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Original Article

Caecum OX40+CD4 T-cell subset associates with mucosal damage and key markers of disease in treated HIV-infection

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KEYWORDS
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Abstract *Background:* Blood OX40-expressing CD4 T-cells from antiretroviral (ART)-treated people living with HIV (PWH) were found to be enriched for clonally-expanded HIV sequences, hence contributing to the HIV reservoir. OX40-OX40L is also a checkpoint regulator of inflammation in multiple diseases. We explored gut mucosal OX40+CD4+ T-cells and their potential significance in HIV disease.

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Thymus;
GALT

Methods: Biopsies of caecum and terminal-ileum of ART-treated PWH (n = 32) were obtained and mucosal damage and HIV reservoir were assessed. Mucosal OX40+ and Ki67+ CD4 T-cell subsets, as well as several tissue T-cell subsets modulating mucosal integrity and homeostasis (Th17, Th22, Treg, Tc17, Tc22, IL17+TCR$\gamma\delta$, IL22+TCR$\gamma\delta$) were quantified. Inflammatory-related markers, T-cell activation and thymic output were also determined in blood samples. Correlations were explored using Spearman rank test and corrected for multiple comparisons by Benjamini-Hochberg.
Results: Compared to healthy controls, a high frequency of mucosal, mainly caecum, CD4 T-cells were OX40+ in PWH. Such frequency strongly correlated with nadir CD4 ($r = -0.836$; $p < 0.0001$), CD4/CD8 ratio ($r = -0.630$; $p = 0.002$), caecum mucosal damage ($r = 0.606$; $p = 0.008$), caecum Th22 ($r = -0.635$; $p = 0.002$), caecum Th17 ($r = 0.474$; $p = 0.03$) and thymic output ($r = -0.686$; $p < 0.001$). It also correlated with Neutrophil-to-Lymphocyte Ratio and blood CD4 T-cell activation and tended to with mucosal HIV reservoir.
Conclusion: High frequencies of caecum OX40+CD4 T-cells are found in people with HIV (PWH) and successful viral control. Interestingly, this cellular subset reflects key markers of disease and peripheral T-cell activation, as well as HIV-driven mucosal damage. OX40+CD4 T-cells deserve further investigation since they could expand because of T-cell homeostatic proliferation and relate to the Th22/Th17 gut mucosal ratio.

Introduction

Antiretroviral therapy (ART) does not avoid the persistence of latent proviruses in CD4 T-cells, constituting the HIV reservoir, that is mainly extended through mucosal tissues.[1] Kuo et al. demonstrated that blood OX40-expressing CD4 T-cells from ART-treated subjects were enriched for clonally-expanded HIV sequences and suggested a potential intervention to reduce the HIV reservoir, using small-molecule antagonists of BIRC5 (Baculoviral Inhibitor of apoptosis Repeat-Containing 5).[2] Remarkably, OX40 (TNFRSF4, CD134), a member of the TNF receptor superfamily, has a protector role in long-term cell viability of T-cells, through the activation of the anti-apoptotic protein BIRC5 and, as a consequence, can promote survival of latently-infected CD4 T-cells, particularly during clonal proliferation.[3]

In contrast to other costimulatory molecules, such as CD27 and CD28, OX40 is absent on resting T-cells, and induced only after T-cell receptor (TCR) engagement.[4] Indeed, OX40 is a known activation-induced marker (AIM) that facilitate the highly sensitive detection of antigen-specific CD4 T-cells in *ex-vivo* assays.[5] The majority of blood OX40+CD4 T-cells express the primary glucose transporter 1 (Glut1) and Glut1+OX40+CD4 T-cells are highly permissive to *in vitro* HIV-infection.[6,7] Consistently, activated cells normally increase their glycolytic activity, which makes the cell more permissive to infection by HIV.[8]

