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To cite this version:
C Horn, M Augustin, Ms Ercanoglu, E Heger, E Knops, et al.. HIV DNA reservoir and elevated PD-1 expression of CD4 T-cell subsets particularly persist in the terminal ileum of HIV-positive patients despite cART. HIV Medicine, Wiley, 2021, 22 (5), pp.397-408. 10.1111/hiv.13031. pasteur-03244353

HAL Id: pasteur-03244353
https://hal-pasteur.archives-ouvertes.fr/pasteur-03244353
Submitted on 1 Jun 2021

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HIV DNA reservoir and elevated PD-1 expression of CD4 T-cell subsets particularly persist in the terminal ileum of HIV-positive patients despite cART

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Objectives
Despite its importance as an HIV anatomic sanctuary, little is known about the characteristics of the HIV reservoir in the terminal ileum (TI). In blood, the immune checkpoint inhibitor programmed-death-1 (PD-1) has been linked to the HIV reservoir and T-cell immune dysfunction. We thus evaluated PD-1 expression and cell-associated HIV DNA in memory CD4 T-cell subsets from TI, peripheral blood (PB) and rectum (RE) of untreated and treated HIV-positive patients to identify associations between PD-1 and HIV reservoir in other sites.

Methods
Using mononuclear cells from PB, TI and RE of untreated HIV-positive (N = 6), treated (n = 18) HIV-positive and uninfected individuals (n = 16), we identified and sorted distinct memory CD4 T-cell subsets by flow cytometry, quantified their cell-associated HIV DNA using quantitative PCR and assessed PD-1 expression levels using geometric mean fluorescence intensity. Combined HIV-1 RNA in situ hybridization and immunohistochemistry was performed on ileal biopsy sections.

Results
Combined antiretroviral therapy (cART)-treated patients with undetectable HIV RNA and significantly lower levels of HIV DNA in PB showed particularly high PD-1 expression in PB and TI, and high HIV DNA levels in TI, irrespective of clinical characteristics. By contrast, in treatment-naïve patients HIV DNA levels in memory CD4 T-cell subsets were high in PB and TI.

Conclusion
Elevated PD-1 expression on memory CD4 T-cells in PB and TI despite treatment points to continuous immune dysfunction and underlines the importance of evaluating immunotherapy in reversing HIV latency and T-cell reconstitution. As HIV DNA particularly persists in TI despite cART, investigating samples from TI is crucial in understanding HIV immunopathogenesis.
Introduction

Despite effective combined antiretroviral therapy (cART), HIV-1 persists in the human body in reservoirs that are a major obstacle to cure [1]. HIV reservoirs can be differentiated into cellular and anatomic reservoirs. In particular, latently infected memory CD4 T-cell subsets, i.e. central memory (T_{CM}), effector memory (T_{EM}) and transitional memory (T_{TM}) cells, are considered an important cellular reservoir [1–3]. These cells circulate in the peripheral blood (PB), but are also found in other anatomic reservoirs. In recent years, the role of gut-associated lymphatic tissue (GALT) as an important reservoir site has been established, in particular the terminal ileum (TI) [4–10].

To date, several markers have been proposed to be associated with cellular HIV reservoirs [11,12], among them the immune checkpoint inhibitor PD-1 [13]. During HIV infection, PD-1 expression is associated with impaired T-cell function, T-cell exhaustion and progression to AIDS [14–16]. As a result of T-cell exhaustion, immune-mediated control of HIV infection and virus elimination are reduced [17]. PD-1 is upregulated on HIV-specific CD4 T-cells in PB [19,21,22]. Moreover, PD-1+ CD4 T-cells in PB correlate directly with viraemia and indirectly with CD4 T-cell count [14]. In particular, blood central memory (T_{CM}) and transitional memory (T_{TM}) cells were found to be a preferential cellular HIV reservoir in patients on cART [2,23–25]. Blockade of PD-1/PDL-1 interaction increases HIV-specific CD4 and CD8 T-cell function [14,18–20] and CD4 proliferation [14,19]. Several in vitro studies also showed that PD-1 blockade could potentially disrupt latency [26–28]. Immunotherapeutic targeting of PD-1 could therefore potentially be used as translational treatment approach for relieving T-cell exhaustion and eliminating cellular reservoirs.

