The effect of timing of antiretroviral therapy on CD4$^+$ T-cell reconstitution in the intestine of HIV-infected patients

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Whether and to what extent gut mucosal CD4$^+$ T cells of HIV-infected patients can be restored by combination antiretroviral therapy (cART) is not yet fully resolved. We studied absolute numbers, differentiation, and activation of mucosal CD4$^+$ T cells at different stages of HIV infection and assessed the effect of timing of cART initiation on this cell population. Mucosal CD4$^+$ T-cell numbers were severely reduced at all stages of chronic infection, but normal in patients with acute infection. In patients with initiation of cART during chronic HIV infection, mucosal CD4$^+$ T cells restored to less than half of the numbers in controls. However, in patients who initiated cART during acute HIV infection, mucosal CD4$^+$ T-cell numbers were fully preserved and markers of microbial translocation and inflammation reversed to normal. The proportion of mucosal effector memory CD4$^+$ T cells normalized only if cART was initiated at >350 CD4$^+$ T cells per μl blood but not with delayed treatment. In conclusion, mucosal CD4$^+$ T-cell numbers can be preserved if cART is initiated in acute HIV infection. In chronically HIV-infected patients, early cART improves mucosal CD4$^+$ T-cell differentiation but cannot prevent the persistent lack of total CD4$^+$ T cells.

INTRODUCTION

In contrast to the progressive decline of CD4$^+$ T cells usually observed in the peripheral blood, the gastrointestinal tract has been shown to be massively depleted of CD4$^+$ T cells at all stages of HIV infection.[1–3] Depletion of CD4$^+$ T cells in the intestinal mucosa has been linked to the immune dysfunction and loss of intestinal barrier function that is thought to trigger systemic immune hyperactivation and disease progression.[4–7] In patients on combination antiretroviral therapy (cART) with suppressed viral load, incomplete CD4$^+$ T-cell recovery and residual immune activation increase the risk of morbidity and mortality.[8,9] According to observations in peripheral blood, efficiency of both CD4$^+$ T-cell recovery and abrogation of T-cell activation varies depending on several factors including the extent of immunodeficiency at the time of cART initiation.[10–12] Consequently, early initiation of cART, before CD4$^+$ T cells counts fall below 350 cells per μl of peripheral blood, is currently recommended by international guidelines.[13] However, treated patients continue to be at risk for diseases commonly associated with age-related immune senescence including non-AIDS-associated cancers, cardiovascular disease, and liver disease.[9] Although the regenerative capacity of CD4$^+$ T cells in the gastrointestinal mucosa may well considerably affect the clinical course of treated HIV disease, the effect of the timing of cART on mucosal CD4$^+$ T cells is still under debate.

Most published data on mucosal immune reconstitution are based on relative CD4$^+$ T-cell percentages and demonstrate wide variation in the degree of CD4$^+$ T-cell recovery, ranging from minimal to complete.[14–19] Data on relative cellular frequencies are always influenced by the size of other cell populations and may therefore not accurately reflect the true efficiency amount of T-cell recovery. For example, previous studies have demonstrated full recovery in absolute numbers of colonic mucosal CD4$^+$ T cells in treated HIV-infected patients, whereas CD4$^+$ T cell percentages, assessed in parallel by flow cytometry, remained reduced.[20,21] Furthermore, data from a clinical trial suggest that the decline in CD8$^+$ T-cell numbers in

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response to cART makes an important contribution to the increase in $CD4^+$ T-cell percentage in duodenal tissue.[22] Although these observations emphasize the need for extensive quantitative studies, published data on absolute numbers of mucosal T cells in the context of HIV infection are sparse.[21–23] Therefore, we conducted a quantitative analysis of mucosal $CD4^+$ T cells in untreated and treated patients at different stages of HIV disease in comparison to HIV-uninfected control subjects.

RESULTS

Definition and characteristics of study patients

We performed a cross-sectional study of the duodenal mucosa in HIV-infected individuals. Paired duodenal biopsies and peripheral blood were collected from 76 HIV-infected subjects either naïve of cART ($n = 48$) or under effective cART ($n = 28$). The treatment-naïve group included patients with the diagnosis of acute HIV infection (acute; $n = 9$) defined by the presence of symptoms compatible with an acute antiretroviral syndrome, a reactive HIV screening ELISA test with incomplete HIV immune blotting (≤ 4 bands) and detectable HIV RNA. Treatment-naïve patients with chronic HIV infection were divided into three groups: peripheral $CD4^+$ T-cell counts greater than 350 per µl of blood (early-stage disease; $n = 11$), $CD4^+$ T-cell counts 200–350 per µl (intermediate-stage disease; $n = 8$), and $CD4^+$ T-cell counts lower than 200 per µl (late-stage disease; $n = 20$). The cART group included patients who started treatment immediately after the diagnosis of acute HIV infection (acute cART; $n = 5$). Patients who started cART during the chronic stage of HIV infection were divided into groups according to their peripheral $CD4^+$ T-cell counts at the time of cART initiation (> 350 per µl, early-stage cART; $n = 5$, 200–350 per µl, intermediate-stage cART; $n = 6$, and < 200 per µl, late-stage cART; $n = 12$). Differences in duration of cART among the treated study groups were not statistically significant. Characteristics of the study participants are summarized in **Table 1**.

Quantitative *in situ* analysis of mucosal $CD4^+$ and $CD8^+$ T cells in patients at different stages of HIV disease

Mucosal $CD4^+$ and $CD8^+$ T cells in the duodenum were quantified by immunohistochemical analysis (**Figure 1**). In acutely HIV-infected patients, mucosal $CD4^+$ T-cell numbers were not significantly different from those of healthy controls, whereas mucosal $CD8^+$ T-cell numbers were higher than in HIV-negative controls (**Figures 1** and **2**). Early accumulation of $CD8^+$ T cells thus caused a relative decrease in the mucosal $CD4^+$ T-cell frequency. Mucosal $CD8^+$ T-cell numbers in acute HIV infection correlated positively with peripheral $CD8^+$ T-cell counts ($r = 0.833$, $P = 0.015$), and negatively with plasma viral loads ($r = -0.893$, $P = 0.012$) indicating a systemic immune response to acute viral infection. In patients with chronic HIV infection, severe depletion of mucosal $CD4^+$ T cells as well as increased mucosal $CD8^+$ T-cell numbers were observed at all stages (**Figure 2a** and **b**). There was no significant correlation of mucosal $CD4^+$ or $CD8^+$ T-cell numbers with plasma viral loads or peripheral T-cell counts in chronically HIV-infected patients.

