliferating memory CD4$^+$ T cells in all studied tissues ($p < 0.001$ for blood, $p < 0.01$ for TI, $p < 0.001$ for RC, $p < 0.001$ for LC, and $p < 0.001$ for SC), and that the magnitude of this increase in memory CD4$^+$ T cell proliferation was comparable in blood and in all intestinal sites ($p =$ NS). We also found that ART-treated HIV-infected individuals showed significantly lower levels of memory CD4$^+$ T cell proliferation when compared with patients with active virus replication ($p < 0.05$ for blood, $p < 0.05$ for TI, $p < 0.001$ for RC, $p < 0.05$ for LC, and $p < 0.05$ for SC). In contrast, we found that HIV infection was associated with significantly increased levels of proliferating memory CD8$^+$ T cells in the blood but not in any of the studied GI sites, where the observed increases in CD8$^+$Ki67$^+$ T cells did not reach significance (Fig. 4B). Fig. 4C shows representative examples of Ki67 staining by immunohistochemistry in the lamina propria of the four examined GI tract sites that confirm the overall increased levels of T cell proliferation associated with HIV infection. We next assessed the relationship between memory T cell proliferation in the blood and GI tract in our entire population of HIV-infected individuals (i.e., untreated and ART-treated). As shown in Fig. 4D, the level of CD4$^+$ T cell proliferation in blood was strongly correlated with those measured in all four GI sites ($p < 0.01$ in all cases). In contrast, the level of memory CD8$^+$ T cell proliferation in blood correlated significantly with that of LC and RC ($p < 0.05$ in both cases) but not with that of the TI and SC (Fig. 4E). Collectively, these data indicate that HIV infection, particularly in patients with active virus replication, is associated with a clear increase in memory CD4$^+$ T cell proliferation in the GI tract, whose magnitude is strongly correlated with the level of memory CD4$^+$ T cell proliferation in peripheral blood.

Depletion of intestinal CD4$^+$ T cells and Th17 cells is directly correlated with increased levels of systemic and mucosal CD4$^+$ T cell proliferation

We next examined the relationship between depletion of intestinal CD4$^+$ T cells and the levels of systemic and intestinal CD4$^+$ T cell

proliferation in our entire population of HIV-infected individuals (both untreated and ART-treated). As shown in Fig. 5A, the level of CD4$^+$Ki67$^+$ in peripheral blood was inversely correlated with the extent of CD4$^+$ T cell depletion in the RC, LC, and SC ($p < 0.001$ in all cases), but not in the TI ($p =$ NS). A similar pattern of inverse correlation was observed, for each individual GI tract site, between the level of CD4$^+$ T cell depletion and CD8$^+$ T cell proliferation at that site (data not shown). Interestingly, we also found a significant inverse correlation between circulating CD4$^+$ Ki67$^+$ T cells and the fraction of IL-17–producing CD4$^+$ T cells (i.e., Th17 cells) (Fig. 5B). Of note, in these experiments of IL-17 production we pooled samples of all collected GI tract sites to analyze a sufficient number of Th17 cells. Finally, we also observed that the severity of CD4$^+$ T cell depletion and the levels of CD4$^+$ and CD8$^+$ T cell proliferation were directly correlated with each other in all examined GI tract sites (data not shown). In all, these data indicate the presence of a clear relationship between the level of systemic and intestinal T cell activation and the severity of intestinal CD4$^+$ T cell depletion (total and of Th17 cells).

Discussion

HIV infection is associated with an early, severe, and progressive depletion of mucosal CD4$^+$ T cells that is thought to result in an overall loss of mucosal immune function, which manifests itself in the GI tract as loss of integrity of the epithelial barrier and microbial translocation (9–11). The mechanisms linking CD4$^+$ T cell depletion in the GI tract and loss of mucosal integrity remain unclear and are likely complex, involving numerous factors including the loss or dysregulation of Th17 cells, NK cells, neutrophils, inflammatory cytokines, and defensins, along with increased epithelial apoptosis (2, 24–29, 46). Although the mucosal immune dysfunction associated with HIV infection and AIDS is a well-established phenomenon, several aspects of the HIV-associated mucosal CD4$^+$ T cell depletion remain controversial, including the level of reconstitution after ART, as well as the relationship between the level of systemic and mucosal CD4$^+$ T cell depletion and the extent of CD4$^+$ T cell activation and proliferation in the same compartments. Clarification of these aspects may have significant implications in terms of clinical management of HIV-infected individuals.