Furthermore, the OX40-OX40L interaction controls the phosphorylation of PI3K and AKT, which are upstream activators of mTOR and hence of inflammatory signals.[9] Moreover, reverse signaling through OX40L to the antigen-presenting cells induces their production of inflammatory cytokines.[10] Consequently, OX40 is emerging as a relevant immune checkpoint regulator in different inflammatory and autoinmmune diseases, being anti-OX40 antibody Rocatinlimab a potent downregulator of Th2, Th1/Th17 and Th22 inflammation.[10–12]

Considering all that, we hypothesized that OX40+CD4 T-cells, particularly those residents at the gut mucosa, as a major HIV reservoir site, could reflect mucosal damage as well as relevant markers of disease and inflammation in chronically-treated PWH.

Methods

This section including additional details of protocols and reagents can be found at Supplementary Material.

Study subjects

People with HIV (PWH) on ART, virologically suppressed at least for three years, were enrolled in the Virgen del Rocío University Hospital. All subjects were on antiretroviral therapy (ART) and virologically suppressed (<20 copies HIV RNA/mL) at the moment of colonoscopy. Subjects suffering cancer, active HCV infection, recent HIV viral rebounds, intestinal infections or inflammatory processes were excluded. Exclusion criteria: cancer, active HCV infection, recent HIV viral rebounds, intestinal infections or inflammatory processes. Peripheral blood and gut biopsies from caecum and terminal ileum were obtained. Clinical data was obtained from the date of starting the last suppressor ART regimen and the date of colonoscopy. The study was approved by the Comité de Ética de la Investigación de los Hospitales Universitarios Virgen del Rocío y Virgen Macarena and informed consents were obtained. Five healthy volunteers were included for reference values.

Gut mucosal cell isolation

Briefly, biopsies were placed in PBS with ethylenediaminetetraacetic acid (EDTA), and then disaggregated and digested with Collagenase II to obtain Mucosal Mononuclear Cells (MMCs).

Immunophenotyping of MMCs and PBMCs

Briefly, cells were stained by surface marker antibodies, followed by fixation and permeabilization, and incubated with intracellular antibodies. Dead cells were stained using LIVE/DEAD Cell Stain. Antibodies used were: anti-CD3, anti-CD4, anti-CD25, anti-CD38, anti-FoxP3, anti-Ki67, anti-CD56, HLA-DR, TCR-$\gamma\delta$ and anti-OX40. A representative example of the $CD4^+$ T-cells gating is shown in Supplementary Fig. 1a. OX40 and the cycling marker Ki67 were determined in CD4 T-cells (Supplementary Fig. 1b). Treg were defined as $CD25^{high}FoxP3^+CD4$ T-cells (Supplementary Fig. 1c). Samples were acquired on a LSR Fortessa and analyzed by FlowJo version 9.2 (Tree Star).

Cytokine production by MMCs

Briefly, MMCs were stimulated with phorbol-12-myristate 13-acetate (PMA) plus ionomycin, in the presence of brefeldin. MMCs were stained with anti-CD3, anti-CD4, anti-CD8, anti-TCR$\gamma\delta$, anti-CD56, anti-IFNγ, anti-IL17a and anti-IL22. Cytokine production was determined in viable CD4 T-cells ($CD4^+CD3^+TCR\gamma\delta^-$), CD8 T-cells ($CD8^+CD3^+TCR\gamma\delta^-$) and TCR$\gamma\delta$ T-cells (CD3+TCR$\gamma\delta$+) (Supplementary Fig. 2a and b). Cellular subsets: Th17 cells ($IFN\gamma^-IL17a^+IL22^{+/-}CD4^+$ T-cells), Th22 cells ($IFN\gamma^-IL17a^-IL22^+CD4^+$ T-cells), Tc17 ($IFN\gamma^-IL17a^+IL22^{+/-}CD8^+$ T-cells), Tc22 cells ($IFN\gamma^-IL17a^-IL22^+CD8^+$ T-cells), $IL17a^+TCR\gamma\delta$ and $IL22^+$ TCR$\gamma\delta$ T-cells.

