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Distribution and Activation of CD8$^+$ T Cells in the Duodenal Mucosa before and after HIV Seroconversion

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CD8$^+$ T cells in the intestinal mucosa influence the HIV-associated pathogenesis, but little is known about the dynamics of mucosal CD8$^+$ T cell counts and activation of these cells during the course of infection. In this study, mucosal CD8$^+$ T cells in the duodenum were studied at different stages of HIV infection, starting from the seronegative phase. In seronegative acute HIV infection, CD8$^+$ T cell counts increased in the epithelium, but not in the lamina propria. Infiltration of the lamina propria by peripherally expanded CD8$^+$ T cells was observed after seroconversion. Highest increase in the expression of perforin, the rate-limiting molecule for cytotoxic CD8$^+$ T cell activity, was evident in the lamina propria of seronegative acutely HIV-infected patients. The number of perforin-expressing cells in the lamina propria of acutely HIV-infected patients was positively associated with biomarkers of enterocyte damage and microbial translocation. After seroconversion, perforin expression was downregulated in the lamina propria, but not in the epithelium. In conclusion, our findings demonstrate that intraepithelial and lamina propria CD8$^+$ T cells exhibit different dynamics of numerical alteration and cytotoxic activity in HIV-infected patients. Moreover, our results suggest that perforin-dependent cytotoxic mechanisms by CD8$^+$ T cells could impair the intestinal mucosal barrier already in the seronegative phase of acute HIV infection, thereby inducing microbial translocation as one of the earliest pathological events in HIV infection. ***The Journal of Immunology*****, 2017, 198: 481–491.**

The gastrointestinal tract is the largest immune organ of the human body and contains more than half of all immune cells (1). In HIV-infected persons, the intestinal mucosa is a major site of viral replication (2–4), massive depletion of CD4$^+$ T cells (5–7), accumulation of CD8$^+$ T cells and macrophages (8, 9), and is highly vulnerable to opportunistic infections and neoplasms (10). Mucosal effector sites that protect the intestinal barriers against dietary Ags and potential pathogens are located within the lamina propria and the surface epithelium (11). At these sites, effector cells after immigration, differentiation, and activation act under tight control by regulatory mechanisms to prevent inflammation-induced tissue injury and neoplasia (12). In HIV infection, overall loss of mucosal immune homeostasis leads to disruption of intestinal barrier functions, enhances the translocation of microbial products from the gut lumen into circulation, and is thus thought to contribute to the development of persistent hyperactivation of the immune system that is directly linked to HIV disease progression (13, 14).

Currently, the most described alteration in the mucosal immune system of HIV-infected persons is the rapid and persistent depletion of lamina propria CD4$^+$ T cells that has been suggested to cause the observed intestinal barrier defect (5–8). We have recently demonstrated that mucosal infiltration and/or local expansion of CD8$^+$ T cells precedes the numerical depletion of mucosal CD4$^+$ T cells and consequently causes the often described early inversion of CD4/CD8 T cell ratio in the intestinal lamina propria (8). Mucosal CD8$^+$ T cells and intraepithelial lymphocytes expressing cytotoxic molecules have previously been described to be associated with the level of intestinal enterocyte death (15–17). Thus, cytotoxic activity of accumulated CD8$^+$ T cells in the gastrointestinal tract of HIV-infected persons could trigger tissue damage similar to HIV-unrelated chronic inflammatory conditions (18, 19).

Although mucosal CD8$^+$ T cells may considerably influence the HIV-associated immune pathogenesis and disease progression, studies on the detailed dynamic of mucosal CD8$^+$ T cells in HIV infection are lacking. Therefore, we conducted a quantitative and phenotypic analysis of mucosal CD8$^+$ T cells in HIV-infected patients at four different stages of untreated disease, from the seronegative phase of acute infection to the advanced chronic stage.

Materials and Methods

Study subjects

Fifty HIV-infected patients without previous antiretroviral therapy 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 this study. Biopsies were obtained from macroscopically unaffected areas in the first part of the duodenum. Patients diagnosed with gastrointestinal neoplasia were excluded from the study. At biopsy procedure of 27 patients, a total of 12 biopsy specimens per patient could be sampled for immune histochemical examination and flow cytometric analysis. From 23 additional patients, only two biopsy specimens could be obtained that were

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Abbreviations used in this article: FasL, Fas ligand; I-FABP, intestinal fatty acid–binding protein; LBP, LPS-binding protein; MAdCAM, mucosal vascular addressin cell adhesion molecule; PD-1, programmed cell death protein 1; sCD14, soluble CD14.

used for immune histological examination. Heparinized blood samples were collected from 43 patients in parallel (Table 1). Patients with the diagnosis of acute HIV infection (n = 11), defined by the presence of symptoms compatible with an acute antiretroviral syndrome and detectable HIV RNA, were divided into two groups: no or indeterminate HIV-specific bands (seronegative acute phase; Fiebig I–IV) (20), and two to four bands in HIV immune blotting (seropositive acute phase; Fiebig V–VI) (20). Serological tests in these patients were performed at the day of biopsy sampling. Patients with established HIV infection were divided into the following two groups: peripheral CD4+ T cell counts >200/μl of blood (chronic stage of HIV disease; n = 17) and CD4+ T cell counts <200/μl (advanced chronic stage; n = 22).

Fourteen healthy HIV-negative subjects without intestinal symptoms who underwent baseline endoscopy in a previous study (i.e., before the elsewhere described study-related vaccination was administered) (21) served as control subjects. Blood samples were collected from 10 control subjects who did not undergo endoscopic biopsy. 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.

Immunohistochemical analysis

Immunostaining on paraffin sections was performed as described previously (8, 9, 15, 22). The primary Abs included mouse anti-CD4 (clone 1F6; Novocastra, Newcastle, U.K.), rabbit anti-CD8 (polyclonal; Spring Bioscience, Fremont, CA), mouse anti-Ki67 (MIB-1; Dako, Hamburg, Germany), mouse anti–mucosal vascular addressin cell adhesion molecule 1 (anti–MAdCAM-1; 17F5; Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-Perforin [KM585(P1-8); Kamiya Biomedical, Seattle, WA], and rabbit anti–Fas ligand (FasL) (polyclonal; LifeSpan Bioscience, Seattle, WA). For detection, the streptavidin-alkaline phosphatase kit with Fast Red as chromogen (Dako) was used according to the manufacturer's instructions. For double immunoenzymatic labeling, slides were incubated with the mouse anti-Ki67 Ab followed by the EnVision peroxidase kit (Dako). Sections were then incubated with the second Ab against CD8, and the streptavidin-alkaline phosphatase kit was used for detection. Nuclei were counterstained with hematoxylin (Merck, Darmstadt, Germany).

Positive cells within the lamina propria were quantified in duodenal tissues per high-power field (0.237 mm^2), and 10 high-power fields were averaged in each case (8, 9, 15, 22). Intraepithelial lymphocytes were quantified in relation to epithelial cells according to Marsh (15, 23). Immunohistochemical evaluations were performed in a blinded manner; that is, the researcher was unaware of the subject's clinical characteristics. Negative controls were performed by omitting the primary Ab 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 (8, 9), and PBMCs were isolated from heparinized venous blood by standard Ficoll gradient centrifugation. The percentage of viable mononuclear cells in the mucosal cell preparations was >97%. Ab clones used in flow cytometric analysis of mucosal mononuclear cells were tested to be suitable for the analysis of collagenase-treated cells.