In this study, we tried to address some of these controversial issues by conducting a cross-sectional examination of the level of CD4$^+$ T cell depletion (total and of specific subsets) as well as the level of CD4$^+$ T cell proliferation in the blood and the GI tract of two groups of HIV-infected individuals (i.e., untreated and ART-treated) as well as healthy controls. A peculiar feature of this study was our ability to sample four different anatomic sites in the GI tract, which enabled us to conclude that the HIV-associated depletion of intestinal CD4$^+$ T cells (total and CCR5$^+$) is overall quite similar at the level of the TI, RC, LC, and SC. Interestingly, our study revealed that, in healthy individuals, there is a higher fraction of total CD4$^+$ T cells in the TI as compared with the sampled sites in the colon, and the presence of a trend toward fewer naive CD4$^+$ T cells when moving from the TI to the RC, LC, and SC.

As expected, our results confirmed a number of previous observations by other groups, including the fact that HIV infection is associated with a more severe depletion of CD4$^+$ T cells (and, in particular, CCR5$^+$ memory cells) in the GI tract than in blood (4–6). Similar to other groups (32, 35, 41), we also found that suppression of virus replication by ART results in levels of intestinal CD4$^+$ T cells that are intermediate between untreated patients and
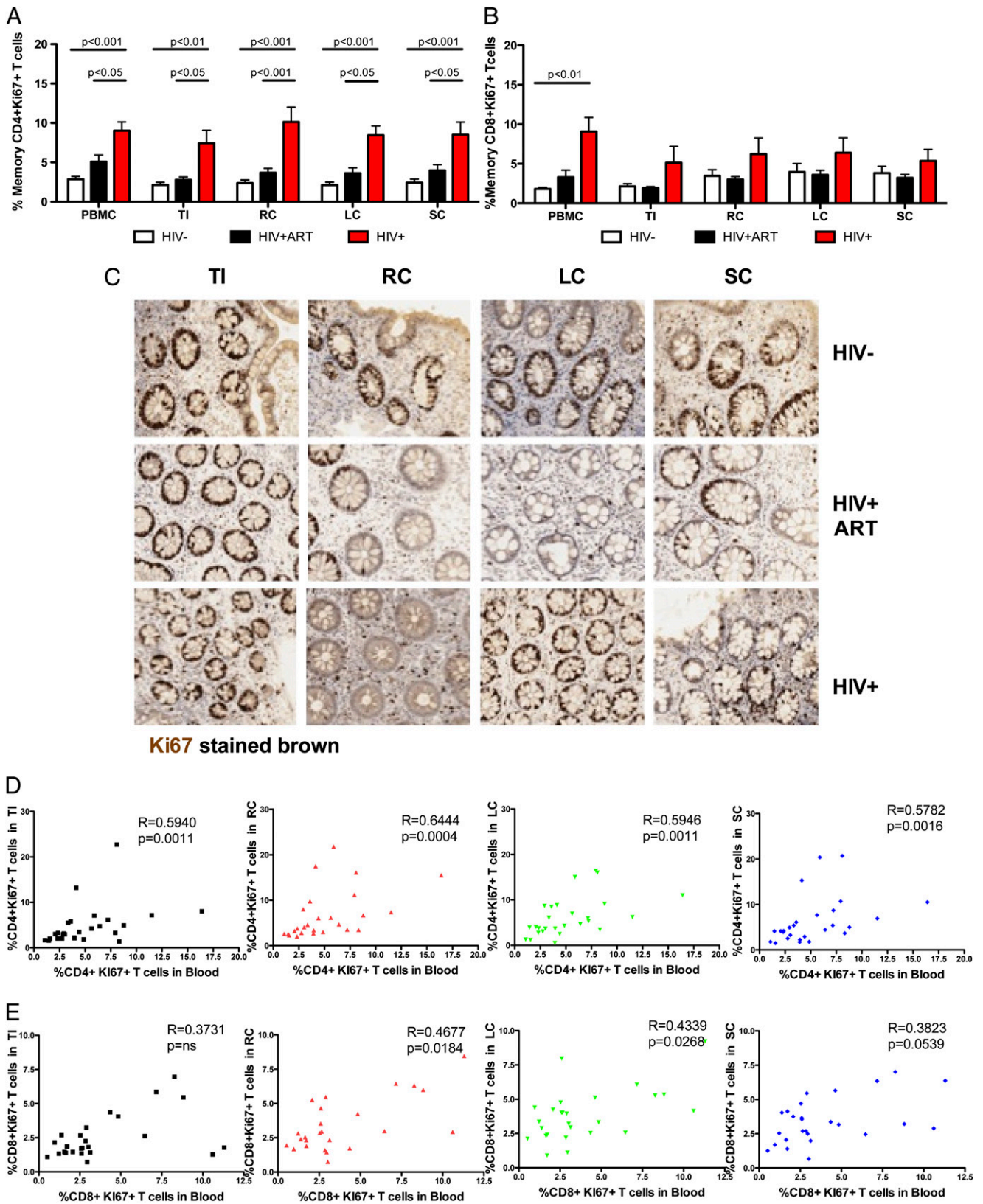
FIGURE 4. HIV induces generalized T cell activation in the blood and all sites of the GI tract. *A*, Average frequency of CD4$^+$ T cells expressing Ki67 in the blood and from four sites along the intestinal tract (TI, RC, LC, and SC). HIV uninfected patients are shown in white, HIV-infected patients on ART are shown in black, and those not on therapy are shown in red. *B*, Average frequency of CD8$^+$ T cells expressing Ki67 in the blood and the intestines. A two-way ANOVA with the Dunn multiple comparison posttest was used to determine the *p* values. *C*, Representative immunohistochemical staining for Ki67 (brown, original magnification ×20) in intestinal tissues from an HIV uninfected individual and from two HIV-infected individuals, one of whom is on ART. *D*, The frequency of Ki67 expression on CD4$^+$ T cells from the intestines correlates with the frequency of Ki67 expression on CD4$^+$ T cells from the blood. *E*, The frequency of Ki67 expression on CD8$^+$ T cells from the colon correlates with the frequency of Ki67 expression on CD8$^+$ T cells from the blood. A Spearman rank correlation was used to determine the *R* and *p* values.
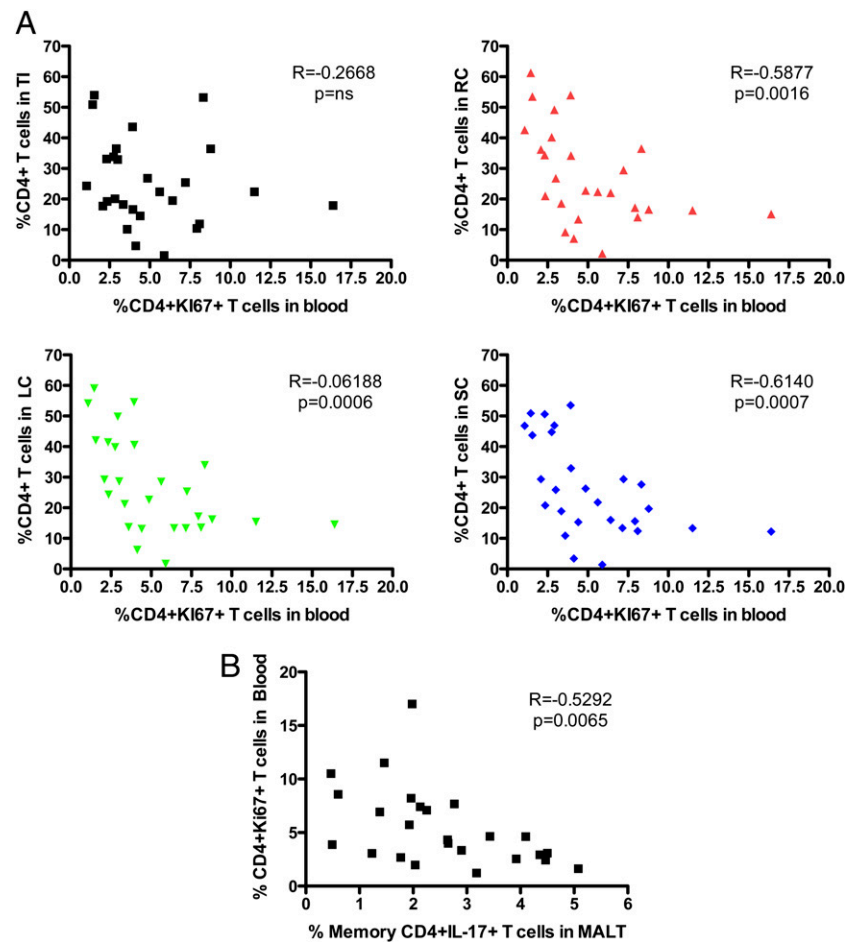
FIGURE 5. Systemic immune activation is a correlate of CD4^{+} T cell loss in the colon and the loss of IL-17–producing cells from the intestine. *A*, The frequency of CD4^{+} T cells in the colon is inversely related to the level of CD4^{+} T cells expressing Ki67 in the blood. *B*, The frequency of CD4 T cells expressing Ki67 in the blood is inversely related to the frequency of intestinal memory cells that express the cytokine IL-17.

