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DOI: 10.1111/hiv.13031
HIV Medicine (2021), **22**, 397–408

ORIGINAL RESEARCH

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

C Horn,[1,2,3,†] M Augustin,[1,2,3,†] MS Ercanoglu,[2,4] E Heger,[4] E Knops,[4] V Bondet,[5] D Duffy,[5] S-H Chon,[6] D Nierhoff,[7] M Oette,[8] H Schäfer,[8] C Vivaldi,[8] K Held,[9,10] J Anderson,[9] C Geldmacher,[9,10] I Suárez,[1] J Rybniker,[1,2,3] F Klein,[3,4] G Fätkenheuer,[1,3] M Müller-Trutwin[11] and C Lehmann[1,2,3]

[1]Division of Infectious Diseases, Department I of Internal Medicine, University of Cologne, Cologne, Germany, [2]Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany, [3]German Center for Infection Research (DZIF), Cologne, Germany, [4]Institute of Virology, Faculty of Medicine and University Hospital of Cologne, University of Cologne, Cologne, Germany, [5]Immunobiology of Dendritic Cells Unit, Inserm U1223, Institut Pasteur, Paris Cedex 15, France, [6]Department of General, Visceral Surgery and Cancer Surgery, University Hospital Cologne, Cologne, Germany, [7]Clinic for Gastroenterology and Hepatology, University Hospital of Cologne, Cologne, Germany, [8]Clinic for Coloproctology, PanKlinik, Cologne, Germany, [9]Division of Infectious Diseases and Tropical Medicine, University Hospital, LMU Munich, Munich, Germany, [10]German Center for Infection Research (DZIF), Munich, Germany and [11]Unité HIV, Inflammation & Persistence, Institut Pasteur, Paris Cedex 15, France

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.

Correspondence: Clara Lehmann, Department I of Internal Medicine, Division of Infectious Diseases, University Hospital Cologne, Kerpener Straße 62, 50937 Cologne, Germany. Tel: +49 221 478 4433; fax: 01149 221 4783470; e-mail: clara.lehmann@uk-koeln.de

[†]These authors contributed equally to this work as first authors.

Keywords: HIV, HIV reservoir, immune dysfunction, memory CD4 T-cell subsets, PD-1
Accepted 4 November 2020

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}) T-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 with ongoing 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, F 1215) 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

Table 1 Clinical characteristics

	HIV-positive cART-treated (n = 18)	HIV-positive treatment-naïve (n = 6)	HIV-negative (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		
NRTI/NRTI/NNRTI	2		
NRTI/NRTI	2		
NRTI/INSTI	2		
NtRTI/NRTI/INSTI	1		
NtRTI/NRTI/NNRTI/PI	1		
NRTI/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; NtRTI, nucleotide reverse transcriptase inhibitor; PI, protease inhibitor; RNA, ribonucleic acid.

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/10^6 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.