Histological assessment of damage on mucosal integrity

Briefly, paraffin embedded tissue-sections were stained with hematoxylin-eosin and histological evaluations were performed by a trained researcher. Five parameters were scored (0—3 scale), and a total score (0—15 scale) was assigned for each location (Supplementary Tables 1 and 2). Images were obtained using a light microscope (Supplementary Fig. 3).

Immunofluorescence

Expression of Caspase-3, Zonulin-1 (ZO-1) and Mucin-2 (Muc2) was assessed by indirect immunofluorescence using different pairs of primary and secondary antibodies (Supplementary Table 3). Briefly, paraffin-embedded sections of gut tissues were mounted on gelatin-coated slides. After permeabilization, sections were blocked and incubated with the primary antibody. After that, slides were rinsed and incubated with the corresponding secondary antibody. Images were acquired on an inverted confocal laser scanning microscope. Immunofluorescence intensity was quantified using Image-J free software package.

Immunohistochemistry

Briefly, gut sections in gelatine-coated slides were treated with hydrogen peroxide in methanol, and incubated in a solution containing TBS and goat serum; firstly with the primary antibody (anti-CD8, anti-CD4 and anti-CD3) and then with biotinylated goat anti-rat or goat anti-rabbit IgG. Sections were then incubated with a peroxidase kit and visualized with a standard diaminobenzidine/hydrogen reaction.

Laboratory measurements and soluble biomarkers

The absolute counts of neutrophils, lymphocytes, CD4 and CD8 T-cell in fresh blood were determined with by conventional flow cytometry. Plasma HIV RNA levels were measured by quantitative polymerase chain reaction (detection limit 20 HIV RNA copies/mL). High sensitive C-Reactive Protein (hsCRP), β2-microglobulin and D-Dimers (DD) were determined by conventional techniques. Lipopolysaccharide Binding Protein (LBP) and Anti-CMV IgG antibody titres were measured by ELISA Kits.

Determination of thymic output

Thymic output was measured as the peripheral δ/β TRECs ratio. Determinations were performed in DNA from PBMCs. Briefly, the six DβJβ-TRECs from cluster one together as well as the δ-TREC were amplified by PCR. All amplicons (DβJβ-and δ-TRECs) were then amplified together in a second PCR round using a quantitative PCR system.

HIV reservoir quantification in gut tissues

Total DNA from biopsies was extracted. Then, HIV-1 DNA frequency was measured by droplet digital PCR (ddPCR). Briefly, we used two different primers/probe sets annealing to the 5′LTR and GAG conserved regions of HIV-1, and RPP30 cellular gene was quantified in parallel to normalize. DdPCR reactions were subsequently analyzed using a QX100™ droplet reader and the QuantaSoft v.1.6 software.

Statistical analysis

Continuous variables are expressed as median and interquartile range [IQR], whereas categorical variables are expressed as number and percentages (%). Wilcoxon rank test was used for paired comparisons. Correlations were assessed using Spearman rank test. A p-value <0.05 was considered statistically significant. The Benjamini-Hochberg adjusted p-values (q) were used to adjust for false discovery rates (FDR) and maintain an $\alpha = 0.05$. Prism, version 8.0 (GraphPad Software, Inc.) and Statistical Package for the Social Sciences software (SPSS 21.0, USA), were used for the generation of the graphs and statistical analysis, respectively.

Results

Characteristics of the study population

The thirty-two subjects had a median age of 51 years with a median CD4 T-cell count of 568 cells/mm^3 and were virally-suppressed from a median of six years, showing a large range of values for nadir CD4 and CD4/CD8 T-cell ratio (Table 1). Several inflammation-related soluble biomarkers,

Table 1 Clinical and epidemiological characteristics of PWH and peripheral biomarkers.