Flow cytometric analysis

Phenotypic analysis was performed using Abs against CD4 (MT310; Dako), CD8 (SK1; BD Biosciences, Heidelberg, Germany), CD38 (1B6; Miltenyi, Bergisch Gladbach, Germany), CD45RO (UCHT1; BD), CD62L (Dreg56; BioLegend, Fell, Germany), HLA-DR [G46-6(L243); BD], and *programmed cell death protein 1* (PD-1; PD1.3.1.3.; Miltenyi). Data were acquired on the FACSCalibur (BD) and analyzed with FlowJo software version 8.8.4. (BD). Lymphocytes were gated on the basis of characteristic forward and side scatter properties. Central memory T cells were classified by coexpression of CD45RO and CD62L, and effector memory T cells were classified by lack of CD62L expression (24). For analysis of cell subset proliferation, surface staining with Abs against CD4, CD45RO, and CD62L was followed by intranuclear immunostaining with anti–Ki-67 mononuclear Ab (Mib1; Dako). Cells were permeabilized and fixed using Fix/Perm buffers (eBioscience, Frankfurt, Germany) according to the manufacturer's instructions.

Quantitation of biomarkers of enterocyte damage and microbial translocation

Commercially available assay kits were used according to manufacturer's protocols to quantify LPS-binding protein (LBP; Hycult Biotech, Uden, the Netherlands), soluble CD14 (sCD14; Diaclone, Besancon Cedex, France), and intestinal fatty acid–binding protein (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 control subjects and each individual HIV-infected patient group and between seronegative and seropositive acutely HIV-infected patients. Bivariate correlations and statistical significance were determined by the Spearman rank correlation test. All data were statistically analyzed with Prism software version 5.0 (GraphPad, La Jolla, CA).

Results

Quantitative in situ analysis of CD8+ T cells in the lamina propria and the epithelial surface

In patients who were in the seronegative peak-viremia phase of acute HIV infection (Table I), CD8+ T cell numbers in the lamina propria as well as the peripheral blood did not differ from those of healthy control subjects (Fig. 1A–C), whereas intraepithelial CD8+ T cells were 3-fold increased (Fig. 1A, 1C). In patients who were in the seropositive phase of acute HIV infection, lamina propria and intraepithelial as well as peripheral median CD8+ T cell numbers were >2- to 3-fold increased over control cell counts (Fig. 1B–D). In chronic HIV infection, median CD8+ T cell numbers were 2.5-fold increased in the lamina propria and the peripheral blood (Fig. 1B, 1C). In patients with advanced HIV disease, CD8+ T cells numbers remained nearly 2-fold increased in the duodenal lamina propria, but not in the epithelium or the peripheral blood (Fig. 1B–D). Peripheral CD8+ T cell counts during disease progression were positively correlated with lamina propria CD8+ T cell numbers (p = 0.0023; r = 0.479), but there was no correlation with intraepithelial CD8+ T cell numbers in the duodenum. In addition, there was no correlation between viral loads and peripheral or mucosal CD8+ T cells.

At both stages of acute infection, no difference in the CD4/CD8 T cell ratio between the lamina propria and peripheral blood was observed (Fig. 1E), which is consistent with the similar dynamic of CD8+ T cells in both compartments (Fig. 1B, 1C) and the previously described preserved mucosal CD4+ T cell numbers in acutely HIV-infected patients (8). In chronically HIV-infected patients, the CD4/CD8 T cell ratio was significantly lower in the lamina propria than in the periphery (Fig. 1E) due to the massive depletion of mucosal CD4+ T cells at this stage of disease (8).

Differentiation and activation of CD8+ T cells at different stages of HIV infection

Mucosal CD8+ T cells in control subjects were mainly of the effector memory subtype, confirming previous findings (Fig. 2A) (25, 26). In HIV-infected patients, the proportion of CD8+ effector memory T cells tended to further increase in duodenal mucosa and was strongly increased in the periphery already in the seronegative phase of acute HIV infection (Fig. 2A).

Activation of CD8+ T cells was assessed by measurement of activation-related surface Ags CD38, HLA-DR, and PD-1 (Fig. 1B–D). At the seronegative phase of acute HIV infection and at all later stages of HIV disease, the $CD38^+HLA\text{-}DR^+$ fraction and the expression density of PD-1 were strongly increased among both mucosal and peripheral CD8+ T cells, indicating an early induction and maintenance of systemic CD8+ T cell immune response post-HIV infection (Fig. 2C, 2D). The proportion of mucosal or peripheral $CD38^+HLA\text{-}DR^-$ CD8+ T cells did not change with disease progression (Fig. 2B).