To assess the efficacy of cART on mucosal $CD4^+$ T-cell recovery, we quantified T cells in biopsy samples from treated patients with undetectable viral load. Only in patients who had started cART at diagnosis of acute HIV infection, mucosal $CD4^+$ T-cell numbers were at similar levels as in the HIV-negative controls (**Figures 1** and **2a**). Mucosal $CD8^+$ T-cell numbers in these patients were not significantly different to those of controls but showed a large variation compared with control mucosal $CD8^+$ T-cell numbers (**Figure 2b**). In contrast, the three groups of patients who had started cART during chronic HIV infection, mucosal $CD4^+$ T cells were reduced by 40–70% relative to control cell numbers (**Figure 2a**). Mucosal $CD4^+$ T-cell numbers correlated with peripheral $CD4^+$ T-cell counts ($r = 0.668$, $P = 0.001$) in treated chronically HIV-infected patients, whereas the extent of persistent $CD4^+$ T-cell depletion was higher in the intestinal mucosa than in the peripheral blood (**Figure 2a** and **Table 1**). Mucosal $CD8^+$ T-cell numbers decreased in all treatment groups but did not normalize in all patients (**Figure 2b**). These results demonstrate that partial depletion of mucosal $CD4^+$ T-cell numbers persists in all patients who started cART during the chronic phase of HIV infection. However, initiating cART during acute HIV-infection enables preservation of mucosal $CD4^+$ T-cell numbers.

Impact of HIV infection and cART on the phenotype of mucosal $CD4^+$ T cells

A characteristic hallmark of the mucosal immune system is the memory phenotype of T cells.[24] To determine the effect of HIV disease progression and cART on mucosal $CD4^+$ T-cell differentiation, we measured the proportionate representation of effector memory (T_{EM}), central memory (T_{CM}), and terminally differentiated memory (T_{TD}) cells among $CD4^+$ T cells. Five to ten individuals of each study group were analyzed.

In both controls and acutely HIV-infected patients, mucosal $CD4^+$ T cells in the duodenum were essentially all T_{EM} cells (**Figure 3a**). In chronic HIV infection, the proportion of T_{EM} phenotype cells within mucosal $CD4^+$ T cells decreased relative to that in controls, with the largest decline in late-stage disease (**Figure 3a**). This reduction of $CD4^+$ T_{EM} cells correlated with both the increase of plasma viral load ($r = -0.567$, $P = 0.018$) and the selective enrichment of mucosal $CD4^+$ T_{TD} cells (**Figure 3b and c**), indicating that HIV-mediated cell death as well as enhanced recruitment of mucosal $CD4^+$ T cells to the terminal stage of differentiation may contribute to the loss of T_{EM} cells from the mucosal $CD4^+$ T-cell pool. There was no correlation between the proportion of T_{EM} cells and the level of T_{CM} cells (**Figure 3d**). In treated patients who started cART during acute or early chronic HIV infection, the phenotype of mucosal $CD4^+$ T cells did not differ significantly from that of healthy controls (**Figure 3a, b** and **d**). In treated patients with less than 350 $CD4^+$ T cells per µl of peripheral blood at initiation of cART, the proportion of T_{EM} cells in the mucosal

Table 1 Characteristics of HIV-infected patients and control subjects in this study

Variable	Treatment-naïve patients				Treated patients				Control subjects
	Acute	Chronic			Acute	Chronic			
		Early	Interm.	Late		Early	Interm.	Late	
n	9	11	8	20	5	5	6	12	14
Sex, male/female	9/0	8/3	7/1	14/6	5/0	3/2	5/1	8/4	14/0
Age, years	38 (22–48)	35 (21–61)	38 (23–64)	42 (34–74)	48 (30–58)	69 (36–75)	53 (31–62)	52 (27–70)	44 (26–77)
$CD4^+$ T-cell count, cells per µl	820 (603–1,633)	552 (277–1,015)	446 (364–657)	273 (200–334)	547 (327–721)	940 (383–1,075)	838 (589–878)	330 (120–548)	62 (4–194)
Viral load, log_{10} copies per ml	6.00(4.9–7.0)	4.8(3.6–6.0)	4.9(3.8–5.2)	5.3(4.1–5.7)	Below limit of detection (20 HIV-RNA copies per ml)				NA
Duration of cART, months	NA	NA	NA	NA	21 (12–60)	82 (36–116)	86 (19–120)	58 (16–108)	NA

Data are median value (range), unless otherwise indicated.
Abbreviations: cART, antiretroviral therapy; Interm., intermediate; NA, not applicable; ND, not determined.