Parameters	PWH (n = 32)
Age (years)	51 [46–57]
Cisgender, male, n (%)	29 (91)
CD4 (cells/mm^3)	568 [276–971]
CD8 (cells/mm^3)	656 [469–841]
CD4/CD8 T-cell ratio	0.93 [0.47–1.46]
Previous AIDS, n/n (%)	11/32 (34)
Previous HBV infection, n/n (%)	6/32 (19)
Time under virological suppression (years)	6.0 [4.7–11.9]
Nadir CD4 (cells/mm^3)	55 [18–400]
CD4 before ART onset (cells/mm^3)	130 [27–423]
CD8 before ART onset (cells/mm^3)	762 [332–916]
CD4/CD8 T-cell ratio before ART onset	0.23 [0.06–0.53]
VL before ART onset (log HIV RNA copies/ml)	4.93 [4.39–5.30]
LBP (ng/mL)	10.8 [9.0–12.4]
B2-microglobulin (μg/mL)	2.0 [1.7–2.3]
D-dimer (μg/L)	244 [170–368]
NLR	1.73 [1.36–2.14]
hsCRP (mg/L)	0.90 [0.60–2.90]
δ/β TRECs ratio	14.1 [0.0–47.9]
CD38+HLADR+ CD4	0.69 [0.44–0.94][a]
CD38+HLADR+ CD8	1.53 [0.83–1.93][a]

Continuous variables are expressed as median values [IQR] and categorical variables are expressed as number of cases (%). HBV, Hepatitis B virus; ART, antiretroviral therapy; VL, viral load; LBP, Lipopolysaccharide binding protein; NLR, Neutrophils to lymphocytes ratio; hsCRP, high sensitive C-reactive Protein.
[a] PBMC phenotypes by flow cytometry were available in 23 subjects. HLA-DR+CD38+CD4+/CD8+ refers to HLA-DR+CD38+ among CD4/CD8 T-cells.

the thymic output and peripheral T-cell activation are also summed up in Table 1.

Higher frequency of OX40+CD4 than Ki67+CD4 T-cells at gut locations, particularly at caecum, but not in PBMCs

Phenotype of mucosal mononuclear cells (MMC), as well as other gut tissue determinations, was analysed depending on the availability of biopsy pinches obtained (Supplementary Table 4). We explored if cells expressing OX40 would simply overlap with the whole pool of activated/proliferating cells, represented by Ki67+ cells. The frequencies of OX40+CD4 T-cells were higher in gut sites, particularly at caecum, than in PBMCs (Fig. 1A). Besides, the frequency of the OX40+CD4 subset was significantly higher than that of the Ki67+CD4 at both gut locations, but both subsets overlapped in PBMCs. Healthy donors showed much lower frequencies of both subsets ($p < 0.05$ for all comparisons) (Supplementary Table 5). Both subsets slightly correlated at caecum (r = 0.478; $p = 0.024$; n = 22), and ileum (r = 0.471; $p = 0.036$; n = 20), and only as a trend in PBMCs (0.373; $p = 0.096$; n = 21) (Fig. 1B). Interestingly, both cellular subsets among PBMCs strongly correlated with the frequency of caecum OX40+CD4+ T-cells (r = 0.606; $p = 0.004$; n = 21 and r = 0.735; $p < 0.001$; n = 21, for peripheral OX40+ and Ki67+ CD4 T-cells, respectively); whereas they correlated weaker with the frequency of ileum OX40+CD4 T-cells (r = 0.438; $p = 0.047$; n = 21 and r = 0.564; $p = 0.010$; n = 20 for peripheral OX40+ and Ki67+ CD4 T-cells, respectively). We also observed tight associations between the same T-cell subsets across the two gut locations (r = 0.740; $p < 0.001$; n = 22 and r = 0.732; $p < 0.001$; n = 21, for mucosal OX40+ and Ki67+, respectively).