Increased PD-1 expression on CD4 T-cells was also observed in other anatomic reservoirs such as lymph nodes [22,29] and rectal tissue (RE) [30,31]. However, so far only one study has analysed PD-1 levels in TI in five cART-treated HIV-positive patients [26]. Therefore, little is known about PD-1 expression in TI and whether it is affected by cART.

In this study, we thus evaluated the expression of PD-1 on memory CD4 T-cell subsets in TI, PB and RE, and investigated whether cART affects PD-1 expression levels. We also determined the levels and cellular distribution of HIV DNA in GALT in long-term cART-treated vs. treatment-naïve HIV-positive patients. As an important HIV anatomic sanctuary withongoing viral replication [32,33], we hypothesized that in TI both HIV DNA levels and PD-1 expression remain high in cART-treated individuals.

Methods

Patients and biopsy acquisition

In all, 18 aviraemic HIV-positive patients on cART, six viraemic, treatment-naïve HIV-positive patients, and 16 uninfected individuals enrolled and signed informed consent approved by the Institutional Review Board of the University of Cologne, Germany (no. 15-180).

Inclusion criteria comprised: (1) age 18–65 years, and (2) infection with HIV-1. Exclusion criteria included clinical contraindications for sedation or endoscopy. All individuals also had to be free of inflammatory or lymphoproliferative bowel diseases on histopathological examination. Patients’ clinical characteristics as well as coinfections at the time of biopsy and blood draws, if present, were documented (Table 1). Fifty millilitres of PB were collected. Six to 10 ileal and rectal biopsies from colonoscopy were immediately placed in 5 ml tissue culture medium (RPMI 1640, Biochrom, United Kingdom, F1215) equipped with 10% penicillin/streptomycin and 25 µg/ml amphotericin B.

Cell isolation and fluorescence-activated cell sorting (FACS)

Peripheral blood mononuclear cells (PBMCs) were separated by density centrifugation on Ficoll gradient (Biochrom, L 6115). To separate lamina propria mononuclear cells (LPMCs) of ileal and rectal mucosae into single cells, the method described by Lehmann et al. [34] was used. Briefly, up to 10 ileal or rectal biopsies stored in 10 ml Hanks’ Balanced Saline Solution (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 40µl EDTA (0.5 M; Sigma-Aldrich, USA) were subjected to rapid shaking at 37°C for 20 min. Subsequently, collagenase type I (1 mg/mL; Sigma-Aldrich), hyaluronidase (2 mg/mL; Sigma-Aldrich) and DNAse type I (0.3 mg/mL; Roche, Germany) were added for digestion and incubated for 35–40 min at 37°C.
37°C. After passage through a blunt 16-gauge needle and washing, MCs were isolated by density centrifugation on Ficoll gradient (Biochrom). PBMCs and LPMCs were then frozen in medium [90% fetal calf serum (Biochrom) and 10% dimethyl sulphoxide (Sigma-Aldrich)]. For analysis, thawed mononuclear cells were incubated without prior culturing with the following antibodies and appropriate isotype controls: CD3-APC-H7 (1:40, 560176; BD Biosciences, San Jose, California, USA), CD4-FITC (1:40, 555346; BD Biosciences), CD45RO APC (1:10, 130-109-430; Miltenyi Biotec, Germany), CCR7 Pe-Cy7 (1:80, 557648; BD Biosciences), CD27 PE (1:40, 560985; BD Biosciences), and PD-1 BV421 (1:20, 329920; Biolegend, San Diego, California, USA). To reduce inter-observer variation and to ensure comparability of results, we measured at least one to two HIV-positive samples and one control sample at a time. Antibody titration was performed and stained cells were thoroughly washed to prevent non-specific binding. Following fluorescence minus one control (FMO) and compensation, flow cytometry was performed on a BD FACSAria Fusion flow cytometer (Fig. 1). A cell strainer (352235; Falcon, Corning, New York, USA) was used to minimize clumping. Expression of PD-1 was assessed by geometric mean fluorescence intensities (gMFI). Dead cells were excluded by forward and side scatter. The freezing and thawing process did not affect cell viability. Detection of membrane proteins by antibodies was not altered by mechanical isolation. The four CD4 memory T-cell subpopulations were sorted into 2 ml collection tubes as illustrated in Fig. 1a. The four-way purity mode guaranteed a contamination and non-target cell free sort in all populations.