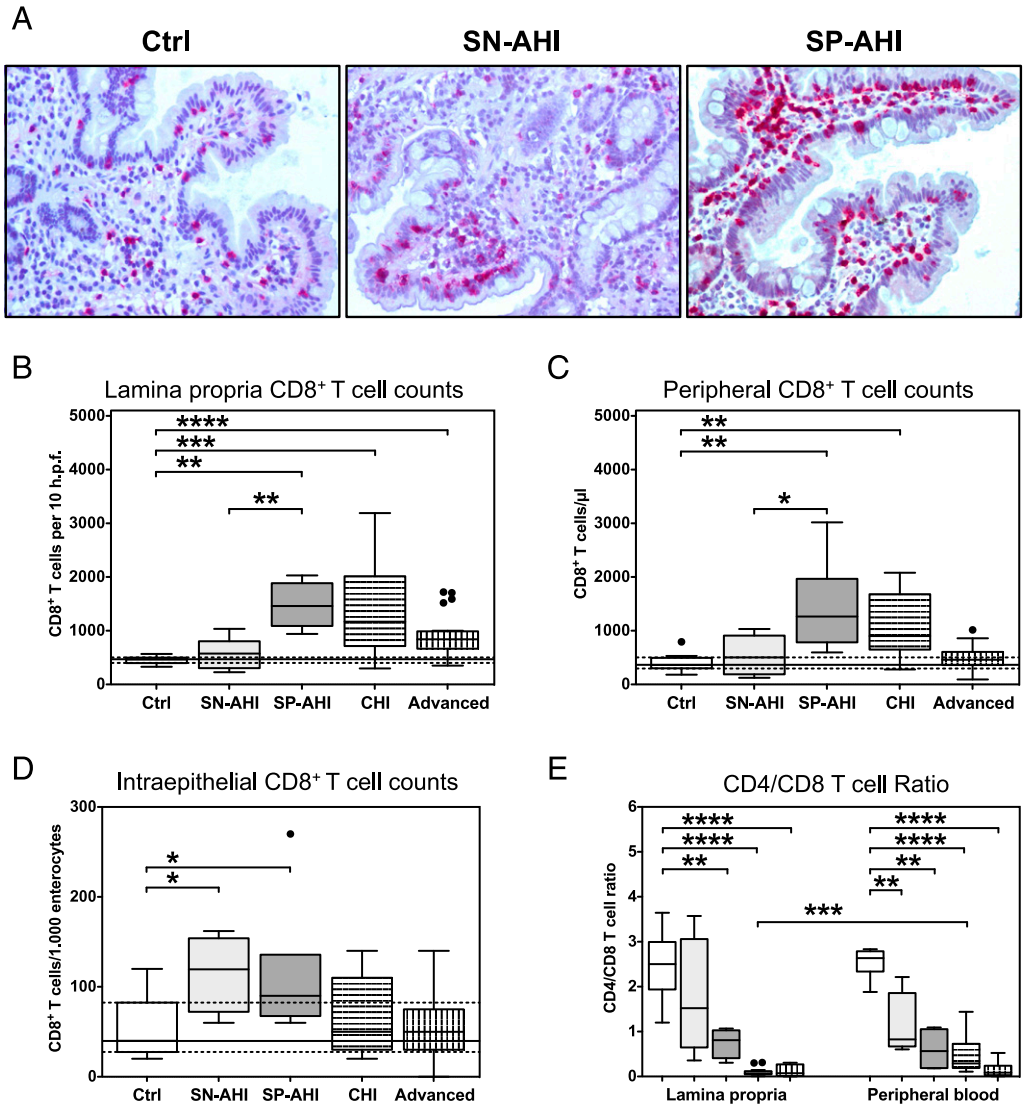

FIGURE 1. Mucosal and peripheral CD8$^+$ T cell numbers in HIV-infected patients at different stages of acute or chronic HIV infection. (**A**) Representative immunohistochemical staining of CD8$^+$ T cells (red) in duodenal mucosa from healthy control subjects and from seronegative or seropositive acutely HIV-infected patients. Original magnification ×200. (**B**) CD8$^+$ T cell numbers in the duodenal lamina propria and (**C**) the peripheral blood from control subjects or from patients. (**D**) CD8$^+$ T cell numbers in the duodenal epithelium. (**E**) CD4$^+$ T cells and CD8$^+$ T cells in the lamina propria and the peripheral blood were quantified by immunohistochemical staining and quantitative flow cytometry, respectively. Ratio of CD4$^+$ T cell numbers to CD8$^+$ T cell numbers in the lamina propria or the peripheral blood of control subjects (white bars), seronegative (light gray bars) or seropositive acutely HIV-infected patients (dark gray bars), and patients with chronic (vertical striped bars) or advanced HIV infection (horizontal striped bars) are shown. Median values of controls are indicated by the thick horizontal lines, and the dashed horizontal lines denote the normal 25th and 75th percentiles in each diagram. Statistical significances are given for comparisons between each patient group and control subjects, between seronegative and seropositive acutely HIV-infected patients, and between the lamina propria and the peripheral blood values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Advanced, advanced chronic stage of HIV infection; CHI, chronic HIV infection; Ctrl, control subjects; SN-AHI, seronegative acute HIV infection; SP-AHI, seropositive acute HIV infection.

Peripheral, but not duodenal, CD8$^+$ T cells proliferate in response to HIV infection

We analyzed intranuclear Ki-67 expression that is present during all active phases of the cell cycle and is closely related to cell proliferation (27). At both the seronegative and the seropositive phase of acute HIV infection, a strong increase in the proportion of proliferating CD8$^+$ T cells was observed in the peripheral blood, but not in duodenal mucosa (Fig. 3A, 3B). Immunohistochemical detection of Ki67-expressing CD8$^+$ T cells confirmed absent CD8$^+$ T cell proliferation in both duodenal effector compartments, the lamina propria and the epithelium (data not shown). In chronic and advanced HIV infection, peripheral CD8$^+$ T cell proliferation remained elevated (Fig. 3A, 3B). The peripheral CD8$^+$ T cell proliferation rate during disease progression was positively correlated with viral loads ($p < 0.0001$; $r = 0.7713$), but there was no correlation with peripheral CD8$^+$ T cell counts. Absent proliferation of mucosal CD8$^+$ T cells suggests that accumulation of activated effector memory CD8$^+$ T cells in the duodenum of HIV-infected patients (Figs. 1B, 2A) is not caused by their local proliferative expansion.

Migratory potential of proliferating CD8$^+$ T cells changes during disease progression

In seronegative acutely HIV-infected patients, the strong increase in peripheral CD8$^+$ T cell proliferation (Fig. 3A) without numerical

Table I. Characteristics of HIV-infected patients and control subjects in this study and numbers of examined samples

Variable	Acute HIV		Chronic HIV	Advanced HIV	Control Subjects
	Seronegative	Seropositive			
Duodenal samples for immune histological analysis, n[a,b]	6	5	17	22	14
Sex, male/female	6/0	4/1	14/3	16/6	14/0
Age, y	44 (21–52)	34 (27–61)	38 (23–74)	47 (26–77)	38 (22–48)
CD4+ T cell count, cells/μl	415 (277–687)	552 (316–1015)	364 (226–657)	36 (3–194)	ND
Viral load, log10 copies/ml	7.1 (6.2–7.5)	5.5 (4.9–6.0)	4.9 (3.6–5.7)	5.3 (4.1–5.9)	NA
Duodenal samples for flow cytometric analysis, n[a,c]	6	5	8	8	10
Sex, male/female	6/0	4/1	6/2	5/3	10/0
Age, y	44 (21–52)	34 (27–61)	51 (23–74)	43 (32–52)	39 (23–47)
CD4+ T cell count, cells/μl	415 (277–687)	552 (316–1015)	299 (226–504)	43 (15–178)	ND
Viral load, log10 copies/ml	7.1 (6.2–7.5)	5.5 (4.9–6.0)	4.6 (3.6–5.7)	5.2 (4.1–5.9)	NA
Peripheral blood samples, n[a,c]	6	5	16	16	10
Sex, male/female	6/0	4/1	13/3	12/4	7/3
Age, y	44 (21–52)	34 (27–61)	40 (23–74)	49 (26–77)	44 (21–56)
CD4+ T cell count, cells/μl	415 (277–687)	552 (316–1015)	349 (226–657)	45 (3–167)	852 (603–1509)
Viral load, log10 copies/ml	7.1 (6.2–7.5)	5.5 (4.9–6.0)	4.9 (3.6–5.7)	5.5 (4.1–5.9)	NA

Data are number of subjects or median value (range), unless otherwise indicated.
[a]In the control group, subjects who underwent endoscopic biopsy and subjects who were included in the analysis of peripheral blood samples did not overlap.
[b]Patients as well as controls were included in both the flow cytometric analysis and the immune histological examinations.
[c]In the patient groups, all subjects who were included in the analysis of blood samples are also included in the analysis of duodenal samples.
NA, not applicable.

increase of peripheral or lamina propria CD8+ T cells (Fig. 1B, 1C) indicates enhanced trafficking of expanding cells to extraintestinal tissues. In these HIV-infected patients, the pool of proliferating CD8+ T cells contained almost 2-fold increased numbers of central memory cells expressing L-selectin (CD62L), which has been described to be associated with lymph node homing potential (28–30) (Fig. 4A). At the same time, the proportion of effector memory cells that lack expression of CD62L and have previously been found to preferentially localize to nonlymphoid tissues (28–30) did not change (Fig. 4B). This phenotypic feature of the proliferating CD8+ T cell pool might indicate that CD8+ T cells with a migratory potential toward peripheral lymph nodes preferentially expand in the seronegative phase of HIV infection. In parallel, the composition of nonproliferating CD8+ T cells remained unaffected in seronegative acutely HIV-infected patients (data not shown). In the later stages of disease, the majority of proliferating CD8+ T cells were CD62L− effector memory cells (Fig. 4B), indicating a persistent enhanced expansion of CD8+ T cells with migratory potential toward nonlymphoid tissues after seroconversion.