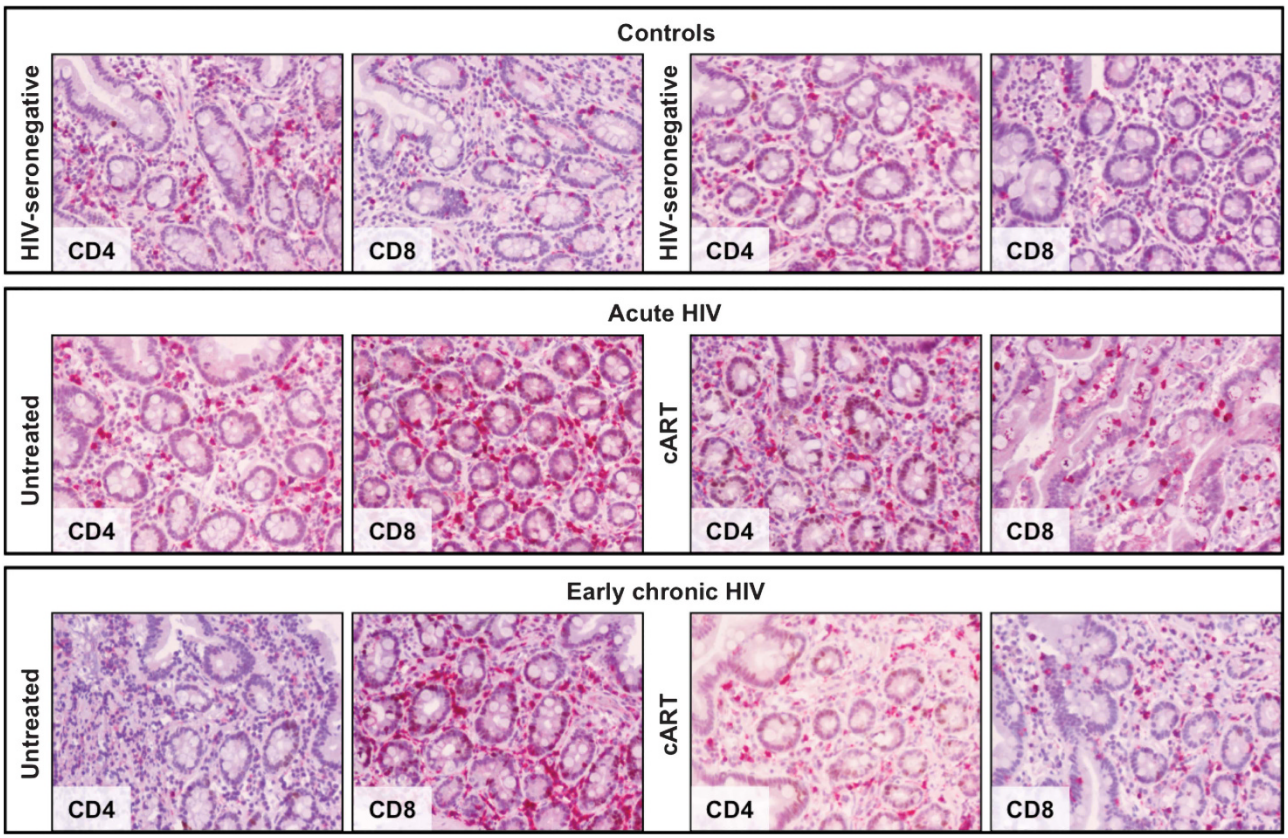

Figure 1 Representative immunohistochemical analysis of mucosal $CD4^+$ or $CD8^+$ T cells (red) in duodenal mucosa from healthy controls and from treatment-naïve and treated acutely HIV-infected or early-stage chronically HIV-infected patients. cART, combination antiretroviral therapy.

$CD4^+$ T-cell compartment was reduced (**Figure 3a**). Thus, start of cART at $CD4^+$ T-cell counts of less than 350 per µl of blood is associated with persistent lack of mucosal $CD4^+$ T_{EM} cells.

Proliferation of mucosal and peripheral $CD4^+$ T-cell subsets

To determine whether or not proliferation of $CD4^+$ T-cell subsets contributes to alterations in the mucosal $CD4^+$ T-cell

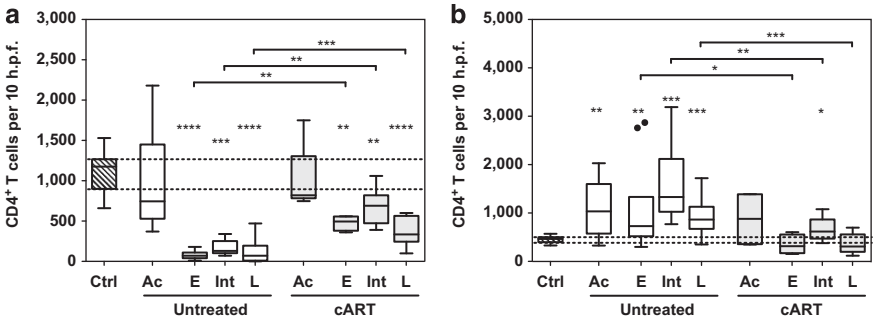

Figure 2 Mucosal CD4$^+$ and CD8$^+$ T-cell numbers in treatment-naïve and treated patients at different stages of HIV infection. (**a**) Mucosal CD4$^+$ or (**b**) CD8$^+$ T-cell numbers in duodenal mucosa from healthy controls and from treatment-naïve and treated HIV-infected patients. Ac, acute stage; cART, combination antiretroviral therapy; Ctrl, healthy HIV-negative controls; E, early-stage; Int, intermediate-stage; L, late-stage HIV disease. *$P<0.05$, **$P\leq0.01$, ***$P<0.001$, ****$P<0.0001$.