**DNA extraction and HIV-1 quantification**

Total HIV DNA was extracted from sorted memory CD4 T-cell subsets using QIAamp DNA Blood Kit (51104; Qiagen, Qiagen, Germany) according to manufacturer’s instructions. Quantitative PCR was performed, and cell-associated HIV-1 DNA copies/mL were normalized to β-globin levels. Results were expressed as HIV copies/10⁶ cells of each sorted cell type. We calculated the contribution of each cell type to the total measured HIV DNA. If HIV-1 DNA was undetectable in memory CD4 T-cell subsets, a value of 0 was attributed to the subset.

Table 1 Clinical characteristics

|                      | HIV-positive | HIV-positive | HIV-negative |
|----------------------|--------------|--------------|--------------|
|                      | cART-treated (n = 18) | treatment-naive (n = 6) | (n = 16) |
| Age (years) [median (IQR)] | 55 (49–60) | 49 (46–51) | 58 (50–65) |
| Gender (%) |            |              |              |
| Female | 11 | 0 | 56 |
| Male | 89 | 100 | 44 |
| HIV infection | |              |              |
| Time since HIV-1 diagnosis (years) | 14 (10–19) | 2.5 (2–5) | |
| CD4 (cells/µL) | 617 (452-890) | 155 (48–263) | |
| CD4 (%) | 33 (26–35) | 17 (6–27) | |
| CD4 nadir (cells/µL) | 244 (137–400) | 140 (40–270) | |
| Plasma viral load (RNA copies/mL) | 40 (20–40) | 792 300 (27 425–1 585 000) | |
| cART | |              |              |
| cART (years) | 8 (5–11) | | |
| Aviraemia (years) | 8 (5–10) | | |
| cART regimen (n) | |              |              |
| NtRTI/NRTI/INSTI | 6 | | |
| NtRTI/NRTI/NNRTI | 3 | | |
| NtRTI/NRTI/NNRTI | 2 | | |
| NtRTI/INSTI | 2 | | |
| NtRTI/NNRTI | 1 | | |
| NtRTI/NNRTI/PI | 1 | | |
| NtRTI/PI/PI | 1 | | |
| Coinfections (n) | |              |              |
| Chronic HBV | 1 | | |
| Acute HCV | 1 | | |
| Gonorrhoea | 1 | 1 | |
| Percentage of total (%) | 16.7 | 16.7 | |

cART, combined antiretroviral treatment; HBV, hepatitis B; HCV, hepatitis C; INSTI, integrase strand transfer inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitors; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; RNA, ribonucleic acid.
Fluorescence HIV-1 RNA ISH was performed using RNA-scope Multiplex Fluorescent Detection Kit v2 and RNAscope Probe-V-HIV1-CladeB probe (both ACD, bio-techne, United Kingdom) according to the manufacturer’s instructions.

Bound probe was detected using the Opal 570 fluorophore (Perkin Elmer, Waltham, Massachusetts, USA). Species-specific positive [human peptidyl-prolyl cis-trans isomerase B (PPIB)] and negative control [bacterial dihydropicolinate reductase (dapB)] probes were run alongside the HIV-1 RNA probe (both ACD, bio-techne). Splenic tissues of HIV-infected and non-infected humanized mice were used as controls for

Combined HIV-1 RNA in situ hybridization (ISH) and immunohistochemistry (IHC)

Fluorescence HIV-1 RNA ISH was performed using RNA-scope Multiplex Fluorescent Detection Kit v2 and RNAscope Probe-V-HIV1-CladeB probe (both ACD, bio-techne, United Kingdom) according to the manufacturer’s instructions.
HIV-1 RNA detection. ISH was followed by IHC with an antibody against human CD3 (CD3-12; Abcam, United Kingdom), detected by a fluorescently labelled anti-rat IgG antibody (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific), and slides were embedded in Fluoromount-G (Thermo Fisher Scientific). The signal was visualized using an inverted Leica DMi8 epifluorescence microscope and an inverted Leica SP8X WLL confocal microscope and analysed with Fiji software [35].

Statistical analysis

Statistical analysis was conducted with GraphPad Prism software v.6 (La Jolla, CA, USA). Significant differences were tested with two-tailed Mann–Whitney test or Wilcoxon matched-pairs test, as applicable. P-values < 0.05 were considered as statistically significant. Spearman’s r was used to describe non-parametric correlations. All values are represented as the median with interquartile range (IQR) unless otherwise stated.