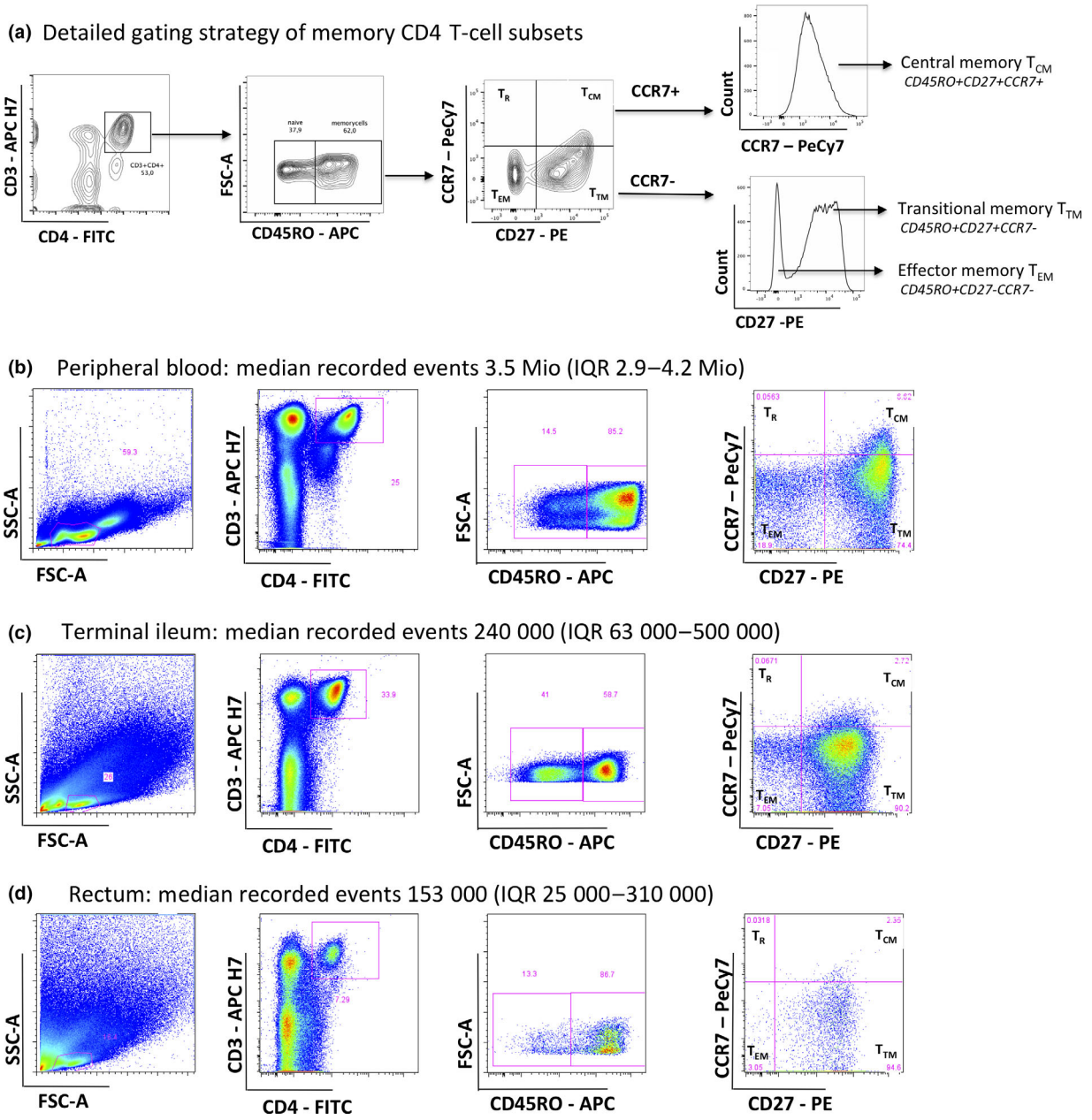

Fig. 1 (a) Gating strategy of memory CD4 T-cell subsets and exemplary flow cytometry dot plots of different anatomic reservoir sites. (b–d) Exemplary flow cytometry dot plots of peripheral blood (PB) (b), terminal Ileum (TI) (c) and rectum (RE) (d) of an HIV-positive patient (ID 4). IQR, interquartile range; Mio, million; TCM, central memory T-cells; TEM, effector memory T-cells; TTM, transitional memory T-cells; TR, remaining memory T-cells. [Colour figure can be viewed at wileyonlinelibrary.com]

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

Fluorescence HIV-1 RNA ISH was performed using RNAscope 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 dihydrodipicolinate 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

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 (T_{TM}) were the largest subset in all three sites, followed by effector memory (T_{EM}) and central memory T-cells (T_{CM}) in PB and TI, and T_{EM} and T_{CM} 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.A). 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, T_{EM} had higher HIV DNA levels per 10^6 CD4 memory T-cells *vs.* T_{TM} ($P = 0.0010$); the difference with T_{CM} was not significant (Fig. 2i). By contrast, T_{TM} comprised more HIV DNA than T_{CM} and T_{EM} 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