CD8+ T cell expression of gut-homing–specific integrin β7 increases during disease progression

The persistent migration potential of peripherally expanded CD8+ T cells to nonlymphoid tissues after seroconversion (Fig. 4A), as well as the absent mucosal CD8+ T cell proliferation (Fig. 3A), suggests that CD8+ T cell accumulation in the intestinal mucosa of HIV-infected persons (Fig. 1) originates from the circulating T cell pool. The major pathway in lymphocyte trafficking to the intestinal mucosa is the integrin α4β7/MAdCAM-1 pathway (31, 32). MAdCAM-1+ vessel density was significantly elevated in seronegative acutely HIV-infected patients and patients with advanced disease, but not in the seropositive acute or chronic stage (Fig. 5A, 5B). This indicates that attraction of immune cells to the duodenal mucosa is most pronounced during high viral replication. Consistent with this, MAdCAM-1 expression was correlated with viral loads (Fig. 5C).

The proportion of integrin β7high-expressing peripheral CD8+ T cells increased during disease progression (Fig. 5D, 5E), indicating a growing trafficking potential of the peripheral CD8+ T cell pool toward intestinal tissues in the course of HIV infection (33, 34). Significantly higher integrin β7high-expressing CD8+ T cell counts than in control subjects were noted in the patient groups who showed significantly increased lamina propria CD8+ T cell counts (Figs. 1B, 5E), indicating that enhanced influx of circulating integrin β7-expressing CD8+ T cells accounts for the increase in mucosal effector memory CD8+ T cells. Consistent with this, lamina propria CD8+ T cell numbers were positively correlated with integrin β7 expression on peripheral CD8+ T cells ($p = 0.0138$; $r = 0.5686$). Lowest integrin β7 expression among peripheral CD8+ T cells was observed in seronegative acutely HIV-infected patients (Fig. 5D), consistent with our data that indicate in these patients a preferential migration of expanding CD8+ T cells to lymphoid tissues (Fig. 4A).

Expression of cytotoxic molecules in the duodenal mucosa at different stages of HIV infection

Previously, we demonstrated that perforin in duodenal mucosa of HIV-infected patients is expressed exclusively by CD8+ T cells, and that increase of perforin-positive CD8+ T cells is limited to the acute phase of infection (15). To assess the cytotoxic activity of mucosal CD8+ T cells, we quantified lymphocytes positive for perforin or FasL, both of which are typically expressed by CD8+ cytotoxic T cells. We found an increase in lamina propria and intraepithelial perforin-expressing CD8+ T cells in patients with seronegative acute HV infection (Fig. 6A, 6B), whereas the number of FasL-expressing cells did not change significantly (Fig. 6C). In these patients, the level of perforin expression in the lamina propria was higher than in seropositive acutely HIV-infected patients (Fig. 6A, 6B). These results indicate that initial mucosal CD8+ T cell activation is associated with local induction of perforin expression. After seroconversion, perforin expression was downregulated in lamina propria CD8+ T cells (Fig. 6B). In the intraepithelial compartment, in contrast, perforin-expressing CD8+ T cells remained increased throughout infection

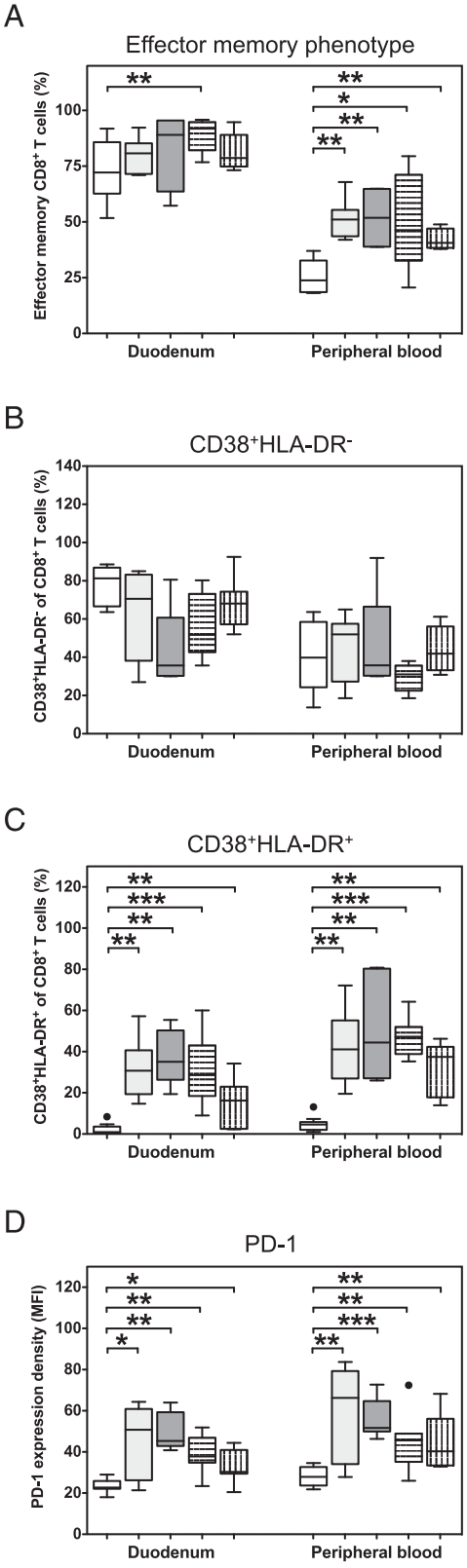

FIGURE 2. Differentiation and activation of $CD8^+$ T cells in duodenal mucosa and the peripheral blood at different stages of acute and chronic HIV infection. (**A**) Frequency of effector memory cells among $CD8^+$ T cells in the duodenum or the peripheral blood of seronegative (light gray bars) or seropositive acutely HIV-infected patients (dark gray bars), and patients with chronic (vertical striped bars) or advanced HIV infection (horizontal striped bars) in comparison with control subjects. (**B**–**D**) Percentages of $CD38^+HLA\text{-}DR^-$ cells (B), $CD38^+HLA\text{-}DR^+$ cells within $CD8^+$ T cells (C), and the expression density of PD-1 on $CD8^+$ T cells (D) in the duodenum or the peripheral blood. Statistical significances are given for comparisons between each patient group and control subjects. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

(Fig. 6B), whereas almost no intraepithelial cells expressed FasL (Fig. 6C).