Results

Patient characteristics and group size

The clinical characteristics of the patients are summarized in Table 1. HIV-positive patients were predominantly male (89% cART-treated; 100% treatment-naïve), while the HIV-negative control group was more gender-balanced (female, 56%; male, 44%). There was no significant age difference between the cART-treated and HIV-negative patients and cART-treated and treatment-naïve patients. However, treatment-naïve patients were significantly younger than the control group (P = 0.0383). At the time of biopsy and blood draw, all cART-treated patients’ viral load was under the level of detection, while all treatment-naïve patients had detectable HIV-1 viral load. ART regimens, duration of treatment and aviraemia, and coinfections at the time of study inclusion are listed in Table 1. Due to technical challenges at different stages in the study design, i.e. insufficient colonoscopy preparation, failure to intubate the TI during endoscopy, or cell clumping during FACS analysis of, in particular, rectal samples, we obtained incomplete data on gastrointestinal biopsies. Differing sample size is listed when appropriate.

Similar distribution of memory CD4 T-cell subsets in HIV-positive and HIV-negative individuals

In PB, TI and RE, we observed similar distribution of memory CD4 T-cell subsets in HIV-positive cART-treated, untreated and HIV-negative patients. Transitional memory T-cells (TEM) were the largest subset in all three sites, followed by effector memory (TEM) and central memory T-cells (TCM) in PB and TI, and TEM and TCM in RE (Fig. 2a–f).

HIV DNA particularly persist in TI despite cART

HIV-1 detection rates in sorted CD4 T-cells subsets were, on average, 89%, 67% and 67% in PB, TI and RE of treatment-naïve patients, respectively, and 80%, 70%, and 53% of cART-treated patients (Table 2). In treatment-naïve patients, we detected similar, high levels of HIV DNA in PB and TI (Fig. 2g; Table 2a,b). By contrast, cART-treated patients had significantly higher levels of HIV DNA in TI than in PB (P = 0.0273; Fig. 2h). Comparison of the two groups revealed that cART-treated patients showed lower levels of HIV DNA in PB and TI compared with treatment-naïve patients. This difference in HIV DNA was statistically significant in PB (P = 0.0077; Figs 2g,h). Comparison to the rectum was not possible due to small sample size (treatment-naïve, n = 1); additional data are required for statistical significance of this comparison.

Level of HIV DNA in memory CD4 T-cell subsets differs by site

In PB of cART-treated patients, TEM had higher HIV DNA levels per 10^6 CD4 memory T-cells vs. TCM (P = 0.0010); the difference with TCM was not significant (Fig. 2i). By contrast, TCM comprised more HIV DNA than TEM and TCM in TI; differences were not statistically significant (P = 0.0645 and P = 0.5195, respectively). In RE, the sample size was small and data were spread (Table 2). Further analysis and comparison of HIV DNA levels per memory T-cell subset of treatment-naïve patients was limited by small sample size.

HIV-1 RNA T-cells are mostly localised in B-cell follicles of GALT

Ileal biopsy tissue sections of five HIV-positive viraemic, treatment-naïve patients were tested for the expression of HIV-1 RNA and CD3 (Fig. 3). In three distinct patients containing visible lymphoid structures, clusters of multiple hybridization signals for HIV-1 RNA were all co-localized with CD3 staining, examples of which are shown in Fig. 3b,c, and localized inside lymphoid structures. In two sections containing discernible germinal centres, these HIV-1 RNA T-cells were found either inside the GC or directly...
adjacent within the inner rim of the T-cell zone (Fig. 3a). No distinct HIV-1 RNA signals were detected outside lymphoid structures, while signal for the positive control probe against human PPIB was detected throughout the tissue sections. No specific staining could be seen in slides stained using the negative control for bacterial dapB.
Table 2 Detection and levels of HIV DNA of memory CD4 T-cell subsets by site