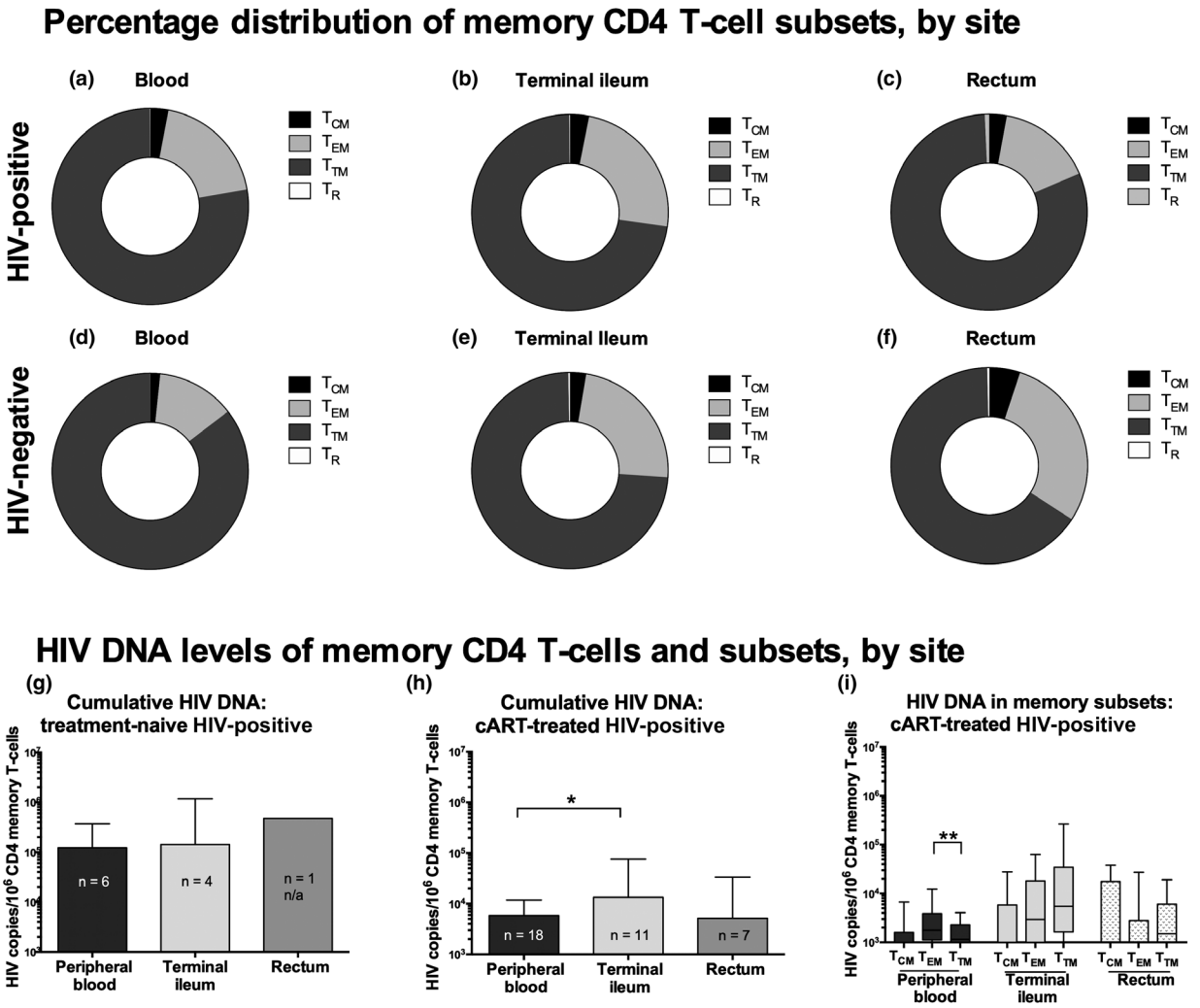

Fig. 2 Distribution of memory CD4 T-cell subsets and HIV DNA in combined antiretroviral treatment (cART)-treated and treatment-naïve patients. (a–f) Relative distribution of memory CD4 T-cell subsets, expressed as a percentage of total memory CD4 T-cells; comparison of HIV-positive (cART-treated and treatment-naïve combined) (a–c) with HIV-negative patients (d–f). Relative distribution of memory CD4 T-cell subsets was similar in HIV-positive treated, HIV-positive untreated and HIV-negative patients in all sites: transitional memory T-cells (TTM) were the largest subset in all three sites for all patient groups. See Table 2 for exact percentages of CD4 T-cell subset distribution and interquartile rages in HIV-positive patients. (g) Cumulative cellular HIV DNA of sorted CD4 T-cell subsets in the peripheral blood, terminal ileum and rectum of treatment-naïve HIV-positive patients by site; no significant difference was seen between peripheral blood and terminal ileum. Comparison to the rectum was not possible ($n = 1$). To determine the cumulative HIV DNA levels per site, normalized HIV DNA copies per million subset memory CD4 T-cells were summed. If HIV-1 DNA was undetectable in a memory CD4 T-cell subset per quantitative polymerase chain reaction, a value of 0 was attributed to the CD4 T-cell subset. (h) Cumulative cellular HIV DNA of sorted CD4 T-cell subsets in the peripheral blood, terminal ileum and