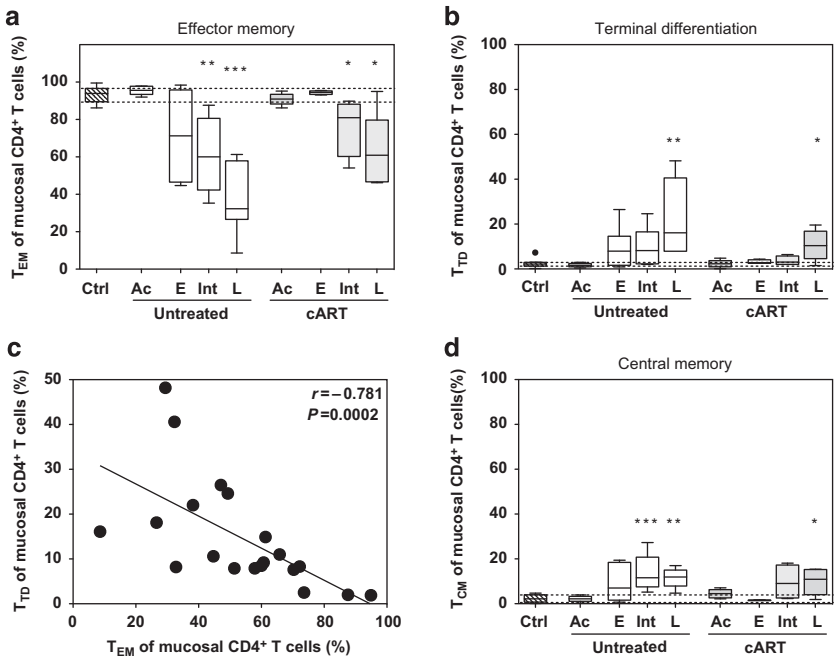

Figure 3 Composition of the mucosal CD4$^+$ T-cell compartment in HIV-infected patients at different stages of disease. Percentages of (**a**) T_{EM} (CD45RO$^+$CD62L$^-$), (**b**) T_{TD} (CD45RO-CD62L-), and (**d**) T_{CM} (CD45RO$^+$CD62L$^+$), cells among CD4$^+$ T cells in the duodenum of treatment-naïve and cART-treated HIV-infected patients in comparison to HIV-negative controls. (**c**) Correlation between CD4$^+$ T_{EM} and T_{TD} cell frequencies in the duodenal mucosa of treatment-naïve chronically HIV-infected patients. Ac, acute stage; cART, combination antiretroviral therapy; Ctrl, healthy HIV-negative controls; E, early-stage; Int, intermediate-stage; L, late-stage HIV disease. *$P<0.05$, **$P\leq0.01$, ***$P<0.001$, ****$P<0.0001$.

composition during HIV disease progression, analysis of CD4$^+$ T cells subset representation was combined with measurement of intranuclear Ki-67 expression, that is present during all active phases of the cell cycle and is closely related to cell proliferation.[25] In controls, the proportion of Ki-67-expressing CD4$^+$ T_{EM} cells was lower in the duodenal mucosa than in the peripheral blood ($P=0.0099$). During chronic HIV infection, proliferation of both mucosal CD4$^+$ T_{EM} cells and peripheral CD4$^+$ T_{EM} cells was increased compared with controls (**Figure 4a** and **b**). Peripheral but not mucosal CD4$^+$ T_{CM} did proliferate during the chronic phase of HIV infection (**Figure 4c** and **d**). CD4$^+$ T_{TD} showed no proliferation in both compartments (data not shown). Together, these results demonstrate that neither impaired proliferation of CD4$^+$ T_{EM} cells nor proliferation of mucosal CD4$^+$ T_{CM} or T_{TD} cells is the driving force behind the contraction of the mucosal T_{EM} cell pool in untreated chronic HIV infection. Proportions of proliferating mucosal CD4$^+$ T_{EM} cells correlated with mucosal CD4$^+$ T_{TD} cell frequencies ($r=0.5646$, $P=0.0112$). Together with the negative correlation between mucosal CD4$^+$ T_{EM} and CD4$^+$ T_{TD} cell frequencies described above (**Figure 3c**), this suggests that enhanced recruitment of dividing CD4$^+$ T_{EM}

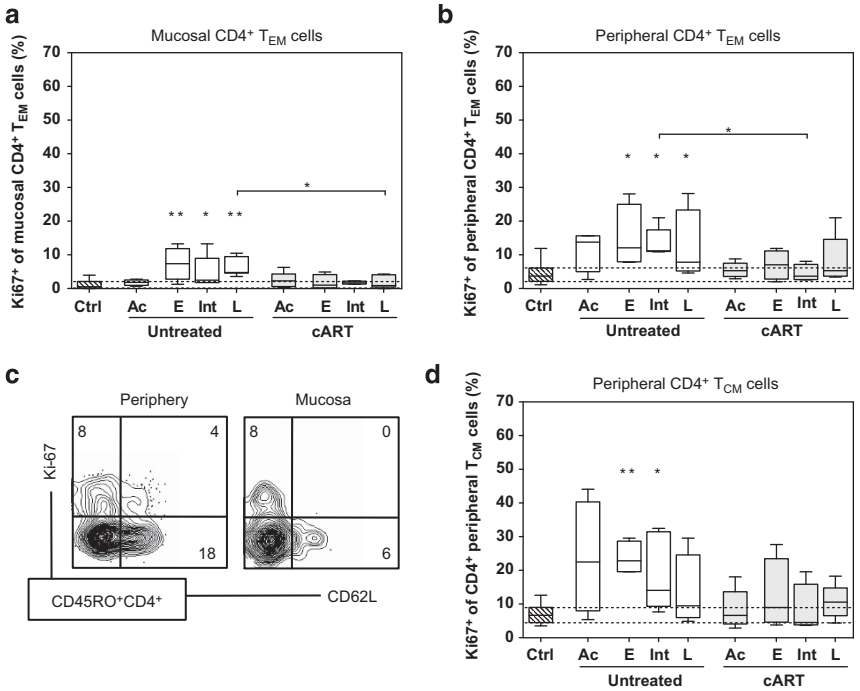

Figure 4 Proliferation of mucosal and peripheral memory CD4$^+$ T-cell subsets. Proliferating fraction within (**a**) mucosal CD4$^+$ T_{EM}, (**b**) peripheral CD4$^+$ T_{EM}, and (**d**) peripheral CD4$^+$ T_{CM} of healthy controls and treatment-naïve or cART-treated HIV-infected patients were assessed by flow cytometric analysis of intranuclear Ki-67 expression. (**c**) CD4$^+$ T_{CM} (CD45RO$^+$CD62L$^+$) proliferate in the peripheral blood but not in the duodenal mucosa, whereas CD4$^+$ T_{EM} (CD45RO$^+$CD62L$^-$) cells proliferate in both compartments. Ac, acute stage; cART, combination antiretroviral therapy; Ctrl, healthy HIV-negative controls; E, early-stage; Int, intermediate-stage; L, late-stage HIV disease. *$P<0.05$, **$P\leq0.01$, ***$P<0.001$, ****$P<0.0001$.

cells into the terminal stage of differentiation contributes to alterations in the mucosal CD4$^+$ T-cell phenotype. With cART, proliferation of mucosal CD4$^+$ T_{EM} cells in all study groups was not different to that of controls (**Figure 4a**).