|                | Peripheral blood | Terminal ileum | Rectum |
|----------------|------------------|----------------|--------|
| (a) HIV-1 detection rate (%) |                  |                |        |
| Treatment-naive |                  |                |        |
| T_{TM}          | 83               | 75             | 100    |
| T_{EM}          | 100              | 75             | 100    |
| T_{CM}          | 83               | 50             | 0      |
| Total           | 89               | 67             | 67     |
| cART-treated    |                  |                |        |
| T_{TM}          | 67               | 91             | 80     |
| T_{EM}          | 94               | 73             | 40     |
| T_{CM}          | 78               | 46             | 40     |
| Total           | 80               | 70             | 53     |
| (b) HIV DNA per memory CD4 T-cells subset (HIV copies/10^6 CD4 memory T-cells) | |
| Treatment-naive |                  |                |        |
| T_{TM} Median   | 21 060           | 724            | n/a    |
| Q1              | 2295             | 133            |        |
| Q2              | 107 025          | 81 979         |        |
| T_{EM} Median   | 15 065           | 4900           | n/a    |
| Q1              | 40908            | 783            |        |
| Q2              | 132 500          | 61 548         |        |
| T_{CM} Median   | 23350            | 43200          | n/a    |
| Q1              | 907              | 0              |        |
| Q2              | 76275            | 1 117 000      |        |
| cART-treated    |                  |                |        |
| T_{TM} Median   | 642              | 5480           | 1510   |
| Q1              | 0                | 1650           | 0      |
| Q2              | 2085             | 34700          | 6080   |
| T_{EM} Median   | 1780             | 2960           | 0      |
| Q1              | 1123             | 0              | 0      |
| Q2              | 3890             | 18200          | 2790   |
| T_{CM} Median   | 873              | 0              | 0      |
| Q1              | 128              | 0              | 0      |
| Q2              | 1615             | 5850           | 17 700 |
| (c) Cumulative HIV DNA (HIV copies/10^6 CD4 memory T-cells) | |
| Treatment-naive |                  |                |        |
| Median          | 123 235          | 141 453        | n/a    |
| Q1              | 9415             | 4672           |        |
| Q2              | 369 225          | 1 176 000      |        |
| cART-treated    |                  |                |        |
| Median          | 5 866            | 13 280         | 5150   |
| Q1              | 2098             | 5490           | 0      |
| Q2              | 11 855           | 75 460         | 33 280 |

cART, combined antiretroviral treatment; Q1, quartile 1; Q3, quartile 3; Q3–Q1 represent the interquartile range (IQR); T_{CM}, central memory T-cells; T_{EM}, effector memory T-cells; T_{TM}, transitional memory T-cells.

Fig. 3 HIV-1 RNA T-cells in lymphoid follicles of the terminal ileum. Exemplary ileal biopsy section from a viraemic HIV-positive patient with 55 762 RNA copies/mL blood and CD4 count of 310 T-cells/μL blood. The combined in situ hybridization/immunohistochemistry for HIV-1 RNA (red), and CD3 (white) of ileal biopsies from five HIV-positive patients revealed several HIV-1 RNA T-cells in ileal lymphoid follicles (red arrows). Yellow arrows indicate examples of extracellular HIV-1 viral particles probably associated with follicular dendritic cells. [a–c] Overview of one lymphoid follicle (a) with close-ups of the regions marked in (a) (b, c). Nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bars, 50 μm (a); 20 μm (b, c). [Colour figure can be viewed at wileyonlinelibrary.com]
Increased expression of PD-1 in HIV-infected patients

Programmed-death-1 expression levels of naïve and memory CD4 T-cells were determined using the gMFI. In all three sites, HIV-positive patients showed higher PD-1 expression levels on naïve CD45RO- CD4 T-cells of cART-treated HIV-positive vs. HIV-negative individuals both in the peripheral blood and in the terminal ileum. (b) PD-1 expression levels (gMFI) on memory CD4 T-cells of HIV-negative and HIV-positive treatment-naïve (cART-) and cART-treated individuals by site. We found no statistical differences between cART-treated and untreated HIV-positive individuals in the peripheral blood and terminal ileum. Comparison of CD45RO- and CD45RO+ CD4 T-cells in HIV-positive individuals showed significantly higher PD-1 expression levels on memory CD4 T-cells in the peripheral blood (P < 0.0001) and terminal ileum (P = 0.0007); data not shown. (c) PD-1 expression levels (gMFI) on memory CD4 T-cell subsets of all HIV-positive individuals (cART-treated and treatment-naïve) by site: In peripheral blood, effector memory cells (TEM) showed the highest PD-1 expression levels; in the terminal ileum PD-1 was upregulated similarly on all memory CD4 T-cells subsets; and in the rectum, central (TCM) and transitional memory (TTL) T-cells showed the highest PD-1 expression levels. Data are expressed as median with interquartile range (IQR). P < 0.05 shows statistical significance: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

Discussion

Latently infected memory CD4 T-cells (TEM, TCM and TTM), are an obstacle for curing HIV and persist in anatomic reservoirs, such as the GALT. This study aimed to analyse their PD-1 expression and interplay with the size of the latent viral reservoir in HIV-positive patients before and during cART in anatomic reservoir sites such as PB, TI and RE.