Enterocyte damage and microbial translocation are already detectable in the seronegative phase of acute HIV infection

Damage of the gut mucosa and subsequent translocation of microbial products from the intestinal lumen into the systemic circulation is considered to be the main cause of persistent immune hyperactivation in HIV disease (13, 35). To determine whether cytotoxic action of mucosal $CD8^+$ T cells (Fig. 6) could contribute to epithelial cell damage and subsequent microbial translocation into the systemic circulation, we quantified plasma concentrations of I-FABP, which is released into the bloodstream upon enterocyte damage, and LBP and sCD14, both of which are produced by the immune system in response to the bacterial product LPS.

In seronegative acutely HIV-infected patients, plasma levels of I-FABP, LBP, and sCD14 were significantly increased (Fig. 7A–C), indicating rapid damage to the epithelial barrier that allows the translocation of immune-activating luminal microbial products into the circulation. Moreover, I-FABP and sCD14 plasma levels in acutely HIV-infected patients were positively correlated with the number of perforin-expressing mucosal cells (Fig. 7C, 7D), which indirectly supports the assumption that the initial damage of the intestinal barrier is mediated by the strong cytotoxic $CD8^+$ T cell activity observed in seronegative acutely HIV-infected patients (Fig. 6A, 6B). In all seropositive stages of disease, significantly increased plasma levels were observed for LBP and/or sCD14, but not for I-FABP (Fig. 7A, 7C), which suggests that microbial translocation persists, whereas enterocyte-damaging processes are less severe after seroconversion.

Discussion

In this study we investigated the dynamics of $CD8^+$ T cells in intestinal effector sites of HIV-infected patients. We demonstrate that early postinfection, that is, before the first Abs against HIV are detectable, the prevalence of $CD8^+$ T cells increases in the intestinal epithelium, but not in the lamina propria. In the lamina propria, $CD8^+$ T cell numbers were highly increased only after seroconversion.

$CD8^+$ T cells in the seronegative peak-viremia phase of acute infection showed strong increase in activation despite no detectable changes in absolute numbers in the lamina propria and the peripheral blood. This marked activation of $CD8^+$ T cells is compatible with a typical primary response to viral infection that has been described along with the occurrence of Ag-specific cytotoxic T cells (36–38) and large $CD8^+$ T cell expansion (39, 40). In our patients, proliferative expansion of peripheral $CD8^+$ T cells was particularly robust in both the seronegative and the seropositive phase of acute HIV infection and was directly related to plasma viral loads. However, although highly proliferative in the peripheral blood, the primary response of $CD8^+$ T cells to HIV infection was nonproliferative in duodenal tissue. $CD8^+$ T cell proliferation was absent during all stages of HIV infection, as has also been shown previously in other regions of the gastrointestinal tract (41, 42). Thus, the intestinal effector tissue is not an immune compartment of proliferative $CD8^+$ T cell expansion in HIV-infected persons. In contrary to this finding in humans, data obtained from *SIV*-infected rhesus macaques showed increased $CD8^+$ T cell proliferation in the jejunum and colon (43, 44),

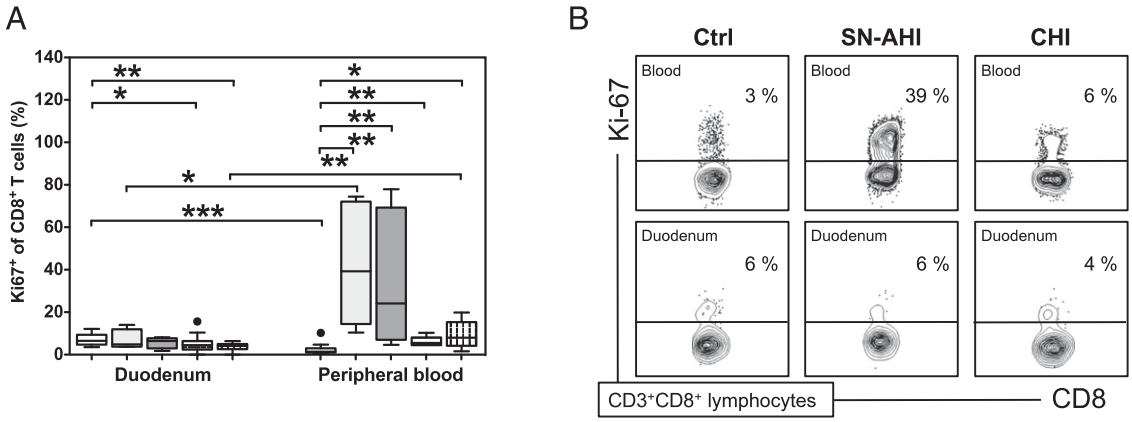

FIGURE 3. Proliferation of mucosal and peripheral CD8+ T cells. (**A**) Proliferating fraction within mucosal and peripheral CD8+ T cells of control subjects (white bars), seronegative (light gray bars) or seropositive acutely HIV-infected patients (dark gray bars), and patients with chronic (vertical striped bars) or advanced HIV infection (horizontal striped bars) were assessed by flow cytometric analysis of intranuclear Ki-67 expression. (**B**) Representative flow cytometric analysis of proliferating CD8+ T cells in the peripheral blood (upper row) and duodenal mucosa (lower row) from healthy control subjects and from seronegative acutely or chronically HIV-infected patients. Statistical significances are given for comparisons between each patient group and control subjects. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CHI, chronic HIV infection; Ctrl, control subjects; SN-AHI, seronegative acute HIV infection; SP-AHI, seropositive acute HIV infection.

suggesting species-specific differences. However, in the duodenum of HIV-infected persons, CD8+ T cells accumulated massively despite persistently absent local proliferation, which indicates enhanced trafficking of peripherally expanded CD8+ T cells to intestinal mucosa.

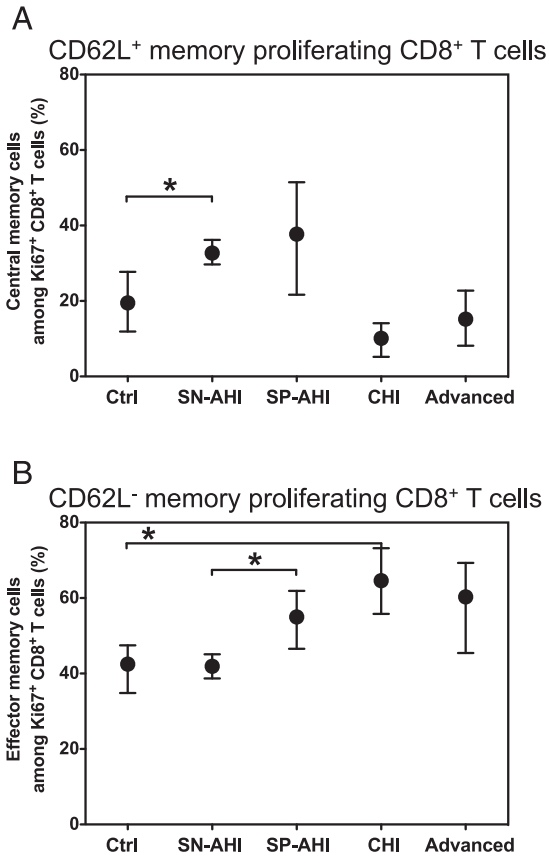

FIGURE 4. Composition of the proliferating CD8+ T cell fraction. To delineate the expanding CD8+ T cell subsets, we combined flow cytometric analysis of Ki-67 expression with that of T cell differentiation molecules. (**A** and **B**) Percentages of central memory ($CD45RO^+CD62L^+$) (A) and effector memory ($CD45RO^+CD62L^-$) cells within Ki-67+ CD8+ T cells (B) were assessed. Statistical significances are given for comparisons between each patient group and control subjects and between seronegative and seropositive acutely HIV-infected patients. *$p < 0.05$, **$p < 0.01$. Advanced, advanced chronic stage of HIV infection; CHI, chronic HIV infection; Ctrl, control subjects; SN-AHI, seronegative acute HIV infection; SP-AHI, seropositive acute HIV infection.