CD4$^+$ T-cell activation during HIV disease progression and in response to cART

HIV infection leads to activation-induced T-cell exhaustion, characterized by the surface expression of various activation antigens. We investigated this activation, by measuring the early-activation-related antigen CD38 (refs 26, 27) and the late-activation-related antigen HLA-DR[27,28] on mucosal CD4$^+$ T cells in comparison to their peripheral counterparts. As shown in **Figure 5a, c,** and **e**, the majority of CD4$^+$ T cells in the duodenum of controls expressed CD38, whereas HLA-DR was present on a minor fraction of mucosal CD4$^+$ T cells. Frequencies of CD38$^+$ HLA-DR$^-$ (early-activation) and CD38$^+$ HLA-DR$^+$ CD4$^+$ T cells (intermediate-activation) were higher in the duodenal mucosa than in the peripheral blood (**Figure 5a–d**), which is compatible with the well-known high level of immune activation in the intestinal mucosa. In acute HIV infection, the CD38$^+$ HAL-DR$^+$ fraction among mucosal, but not peripheral, CD4$^+$ T cells was increased indicating a strong T-cell response in the intestinal mucosa (**Figure 5c** and **e**). During untreated chronic HIV infection, decreasing frequencies of CD38$^+$HLA-DR$^-$ CD4$^+$ T cells were associated with an increase of CD38$^-$HLA-DR$^+$-expressing CD4$^+$ T cells (late-activation) in the duodenum ($r=-0.611$, $P=0.0009$) indicating that activated mucosal CD4$^+$ T cells progress to late-stage activation (**Figure 5a** and **f**). On cART, activation pattern of mucosal CD4$^+$ T cells remained altered in all groups.

cART-induced reduction of systemic inflammation

Enhanced translocation of microbial products across the damaged epithelial barrier is thought to trigger systemic immune hyperactivation in HIV-infected persons.[5,29] To determine whether preservation of mucosal CD4$^+$ T cells in treated acutely HIV-infected patients is associated with reversion of epithelial barrier defect and systemic immune activation, we measured plasma concentrations of lipololysaccharide (LPS)-binding protein (LBP) and soluble CD14 (sCD14), both of which are produced in response to the microbial product LPS, interleukin 6 (IL-6) that can be secreted upon stimulation by several factors including LPS and other microbial products, the inflammation biomarkers C-reactive protein and D-dimer, and intestinal fatty acid-binding protein (I-FABP), which is released into the bloodstream upon enterocyte damage.

In acutely as well as chronically HIV-infected patients, plasma levels of LBP, sCD14, and IL-6 were increased (**Figure 6a–c**). Levels of LBP and sCD14 correlated with

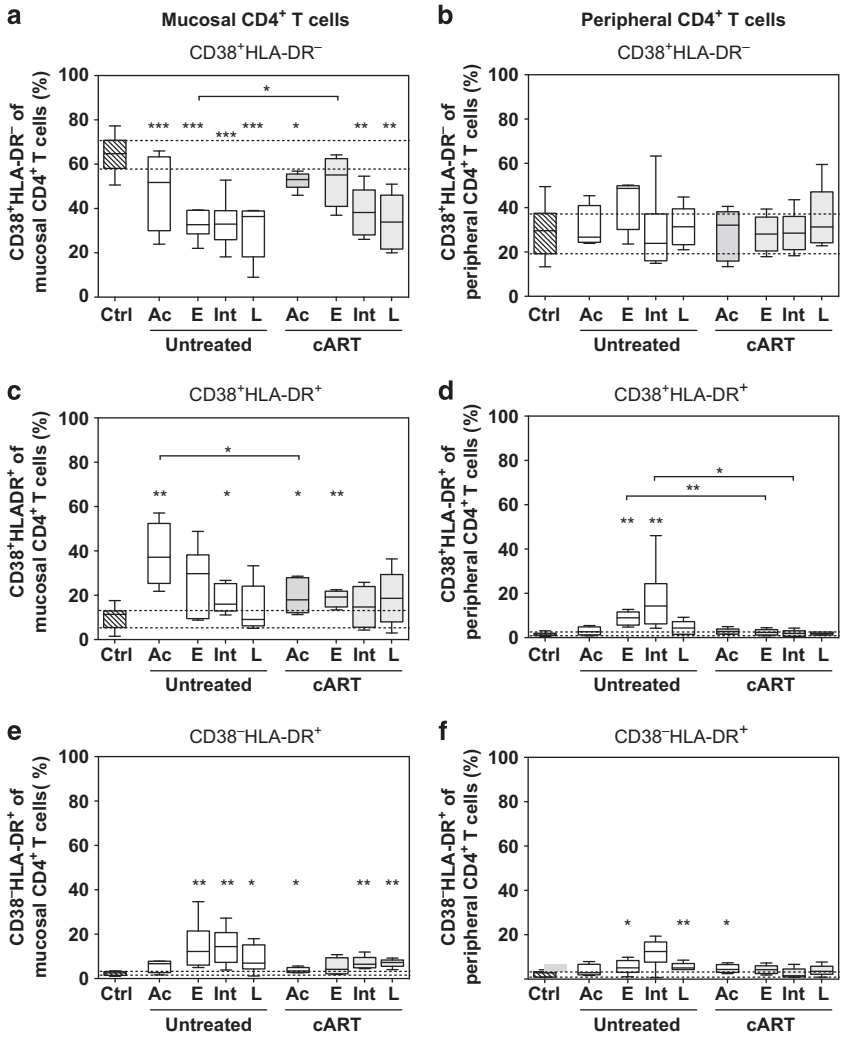

Figure 5 Expression of antigens associated with T-cell activation on mucosal or peripheral CD4$^+$ T cells of HIV-infected patients at different stages of disease. Percentages of (**a**) CD38$^+$HLA-DR$^-$, (**b**) CD38$^+$HLA-DR$^+$, and (**c**) CD38$^-$HLA-DR$^+$ within CD4$^+$ T cells in the duodenum and (**d–f**) in the peripheral blood of HIV-negative controls and treatment-naïve or cART-treated HIV-infected patients. Ac, acute stage; cART, combination antiretroviral therapy; Ctrl, healthy HIV-negative controls; E, early-stage; Int, intermediate-stage; L, late-stage HIV disease. *$P<0.05$, **$P\leq0.01$, ***$P<0.001$, ****$P<0.0001$.