In our study, we observed that cART-treated patients showed similar PD-1 levels on memory CD4 T-cells and those on treatment-naïve patients in PB. Importantly, elevated PD-1 levels also persisted in TI of cART-treated patients and were significantly elevated in comparison to non-infected individuals. While persisting PD-1 elevation
in PB was previously observed [26,36,37], our study provides evidence that PD-1 expression on memory CD4 T-cells is also elevated in TI, and persists despite cART. Indeed, patients in this study had been treated for a median (IQR) of 8 (5–11) years, longer than in a previous study [36]. This suggests that even under prolonged treatment, PD-1+ memory CD4 T-cells remain stable in both PB and TI. As the up-regulation of counter-regulatory immunosuppressive responses in PB despite treatment is associated with lower HIV-specific T-cell responses [37], causative mechanisms need to be explored further. First, a higher presence of T follicular helper cells (Tfh) in lymphoid tissue may contribute to a general increase in PD-1 expression in TI, also noted in uninfected individuals (Fig. 4) [29,38–40]. However, as our panel did not include Tfh markers, i.e. CXCR5, it cannot be determined here to what extent increased PD-1+ in HIV-positive patients is indeed due to Tfh, which needs exploring in the future. Second, persisting PD-1 expression in TI suggests that T-cell exhaustion and, as a result, immune-mediated control of HIV infection and virus elimination described in PB [14–16] are also impaired in TI. This links to chronic immune activation and low level of continuous viral replication in GALT [6], which may contribute to high PD-1 expression despite cART. Third, insufficient drug penetration in lymphoid structures, in particular in GALT, have been previously reported [41–44] and correlated with continued low-level replication in the gut compared with PB [6]. Here, we did not examine drug levels in TI, which would be an important step in the future to correlate biomarker expression with drug penetration. However, even in PB, where cART concentration is high and effectively suppresses viraemia, PD-1 expression remains elevated. Low cART concentration in TI may contribute, but is unlikely to be a primary cause of persisting PD-1 expression.

Recently, it has been suggested that PD-1 expression may provide latently infected cells with a selective advantage to persist during cART [26]. We therefore tested for correlations between PD-1 expression and HIV DNA levels in memory CD4 T-cell subsets. Intriguingly, we found no significant correlation between PD-1-expressing CD4 T-cells and HIV DNA in PB or TI, although we expected blood PD-1+ CD4+ T-cells to be preferentially infected, in particular TCM and TTM [2,13,23,24]. Similarly, high levels of HIV DNA and PD-1 expression were not associated with specific cART regimens or other clinical characteristic, i.e. CD4 nadir or cART duration (Table 1). However, given the small number of subjects with detectable HIV DNA, in particular of treatment-naïve patients, and the large spread of measured HIV DNA, the power to observe a correlation in our study was limited. Furthermore, in contrast to recent studies [26,37], we did not differentiate between high and low PD-1 expression, which may have facilitated detection of correlations between highly PD-1 expressing cells and HIV DNA. Further research is required to ascertain correlations, in particular including longitudinal data from pre- and post-treatment initiation in HIV-positive patients, given the immense potential of immunotherapy to reverse HIV latency and T-cell reconstitution.

Our study demonstrated that, overall, treatment-naïve patients harboured more HIV DNA than did cART-treated patients. In treatment-naïve patients, we could not identify a preferential HIV reservoir, as HIV DNA levels were similar in all anatomic sites. By contrast, cART-treated patients had significantly higher HIV DNA levels in TI than in PB, as seen in previous studies [4–10]. Similarly, it was recently shown in a longitudinal study that in PB and lymph nodes, each additional year on cART significantly lowered the proportion of HIV DNA in CD4+ T-cells, while the decrease in the gut was not statistically significant [46]. In contrast to Lee et al. [46], we did not find a correlation between the duration on cART and the level of HIV DNA, which is most likely attributable to the fact that our data was not longitudinal. Additionally, cell-associated HIV RNA or intact proviral DNA was not measured. We cannot provide information on whether HIV is replication-competent [2,45], and, due to the small size of sorted samples, no viral outgrowth assay could be performed. However, in line with recent findings [46,47], our results suggest that cART may lead to a detectable reduction of HIV DNA levels in PB, while in TI, HIV DNA levels remain higher. Therefore, our observations provide additional evidence for the important role of the TI as a preferential anatomic HIV reservoir in HIV-positive patients despite cART [7,43].