Based on our phenotypic data of CD8+ T cells obtained from seronegative acutely HIV-infected patients, it can be presumed that early-expanding CD8+ T cells in the circulation exhibit enhanced migratory potential to lymph nodes, the sites of Ag deposition and induction of adaptive and humoral responses. Consistent with this, marked lymphadenopathy associated with CD8+ T cells and B cell lymphoid infiltrates in lymph nodes is an early sign of recent HIV infection. The analysis of lymphoid tissues from seronegative acutely HIV-infected patients would therefore be interesting to address in future studies of CD8+ T cell homing in HIV infection. However, accumulation of nonproliferating CD8+ intraepithelial cells in the seronegative phase of acute HIV infection suggests that a subset of cells could traffic to the gut mucosa and preferentially locate to the epithelium. Alternatively, intraepithelial CD8+ T cells may also be generated from resident progenitor cells located in the intestinal epithelium (45).

After seroconversion, the proportion of circulating CD8+ T cells guided to infiltrate the intestinal mucosa increased continuously during HIV infection as demonstrated by the increasing expression of integrin β7 on peripheral CD8+ T cells. These cells may have been primed in GALTs and migrate to mucosal effector sites, including the lamina propria, by binding to its ligand MAdCAM-1 (33, 46, 47). In the duodenal lamina propria, the levels of MAdCAM-1+ endothelium were particularly high in the seronegative phase, as well as the advanced stage, of HIV infection and correlated with viral loads, which implies that recruitment of integrin β7-expressing cells from the blood to intestinal tissue is induced by viral factors or HIV-related inflammatory processes. This is supported by a previous study showing an association between effective HIV suppression and MAdCAM-1 reduction in the duodenal lamina propria of patients with advanced HIV disease (48). However, this HIV-associated cell trafficking is not restricted to CD8+ T cells because MAdCAM-1–mediated adhesion may also recruit integrin β7-expressing CD4+ T cells, monocytes, and/or dendritic cells, and probably others.

The two main mechanisms by which cytotoxic CD8+ T cells induce cell death are FasL-induced apoptosis and the perforin pathway (49). A previous study in SIV-infected rhesus macaques had demonstrated virus-specific antiviral cytotoxic activity of jejunal intraepithelial lymphocytes (50), which suggests that infiltrating intraepithelial CD8+ T cells are involved in primary