each other ($r=0.611$; $P<0.0001$) and with plasma concentrations of C-reactive protein ($r=0.647$; $P<0.0001$ and $r=0.370$; $P=0.017$, respectively) and D-dimer ($r=0.396$; $P=0.015$ and $r=0.372$; $P=0.016$, respectively) confirming the well-known association of host response to microbial products with systemic inflammation.[5] In treated patients who had started cART during chronic HIV infection, plasma levels of IL-6 and LBP remained significantly increased (**Figure 6a** and **c**), which suggests that immune activation induced by microbial products persists in patients with insufficient CD4$^+$ T-cell reconstitution. Consistent with this, plasma levels of I-FABP indicate enhanced intestine epithelial damage in these patients (**Figure 6f**). In contrast, in treated patients who had started cART at diagnosis of acute HIV infection, levels of all biomarkers were in the range of control values (**Figure 6a–f**). Mucosal CD4$^+$ T-cell numbers inversely correlated with LBP ($r=-0.2845$; $P=0.0335$), sCD14 (**Figure 6g**), and D-dimer (**Figure 6h**). These data suggest that preservation of mucosal T cells by early start of cART may reduce systemic spread of bioactive microbial products and systemic inflammation.

DISCUSSION

We studied quantitative and phenotypic alterations of CD4$^+$ T cells in the duodenal mucosa of HIV-infected patients in the context of different stages of HIV disease and timing of cART initiation. One major finding was that absolute CD4$^+$ T-cell numbers in the intestinal mucosa of HIV-infected patients were preserved, if cART was initiated during the acute phase of the disease. According to our quantitative analysis, decline in mucosal CD4$^+$ T-cell percentages that has often been described for untreated acute HIV infection[1,2,14,15,20] is largely

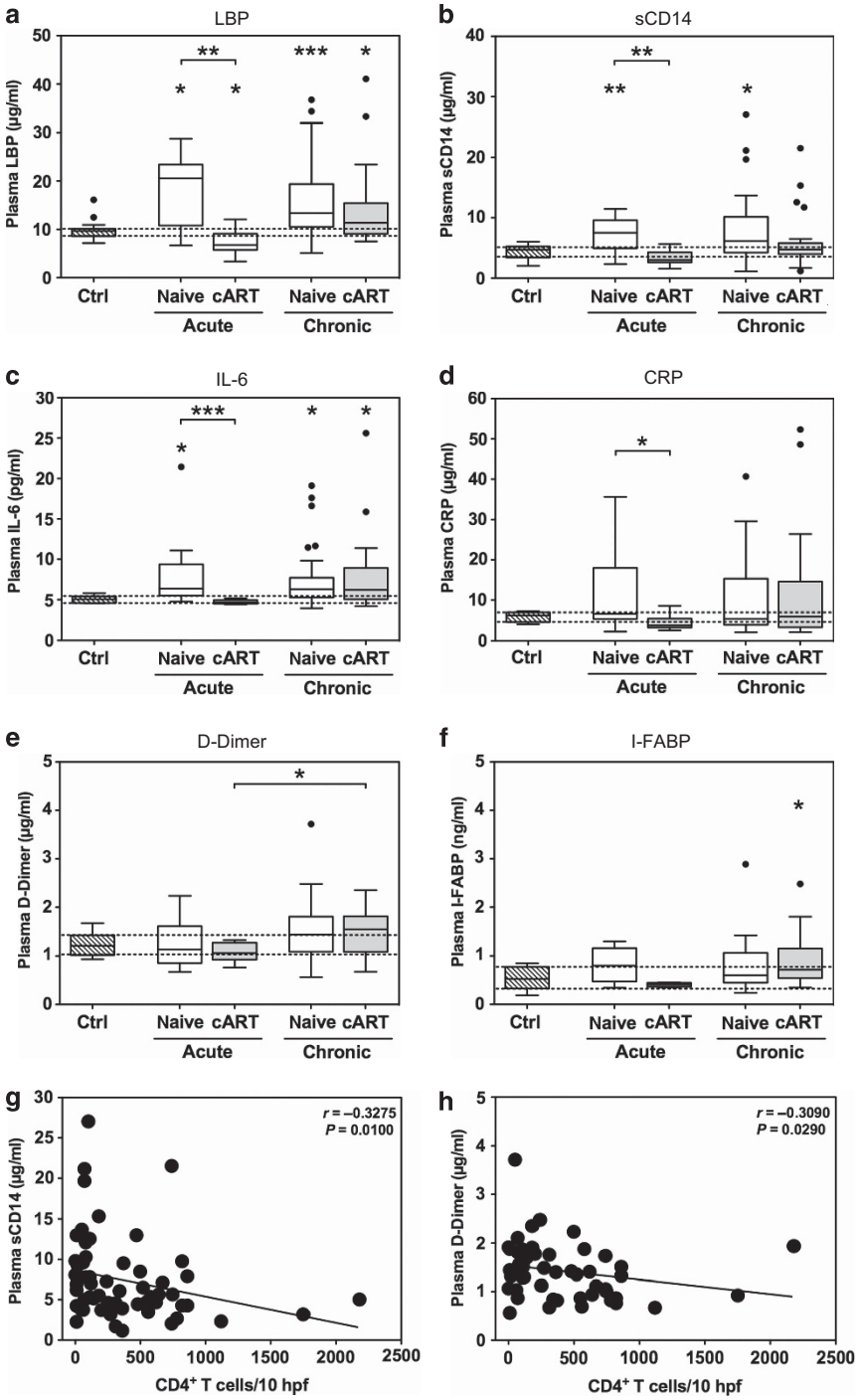

Figure 6 Biomarkers of microbial translocation and systemic inflammation. Plasma concentrations of (**a**) lipololysaccharide (LPS)-binding protein (LBP), (**b**) sCD14, (**c**) interlukin (IL)-6, (**d**) C-reactive protein (CRP), (**e**) D-Dimer, and (**f**) intestinal fatty acid-binding protein (I-FABP) in treatment-naïve and cART-treated acutely or chronically HIV-infected patients in comparison to HIV-negative controls. Correlations of mucosal $CD4^+$ T-cell numbers with plasma levels of (**g**) sCD14 and (**h**) D-Dimer in HIV-infected patients. cART, combination antiretroviral therapy. $^*P<0.05$, $^{**}P\leq0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

caused by an early accumulation of $CD8^+$ T cells in the intestinal mucosa, which probably occurs as part of the systemic antiviral immune response. In treated acutely HIV-infected patients, we observed a large variation in mucosal $CD8^+$ T-cell numbers when compared with those in controls and the other treated patient groups indicating that mucosal $CD8^+$ T-cell accumulation can be partially maintained. Preservation of mucosal $CD4^+$ T cells in treated acutely