rectum of cART-treated HIV-positive patients by site; here, significantly more HIV DNA was detected in the terminal ileum than in peripheral blood. In comparison to treatment-naïve patients (b), level of HIV DNA was lower in cART-treated patients in peripheral blood ($P = 0.0077$), but statistically not in TI. (i) Cellular HIV DNA of distinct memory CD4 T-cell subsets in the peripheral blood, terminal ileum and rectum of cART-treated patients, by site. Data are expressed as median (interquartile range) in (a)–(c), and minimum to maximum in graph D. $P < 0.05$ shows statistical significance: $*P \leq 0.05$, $**P \leq 0.01$. TCM, central memory T-cells; TEM, effector memory T-cells; TTM, transitional memory T-cells; TR, remaining memory T-cells.

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-naïve	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	4960	n/a
		Q1	4908	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	5490	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-naïve	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.

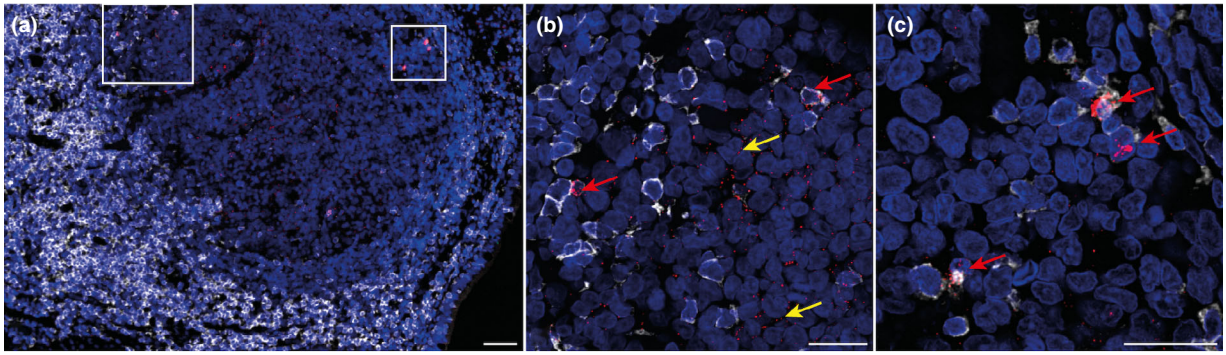

Fig. 3 HIV-1 RNA T-cells in lymphoid follicles of the terminal ileum. Exemplary ileal biopsy section from a viraemic HIV-positive patient with 55 762 RNA copies/mL blood and CD4 count of 310 T-cells/μL blood. The combined *in situ* hybridization/immunohistochemistry for HIV-1 RNA (red), and CD3 (white) 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]