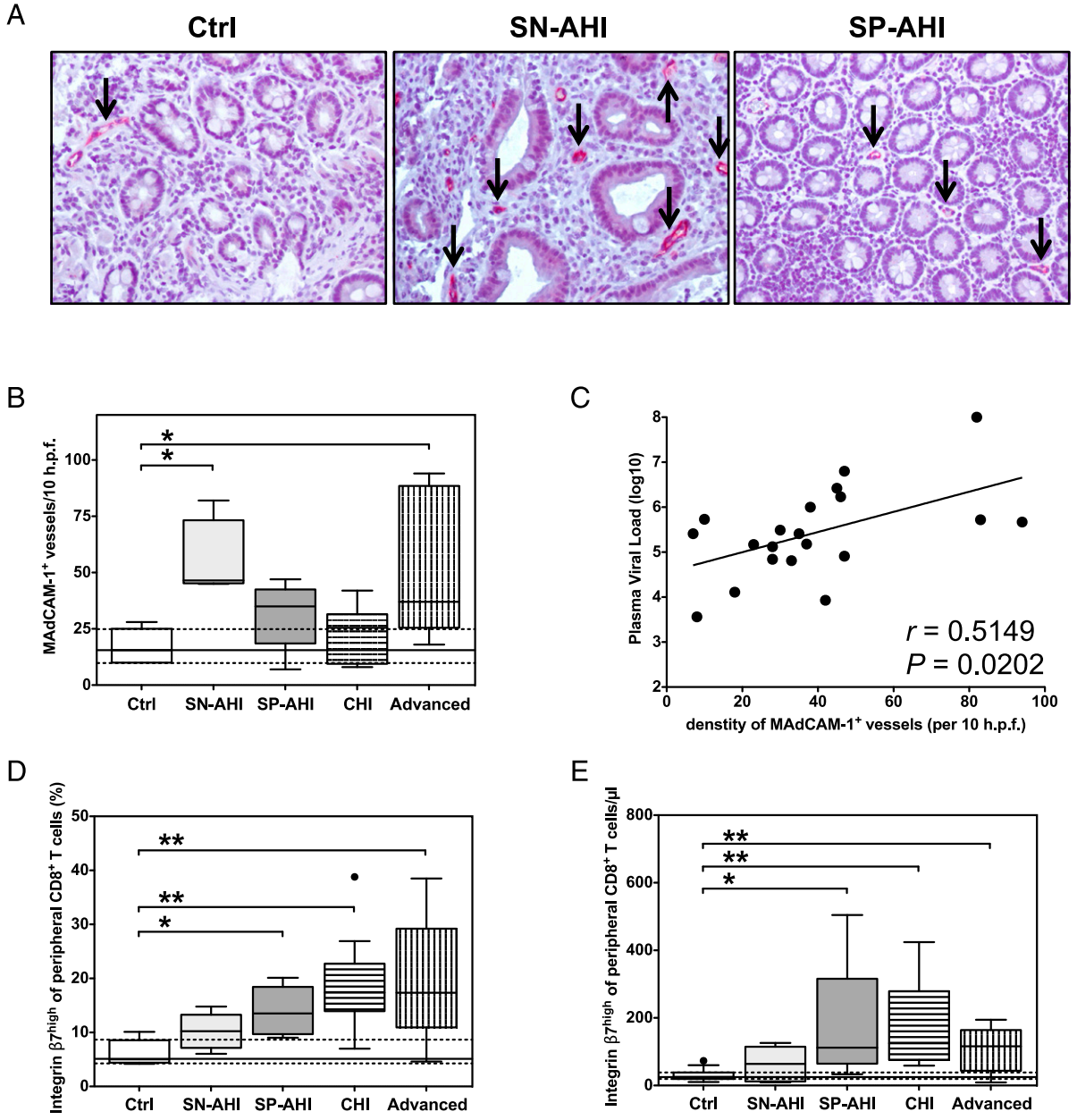

FIGURE 5. Density of MAdCAM-1$^+$ vessels in the duodenum and expression of integrin β7 on peripheral CD8$^+$ T cells. (**A**) Representative immunohistochemical staining of MAdCAM-1 (red, arrows) in duodenal lamina propria from healthy control subjects and from seronegative or seropositive acutely HIV-infected patients. Original magnification ×400. (**B**) MAdCAM-1$^+$ vessel densities in duodenal lamina propria and (**C**) correlation between duodenal MAdCAM-1 density and plasma viral load. (**D**) Expression density of integrin β7 on peripheral CD8$^+$ T cells and (**E**) integrin β7-expressing CD8$^+$ T cell counts. Five to six subjects of each comparison group were analyzed. Median values of control subjects are indicated by the thick horizontal lines, and the dashed horizontal lines denote the normal 25th and 75th percentiles in each diagram. Statistical significances are given for comparisons between patients in each group and control subjects and between seronegative and seropositive acutely HIV-infected patients. *$p < 0.05$, **$p < 0.01$. Advanced, advanced chronic stage of HIV infection; CHI, chronic HIV infection; Ctrl, control subjects; SN-AHI, seronegative acute HIV infection; SP-AHI, seropositive acute HIV infection.

antiviral immune response. The perforin-dependent response of mucosal CD8$^+$ T cells is restricted to the acute phase of HIV and SIV infection (15, 25, 51) and is linked to the apoptosis rate of epithelial cells and the related mucosal barrier defect (15). Consequently, action of mucosal CD8$^+$ T cells may be beneficial in early antiviral defense by recognizing and killing of HIV-infected cells, however potentially detrimental by damaging mucosal structures. We found only rare expression of FasL in the lamina propria or epithelium, indicating that FasL-induced apoptosis is not involved in duodenal antiviral defense or tissue destruction. In contrast, perforin expression was induced in both effector compartments already in the seronegative peak-viremia phase of HIV infection. Increased plasma concentrations of I-FABP in seronegative acutely HIV-infected patients and the observed positive correlation between I-FABP levels and mucosal perforin expression in acute HIV infection suggest that perforin-dependent cytotoxicity could cause enterocyte damage, and thus initially triggers the mucosal barrier defect. Furthermore, plasma levels of sCD14 and LBP were increased in seronegative acutely HIV-infected patients, indicating that subsequent microbial translocation occurs as

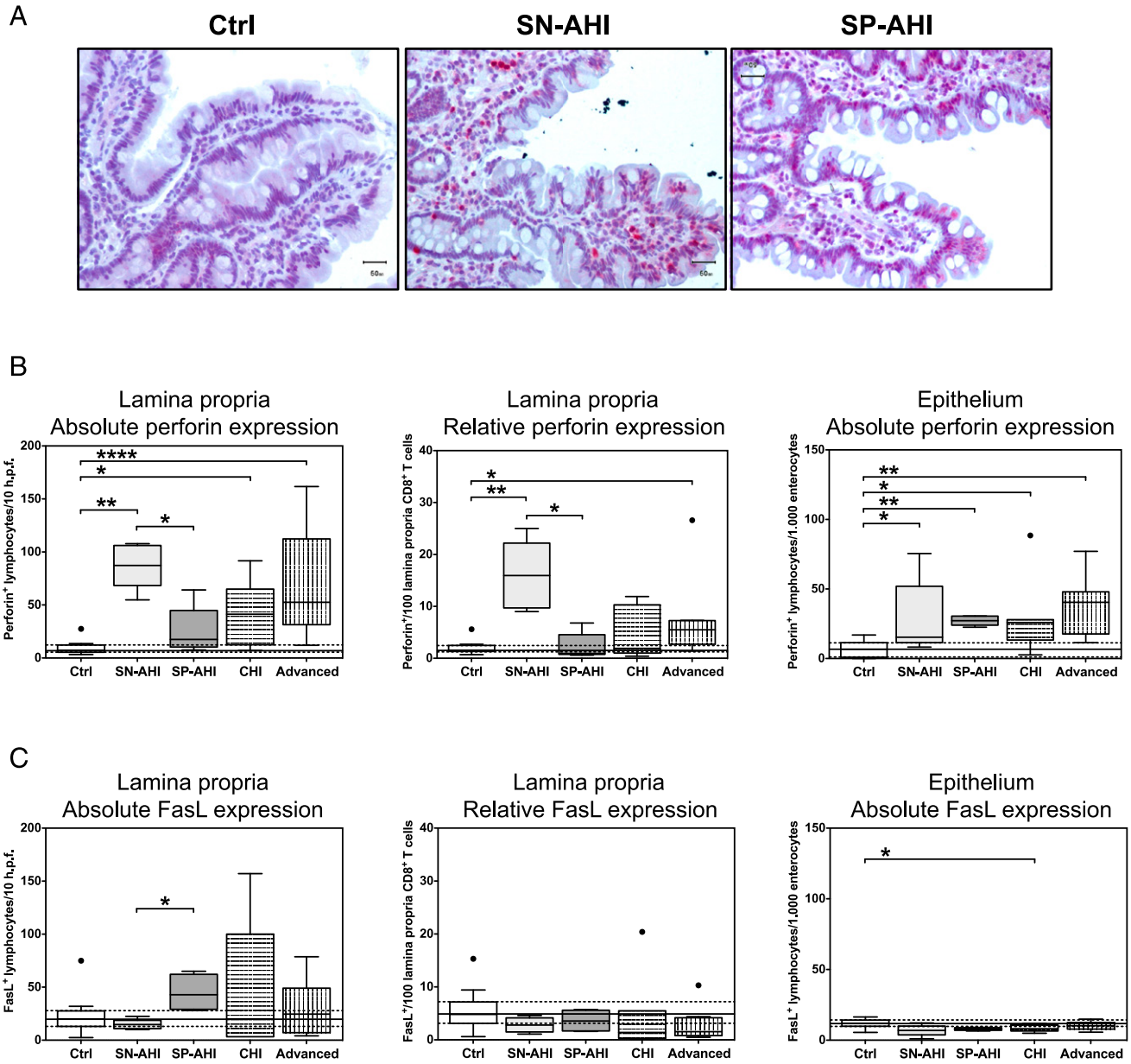

FIGURE 6. Expression of perforin and FasL in the duodenal mucosa of HIV-infected patients. (**A**) Representative immunohistochemical staining of perforin$^+$ lymphocytes (red) in duodenal mucosa from healthy control subjects and from seronegative or seropositive acutely HIV-infected patients. (**B**) Density or relative frequency of perforin-expressing lymphocytes in the duodenal lamina propria or epithelium from control subjects or from HIV-infected patients. (**C**) Density or relative frequency of FasL-expressing lymphocytes in the duodenal lamina propria or epithelium. The frequency of perforin or FasL per mucosal CD8$^+$ T cells was determined by dividing the number of perforin$^+$ or FasL$^+$ lymphocytes by the number of mucosal CD8$^+$ T cells. Median values of control subjects are indicated by the thick horizontal lines, and the dashed horizontal lines denote the normal 25th and 75th percentiles in each diagram. Statistical significances are given for comparisons between patients in each group and control subjects and between seronegative and seropositive acutely HIV-infected patients. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. Advanced, advanced chronic stage of HIV infection; CHI, chronic HIV infection; Ctrl, control subjects; SN-AHI, seronegative acute HIV infection; SP-AHI, seropositive acute HIV infection.

an initial pathological event. This is supported by recent data from SIV-infected cynomolgus macaques that show hyperacute microbial translocation within the first days of infection (52).