HIV-infected patients may therefore be missed if their frequency is determined as a percentage of total mucosal T cells. Furthermore, levels of biomarkers of systemic inflammation and intestine epithelial damage were comparable between treated acutely HIV-infected patients and controls, which suggests that the well-preserved mucosal $CD4^+$ T cell pool may help to attenuate inflammatory disease progression.

In treatment-naïve chronically HIV-infected patients, mucosal $CD4^+$ T cells were severely depleted at all stages of the disease. Maximum severity of loss of mucosal $CD4^+$ T-cell numbers was already achieved in the early stage of chronic HIV disease, that is, when $CD4^+$ T-cell count in peripheral blood is still over 350 per µl. In contrast to peripheral blood, where T_{EM} cell frequencies among peripheral $CD4^+$ T cells rather increase during chronic disease progression,[30] T_{EM} cells among $CD4^+$ T cells in the intestinal mucosa progressively declined. Our results suggest that enhanced recruitment of mucosal $CD4^+$ T cells into the terminal stage of differentiation is one mechanism behind this loss of mucosal T_{EM} cells.

With regard to the $CD4^+$ T-cell subset proliferative potential, we identified a major difference between the mucosal and peripheral immune compartment. Specifically, whereas $CD4^+$ T_{CM} cells in peripheral blood of HIV-infected patients exhibit a higher proliferation rate than other T-cell subsets,[30] we found no proliferation within their mucosal counterparts, which is in agreement with previous observations in SIV-infected rhesus macaques.[31] This suggests that mucosal $CD4^+$ T_{CM} cells are in a resting, non-activated state, a property that potentially makes them less susceptible to HIV infection. Moreover, in this resting cellular state, $CD4^+$ T_{CM} cells are most likely not able to differentiate into new effector cells, which may enhance the preferential loss of T_{EM} cells from the mucosal $CD4^+$ T-cell compartment. Because $CD4^+$ T_{EM} cells have a pivotal role in maintaining protective immunity, their severe contraction in patients with late-stage disease may be one determinant of the clinically evident mucosal immune dysfunction (that is, opportunistic infections and cancers at the level of the gastrointestinal tract) that usually occurs after years of untreated HIV infection. Consistent with this assumption, studies performed in SIV-infected rhesus macaques indicate a close association between the host susceptibility to opportunistic infections and the depletion of effector site $CD4^+$ T_{EM} cells below a critical threshold level.[6,32] In addition, we found that semi-activated mucosal $CD4^+$ T cells shift toward a fully-activated state in chronically HIV-infected patients. This is likely triggered by persistent exposure to HIV antigens, different luminal antigens, and/or inflammatory effector molecules, which usually leads to activation-induced T-cell exhaustion with ineffective responsiveness to stimulation.[33–35]

Incomplete regeneration of the mucosal immune system during treated HIV infection likely promotes disease progression. In patients with initiation of cART during chronic HIV infection, repopulation of the intestinal mucosa with $CD4^+$ T cells was insufficient to achieve full reconstitution. At all stages of chronic disease, $CD4^+$ T cells in the duodenum restored to less than half of the numbers observed in HIV-negative controls. Thereby, levels of $CD4^+$ T-cell intermediate/late-stage activation remained elevated in the gastrointestinal tract of these patients, suggesting a link between insufficient downregulation of local immune hyperactivation and persistent lack of mucosal $CD4^+$ T cells. Inflammatory conditions can prevent sufficient cell trafficking and thus limit $CD4^+$ T-cell repopulation with cART. A previous study demonstrated an association of enterocyte damage and consequent dysregulated chemokine expression with defective homing of $CD4^+$ T cells to the gut.[36] Consistent with this study, we found increased plasma concentrations of I-FABP in our treated patients with chronic HIV infection reflecting persistent damage to enterocytes. Fibrotic damage to gut inductive sites, as has recently described for ileal Peyer's Patches, may additionally limit mucosal T-cell survival and expansion.[37]

The clinical consequences of this persistent defect in the mucosal immune system are currently unknown. An association between impaired mucosal $CD4^+$ T-cell reconstitution and non-specific enteritis has recently been described.[23] Moreover, evidence for increased incidence of adenomas and intestinal malignancies in treated chronically HIV-infected patients[38,39] suggests that mucosal immune defects increase the risk of adverse outcome despite otherwise effective long-term cART. Our findings of increased systemic immune activation and enterocyte damage indicate ongoing inflammatory disease progression in treated patients with persistent mucosal $CD4^+$ T-cell depletion.

Interestingly, the proportion of T_{EM} cells among $CD4^+$ T cells normalized in chronically HIV-infected patients who had initiated cART with peripheral $CD4^+$ T-cell counts of >350 per µl but not in patients whose treatment was started later. A recent study revealed that secondary lymphoid structures and functions are preserved in early treated HIV-infected patients, which suggests that gut inductive sites still have a potent capability to induce T-cell differentiation.[19] By contrast, insufficient restoration of inductive lymphoid tissue described for patients with later start of cART[19] may hamper T-cell differentiation and thus might be linked to the persistent lack of mucosal $CD4^+$ T_{EM} cells observed in our patients with later start of cART. Together, these observations suggest a beneficial effect of early cART on mucosal protective immunity. For example, the improved restoration of effector site T_{EM} cells may help reduce the incidence of secondary infections and cancers and thus might account for the lower morbidity that has often been described among patients initiating cART at above 350 $CD4^+$ T cells pet µl compared with patients with delayed treatment.[40–42] Therefore, whether or not mucosal protective immune function can be restored in chronically HIV-infected patients despite the persistent overall reduction in total mucosal $CD4^+$ T cells would be interesting to address in future studies. Such functional analyses were not possible in the present study because of the limited amount of cells that can be isolated from the available human tissue specimens.