To identify the microanatomical localization of HIV in TI, we used ileal biopsy sections of treatment-naïve patients to detect HIV RNA and, thus, potentially productively infected cells. This analysis revealed that TI in viraemic treatment-naïve patients not only contains high amounts of viral DNA, but also HIV-1 RNA, producing potentially productively infected cells. HIV RNA cells in TI were virtually all CD3+ and exclusively located inside lymphoid structures. The relatively low number of HIV-1 RNA cells detected within the ileal biopsies of highly viraemic human subjects is in line with Estes et al. [6], where a significantly lower number of HIV-1 RNA clls was also detected within lymphoid tissues of humans compared with SIV-infected non-human primates (NHPs). Future studies with more human lymphoid tissue samples need to address whether the latter observation primarily
reflects the overall lower number of viral RNA cells in chronically infected humans vs. NHPs, or whether HIV-positive RNA cells are truly more confined to the ‘B-cell follicular immune sanctuary’ and whether this is affected by cART.

Focusing on memory CD4 T-cell subsets in HIV-positive and HIV-negative individuals, we observed no differences in relative subset frequencies between treatment-naive, cART-treated HIV-positive and HIV-negative patients in any site. However, absolute CD4 T-cells were significantly lower in treatment-naive than in cART-treated patients (Table 1), which suggests that depletion of memory subsets occurs across all memory subsets to a similar extent. Further, similar relative distributions are not indicative of functional recovery, which may prevail between HIV-negative, cART-treated and treatment-naive patients.

With T\textsubscript{TM} being the largest subset in all three sites, our findings contrast with previous research that found increased frequencies of T\textsubscript{TM} in PB and of T\textsubscript{EM} in TI [2,7]. In PB, we observed that T\textsubscript{TM} harbour more HIV DNA, whereas previously, T\textsubscript{CM} were found to be the largest reservoir in PB [2]. Similarly, we identified T\textsubscript{TM} as the largest reservoir in GALT, while Yukl \textit{et al.} [7] found T\textsubscript{EM} to be the most frequent subset and largest reservoir. First, these differences may be due to clinical characteristics: cohorts differed in particular regarding time since HIV diagnosis and average time on cART. Total time since HIV infection, time until cART initiation, and time on cART are discussed to impact the size of HIV reservoir and contribute to the divergences in observations [25,33]. Second, antibodies, markers, i.e. CD45RA instead of CD45RO, and gating strategies differed [2]. Third, in addition to limitations of our study listed earlier (small sample size, few biopsies, low cell yield, detection rates, spread of HIV DNA, no viral outgrowth assay), discrete CD27 and CCR7 expression in TI and RE impede the clear spread of HIV DNA, no viral outgrowth assay), discrete ple size, few biopsies, low cell yield, detection rates, proliferation.

In conclusion, our study provides evidence that PD-1 expression level is increased in PB and TI in HIV-positive patients compared with HIV-negative individuals. Although cART achieves an overall decrease of the HIV reservoir in PB and TI, high PD-1 expression and HIV DNA particularly persists in the latter, which therefore remains an important HIV DNA reservoir. Efforts to reverse latency and eradicate HIV should therefore focus on assessing mucosal sites, particularly in TI, and evaluating checkpoint inhibitors as additional treatment options to cART.

Acknowledgements
The authors thank the study participants and collaborating clinical sites and laboratories. Thanks to Ute Sandaradura de Silva for her expertise and constant support and to Anne Wegner for language improvements. Open Access funding enabled and organized by ProjektDEAL. WOA Institution: Uniklinik Koln Blended DEAL: ProjektDEAL.

Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Financial disclosure: This work was supported by the German Center for Infection Research (grant nos 80185MDCAH to CH and 8018804915 to CL) and the French Agency for Research on AIDS and Viral Hepatitis (ANRS) (grant no. 17066 to MMT). For the remaining authors none are declared.

Author contributions
CL, MA and CH designed the experiments; MA, CH, MSE, DD, VB, EH, KH, JA, CG and FK performed the experiments; DN, SC, CV and MO collected mucosal biopsies; JR, IS and CL selected the patients; CL, MA, CH, GF and MMT discussed and analysed the data; and CH, MA and CL drafted the manuscript. All authors provided valuable input and critically reviewed manuscript.

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