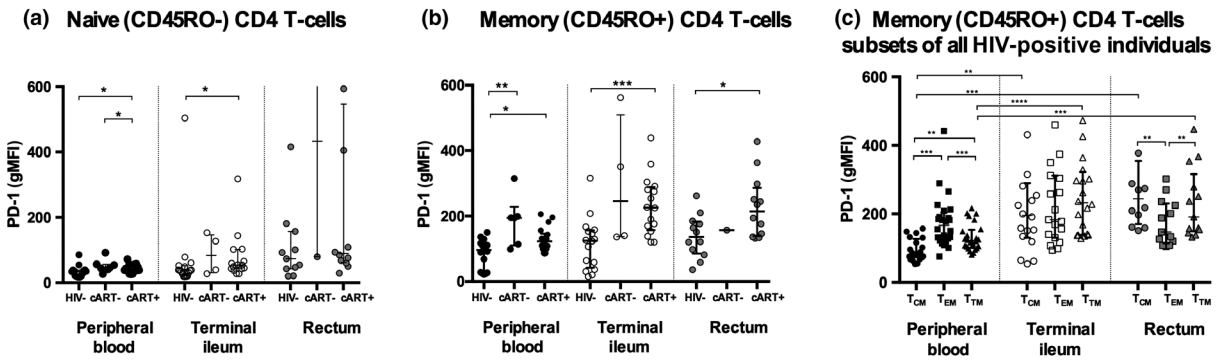

Fig. 4 Comparison of programmed-death-1 (PD-1) expression levels. (a) PD-1 expression levels as geometric mean intensity of fluorescence (gMFI) on naïve (CD45RO-) CD4 T-cells of HIV-negative, HIV-positive treatment-naïve (=cART-) and cART-treated individuals by site: PD-1 expression levels were significantly higher 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-naive) 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 (TTM) T-cells showed the highest PD-1 expression levels. Data are expressed as median with interquartile range (IQR). $P < 0.05$ shows statistical significance: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

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 antigen-naïve (CD45RO–) and memory (CD45RO+) CD4 T-cells than did HIV-negative controls (Figs. 4a and b; average increase from HIV-negative across all sites: antigen-naïve, 1.3-fold; and memory CD4 T-cells, 1.5-fold); only the RE antigen-naïve CD4 T-cells did not exhibit this pattern (Fig. 4a). When we focused on HIV-positive individuals, memory CD4 T-cells showed significantly higher PD-1 expression than did antigen-naïve CD4 T-cells in PB (three-fold increase from HIV-, $P < 0.0001$) and TI (four-fold increase from HIV-negative; $P = 0.0007$). In addition, PD-1 was significantly upregulated in TI (1.8-fold from HIV-negative, $P < 0.0001$) and RE (1.5-fold from HIV-negative, $P = 0.0034$) compared with PB of HIV-positive individuals, whereas the expression was similar in all sites for the control patients. Of note, PD-1 expression levels on memory CD4 T-cells and subsets were similar in treatment-naïve and cART-treated patients (Fig. 4b).

We then looked at the PD-1 expression on distinct memory CD4 T-cell subsets (Fig. 4c). The PD-1 expression of memory subsets differed between the sites: in PB, T_{EM} showed the highest PD-1 expression (*vs.* T_{CM}: two-fold increase, $P = 0.0010$; *vs.* T_{TM}: 2.4-fold increase, $P = 0.0006$; $T_{TM} > T_{CM}$: 1.5-fold increase, $P = 0.0055$); in TI, PD-1 expression was overall increased without significant differences between memory CD4 subsets. In RE, T_{TM} and T_{CM} showed significantly higher PD-1 expression than did T_{EM} (T_{TM}: 1.3-fold increase, $P = 0.0026$; T_{CM}: 1.7-fold increase, $P = 0.0034$).

We found no correlation between PD-1 expression on memory CD4 T-cell subsets and measured HIV DNA. Importantly, clinical characteristics such as CD4 nadir, viral load, duration on cART, and coinfections (Table 1) did not impact on HIV DNA and PD-1 expression.

Discussion

Latently infected memory CD4 T-cells (T_{CM}, T_{EM} and T_{TM}), 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 supresses 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 T_{CM} and T_{TM} [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 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-naïve, cART-treated HIV-positive and HIV-negative patients in any site. However, absolute CD4 T-cells were significantly lower in treatment-naïve 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-naïve patients.

With T_{TM} being the largest subset in all three sites, our findings contrast with previous research that found increased frequencies of T_{CM} in PB and of T_{EM} in TI [2,7]. In PB, we observed that T_{EM} harbour more HIV DNA, whereas previously, T_{CM} were found to be the largest reservoir in PB [2]. Similarly, we identified T_{TM} as the largest reservoir in GALT, while Yukl *et al.* [7] found T_{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 distinction of T_{EM}, T_{TM} and T_{CM} (Fig. 1) [7]. Overall, these differences in findings underline that cell-associated HIV DNA is not easily quantifiable and depends on multiple factors.

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, EK, 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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