In conclusion, our study demonstrates that preservation of mucosal $CD4^+$ T cells at both the quantitative and the

differentiation levels is possible if cART is initiated during the acute phase of HIV infection. In chronically HIV-infected persons, restoration capacity of mucosal $CD4^+$ T-cell counts is irreversibly compromised. Both persistent depletion of mucosal $CD4^+$ T cells and enhanced levels of $CD4^+$ T-cell activation in the largest immunological organ are characteristic features of treated chronic HIV infection, regardless of the timing of cART initiation. Taken together, our findings highlight the importance of the development of adjunctive immunomodulatory therapies[43,44] aiming at immune restoration in the intestinal mucosa of chronically HIV-infected patients.

METHODS

Study subjects. 76 HIV-infected patients without previous antiretroviral therapy or receiving cART underwent diagnostic endoscopy for various symptoms related to the gastrointestinal tract such as nausea, abdominal pain, heartburn, diarrhea, and weight loss. With the patients' informed consent for this procedure, additional duodenal biopsy specimens were collected for the purpose of the present study. Biopsies were obtained from macroscopically unaffected areas in the first and second part of the duodenum. Patients diagnosed with gastrointestinal neoplasia were excluded from the study.

Fourteen healthy HIV-negative subjects without intestinal symptoms who underwent baseline endoscopy in a previous study (that is, before the elsewhere described study-related vaccination was administered[45]) and were with no current medication served as controls. Patient and control characteristics are given in **Table 1**. The study was approved by the Charité - Universitätsmedizin Berlin institutional review board, and all participants gave written informed consent to study participation.

***In situ* quantitation of mucosal T cells**. All biopsy samples used for immunohistochemical analysis were embedded in paraffin. Immunostaining on paraffin sections was performed as described previously.[46–48] Antibodies used were mouse anti-CD4 (clone 1F6; Novocastra, Newcastle, UK) or mouse anti-CD8 (polyclonal; Spring Bioscience, Fremont, CA). For detection of CD4 or CD8 labeling, a streptavidin alkaline phosphatase-kit with Fast Red as chromogen (Dako, Hamburg, Germany) was used according to the manufacturer's instructions. Positive cells within the lamina propria were quantified in duodenal tissues per high-power field (h.p.f.; 0.237 mm^2), and 10 h.p.f. were averaged in each case.[46–48] Immunohistochemical evaluations were performed in a blinded manner, i.e., the researcher was unaware of the subject's clinical characteristics. Negative controls were performed by omitting the primary antibody and by using the appropriate isotype control.

Mucosal and peripheral cell isolation. Mucosal mononuclear cells were isolated from duodenal biopsy specimens by collagenase type II (Sigma, Hamburg, Germany) digestion as described previously,[46,49] and peripheral blood mononuclear cells were isolated from heparinized venous blood by standard Ficoll gradient centrifugation. The percentage of viable mononuclear cells in the mucosal cell preparations was $>97\%$ as assessed flow cytometrically, using propidium iodide (Sigma) for dead cell staining. Antibody clones used in flow cytometric analysis of mucosal mononuclear cell were tested to be suitable for the analysis of collagenase treated cells.

Flow cytometric analysis. $CD4^+$ T-cell counts were determined in fresh whole blood by the use of Trucount Tubes and CD3/CD4/CD8 TriTest (Becton Dickinson, BD, Heidelberg, Germany) according to the manufacturer's protocol. Phenotypic analysis of $CD4^+$ T cells was performed with isolated mucosal mononuclear cells or peripheral blood mononuclear cells using antibodies against CD4 (RTA-T4; BD Biosciences, Heidelberg, Germany), CD8 (SK1; BD), CD38 (1B6; Miltenyi, Bergisch-Gladbach, Germany), CD45RO (UCHT1; BD), CD62L (Dreg56; BioLegend, Fell, Germany), and HLA-DR (G46-6(L243); BD). Antibodies were conjugated to fluorescein isothiocyanate, phycoerythrin, peridin chlorophyll protein, allophycocyanin, or Alexa Fluor 647. Data were acquired on the FACS Calibur (BD) and analyzed with FlowJo software version 8.8.4. (BD). Lymphocytes were gated on the basis of characteristic forward and side scatter properties. T_{CM} cells were classified by co-expression of CD45RO and CD62L, and T_{EM} cells were classified by lack of CD62L expression.[30] T_{TD} cells were identified by the lack of CD62L expression on $CD45RO^-$ $CD4^+$ T cells.[30] For analysis of cell subset proliferation, surface staining with antibodies against CD4, CD45RO, and CD62L was followed by intranuclear immunostaining with anti-Ki-67 mononuclear antibody (Mib1; Dako). Cells were permeabilized and fixed using Fix/Perm buffers (eBioscience, Frankfurt am Main, Germany) according to the manufacturer's instructions.

Quantitation of biomarkers of inflammation, LPS bioactivity, and enterocyte damage. Commercially available assay kits were used according to the manufacturer's protocols to quantify LBP (Hycult Biotech, Uden, The Netherlands), sCD14 (Diaclone, Besancon Cedex, France), IL-6 (Diaclone), C-reactive protein (Hölzel Diagnostika, Cologne, Germany), D-Dimer (RayBiotech, Norcross, GA), and I-FABP (Hycult Biotech) in plasma. Each test was determined in duplicates and the average of each marker was calculated.

Statistical analysis. Data are represented as medians with interquartile ranges, minimum and maximum values, and outliers, and were analyzed using Mann–Whitney *U*-test. Multiple independent tests were performed for comparisons between controls and each individual HIV-infected patient group and between treatment-naïve patients and treated patients for each stage of disease. Significance is denoted with asterisks (i.e., $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$). Bivariate correlations and statistical significance were determined by the Spearman rank correlation test. All data were statistically analyzed with Prism software version 5.0 (Graph Pad Inc., La Jolla, CA).

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DISCLOSURE

The authors declare no conflict of interest.

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