After seroconversion, perforin expression in CD8$^+$ T cells decreased in the lamina propria, but not in the epithelium, which implies that lamina propria and intraepithelial CD8$^+$ T cells differ in their cytotoxic activity. The persistent increased prevalence of cytotoxic T cells in the epithelium could prevent restoration of the epithelial integrity in chronic HIV infection. Furthermore, loss of helper function caused by the massive lack of mucosal CD4$^+$ T cells in chronic HIV infection (8) further impairs the gut mucosal defense and, consequently, contributes to the translocation of microbial products into the systemic circulation (8, 13, 53). This is in line with our data showing that plasma levels of sCD14 and/or LBP, signs of systemic triggering by bacterial ligands, remained increased throughout infection.

In conclusion, increase of CD8$^+$ T cells in the intraepithelial compartment, induction of perforin expression, as well as enterocyte damage in the duodenum are initial events in HIV infection.

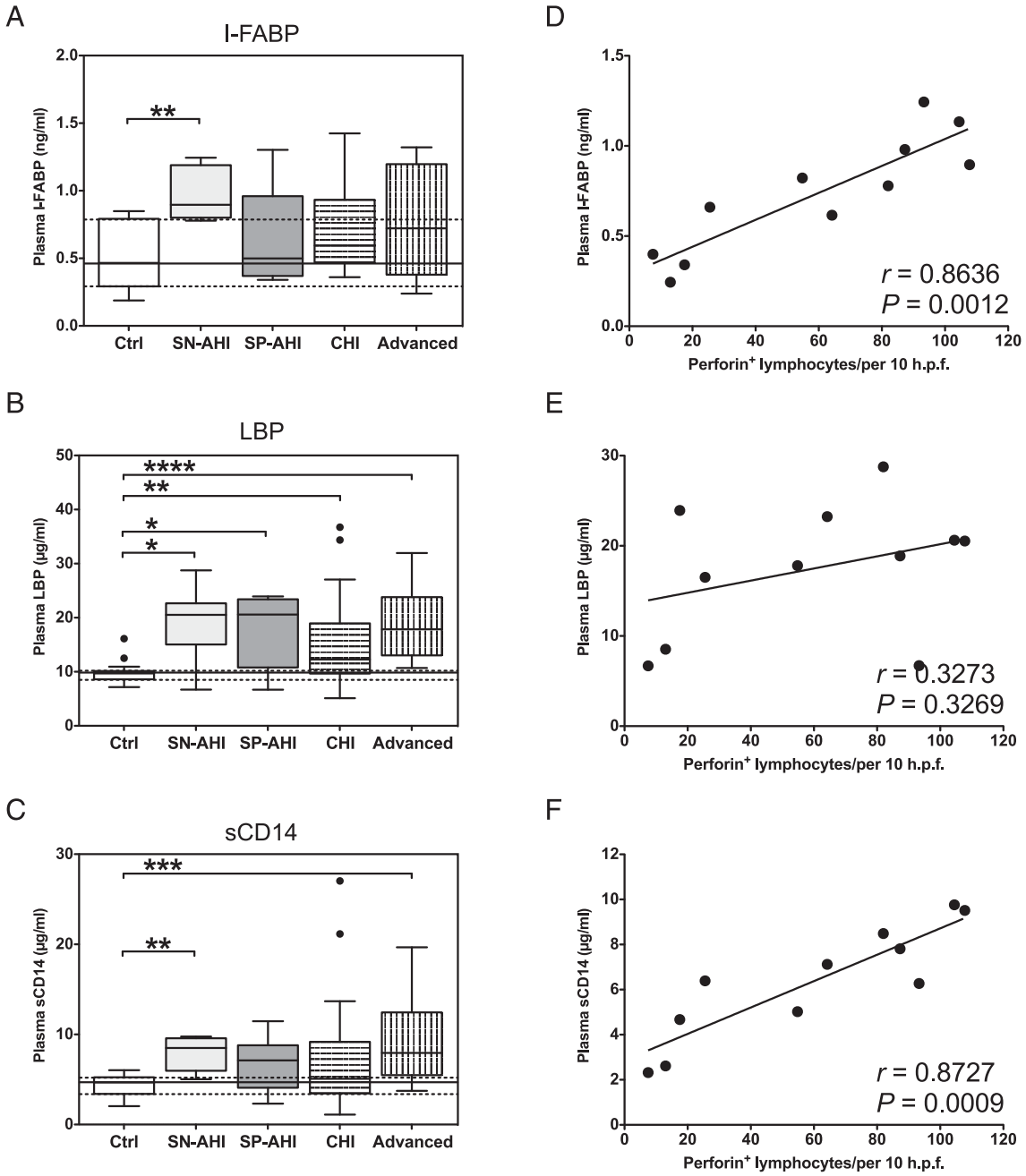

FIGURE 7. Biomarkers of enterocyte damage and microbial translocation. (**A**–**C**) Plasma concentrations of I-FABP (A), LBP (B), and sCD14 (C) in HIV-infected patients in comparison with control subjects. (**D**–**F**) Correlations between plasma I-FABP (D), LBP (E), or sCD14 (F) and the number of perforin-expressing lymphocytes in the duodenum of seronegative or seropositive acutely HIV-infected patients. Median values of control subjects are indicated by the thick horizontal lines, and the dashed horizontal lines denote the normal 25th and 75th percentiles in each diagram. Statistical significances are given for comparisons between patients in each group and control subjects and between seronegative and seropositive acutely HIV-infected patients. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Advanced, advanced chronic stage of HIV infection; CHI, chronic HIV infection; Ctrl, control subjects; SN-AHI, seronegative acute HIV infection; SP-AHI, seropositive acute HIV infection.

Our findings indicate that perforin-dependent $CD8^+$ T cell cytotoxicity destroys enterocytes already in the seronegative phase, and thereby rapidly triggers the mucosal barrier defect and hyperacute microbial translocation. Massive infiltration of the lamina propria by $CD8^+$ T cells that becomes first detectable after seroconversion is caused by enhanced cell trafficking from the periphery, precedes the mucosal $CD4^+$ T cells depletion, and is associated with downregulation of perforin expression in the lamina propria. In the intraepithelial compartment, perforin-dependent cytotoxicity persists after seroconversion and may contribute to the maintenance of the mucosal barrier defect in chronic HIV infection. Consequently, lamina propria and intraepithelial $CD8^+$ T cells may differentially affect the immune pathogenesis in HIV-infected persons.

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Disclosures

The authors have no financial conflicts of interest.

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