healthy controls. This latter finding suggests that ART induces a significant, albeit incomplete, repopulation of CD4^{+} T cells (including CD4^{+}CCR5^{+} T cells) in the intestinal mucosa. Note, however, that in this study no baseline (i.e., pre-HIV infection) level of intestinal CD4^{+} T cells was available, thus making it impossible to assess the actual extent of CD4^{+} T cell reconstitution in each individual HIV-infected patient treated with ART. Surprisingly, we observed levels of intestinal CD4^{+} T cell depletion, both total and that of CD4^{+}CCR5^{+} T cells, that appeared to be less severe than those reported in other studies (4). This difference could be easily attributed to specific features of the studied patient population. Having said this, our present set of data also indicates that the level of HIV-associated depletion of CD4^{+}CCR5^{+} cells in the GI tract is much lower than what has been reported in SIV-infected macaques by us as well as by other groups (2, 3, 19), thus raising the possibility of substantial differences in the mucosal pathophysiology in these two models of pathogenic lentiviral infection. Consistent with the known association between progressive HIV infection and chronic immune activation (14–18), we also found that HIV infection with active virus replication is associated with an increased fraction of proliferating CD4^{+} T cells in both blood and GI tract, with ART-treated HIV-infected individuals showing levels of CD4^{+} T cell proliferation that were intermediate between those of untreated HIV-infected patients and healthy controls.

We next focused our analysis on the impact of HIV infection on the overall CD4^{+} T cell homeostasis by measuring the levels of both CD4^{+} T cell depletion and proliferation in the blood and the GI tract, and by examining how these immunological markers are correlated with each other. The goal of this analysis was to assess whether the relationship between CD4^{+} T cell depletion and CD4^{+} T cell activation was similar and interrelated in the various anatomic compartments, thus enabling us to determine whether distinct pathophysiologic events may be responsible for the HIV-induced immunopathogenesis at the level of blood and GI tract. We observed that 1) the levels of CD4^{+} T cell depletion in blood were directly correlated with the level of CD4^{+} T cell depletion in all examined GI tract sites, 2) the level of memory T cell proliferation in blood was also directly correlated with the level of memory T cell proliferation in the GI tract, and 3) the levels of CD4^{+} and CD8^{+} T cell proliferation in blood were directly correlated with the severity of CD4^{+} T cell depletion in the GI tract (both total and of Th17 cells). These observations are consistent with a model of HIV/AIDS pathogenesis wherein similar mechanisms (or at least mechanisms that act with similar temporal kinetics) induce CD4^{+} T cell depletion and increased CD4^{+} T cell proliferation in the blood and intestinal mucosal tissues. In other words, our results suggest that the immunological effects of HIV infection are not compartmentalized between these two distinct anatomic sites (i.e., peripheral blood and intestine). In this context, the biologic link between CD4^{+} T cell depletion and increased fraction of proliferating CD4^{+} T cells may be complex, and it may reflect both the known phenomenon that progressive HIV infection is associated with hyperimmune activation, and the fact that CD4^{+} T cell depletion may activate homeostatic mechanisms aimed at repopulating this cellular compartment. We favor the hypothesis that a predominant role in inducing the increased CD4^{+} T cell proliferation is played by the HIV-associated hyperimmune

activation, since other nondepleted circulating cell types (i.e., CD8$^+$ T cells) are also recruited into cycles of activation and proliferation during HIV infection. On the other hand, we emphasize that our study did not reveal an increase in CD8$^+$ T cell proliferation in the GI tract of HIV-infected individuals, perhaps consistent with the presence of local, lineage-specific and homeostasis-driven factors responsible for the selective increase of CD4$^+$Ki67$^+$ T cells. Collectively, our results suggest that AIDS pathogenesis is associated with a pathophysiological vicious cycle that starts with the loss of mucosal CD4$^+$ T cells caused by both virus-mediated killing as well as bystander apoptosis (as suggested by the lack of significant correlation between plasma viral load and level of intestinal CD4$^+$ T cells). This mucosal CD4$^+$ T cell loss would then result in an impairment of mucosal immune function, increased mucosal and systemic immune activation that is (at least in part) caused by microbial translocation, increased virus replication due to higher numbers of activated CD4$^+$ T cells that act as targets for HIV, and further systemic and mucosal CD4$^+$ T cell depletion.

A key question in terms of clinical management of HIV-infected individuals is whether relying only on peripheral blood CD4$^+$ T cell counts to assess the level of immunodeficiency (and thus the risk of opportunistic infections) represents a sufficiently informative approach or, alternatively, is there added clinical benefit in periodically sampling the GI tract of HIV-infected individuals to directly assess the impact of the infection on mucosal immunity. In this regard the present study, while confirming the presence of specific differences in the immunological impact of HIV infection in the blood and gut (i.e., the severity of CD4$^+$ T cell depletion), indicated that the level of CD4$^+$ T cell depletion and proliferation in the blood is by and large predictive of the level of the same markers in the GI tract. As such, this study does not provide substantial evidence in favor of the concept that periodic sampling of the intestinal mucosa will translate into a tangible benefit in the clinical management of HIV-infected individuals. In contrast, our data suggest that the immunopathogenesis of HIV infection and AIDS is related to pathophysiological mechanisms that appear to act concomitantly in the blood and the GI tract.

Acknowledgments

Disclosures

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