The frequency of caecum OX40+CD4 T-cells strongly correlated with key markers of disease and inflammation

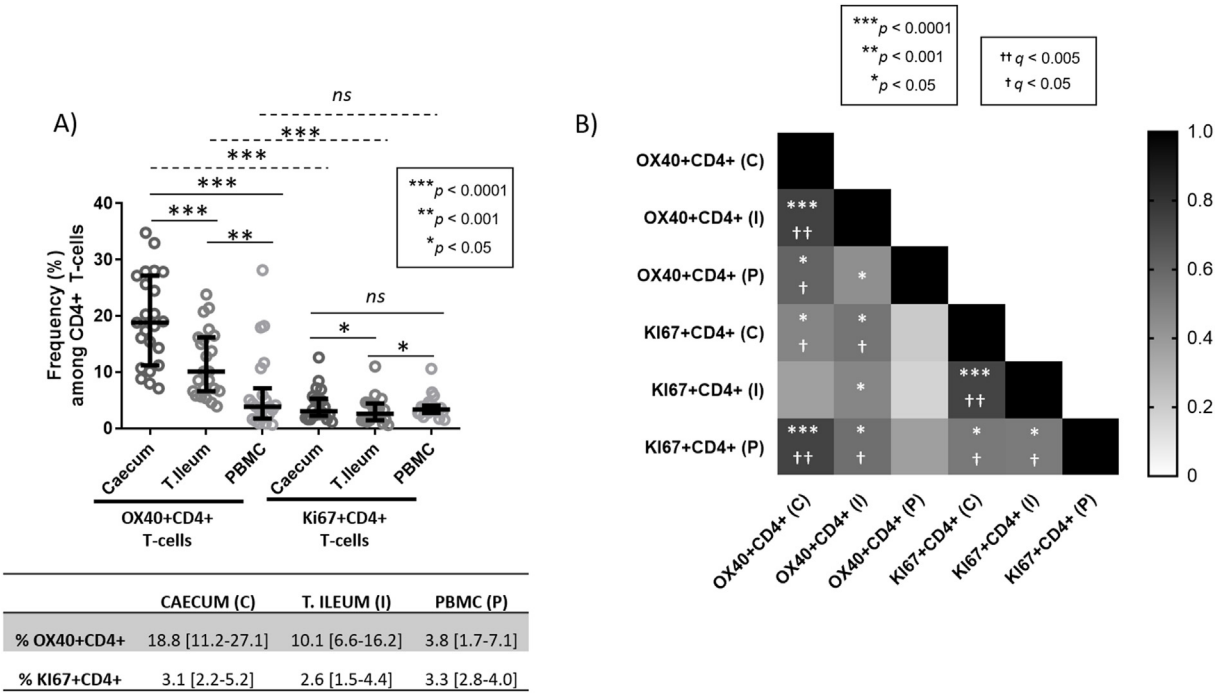
Potential associations with relevant clinical, immunological and inflammation/activation-related parameters were explored (Fig. 2). The frequency of caecum OX40+CD4 T-cells strongly correlated with nadir CD4 (r = −0.836; $p < 0.0001$; n = 23) and CD4/CD8 ratio (r = −0.630; $p = 0.002$; n = 23). Moreover, caecum and ileum OX40+CD4 T-cells correlated with neutrophil-to-lymphocyte ratio (NLR) (r = 0.518; $p = 0.011$; n = 23 and r = 0.554; $p = 0.007$; n = 22, respectively). A strong inverse relationship was also observed between caecum OX40+CD4 T-cells and thymic output (r = −0.686; $p < 0.001$; n = 22). Furthermore, all subsets except circulating OX40+CD4 T-cells, strongly correlated with blood activated CD4 T-cells, but not with activated CD8 T-cells.

Caecum OX40+CD4 T-cells were associated to caecum mucosal damage

Mucosal integrity damage was assessed by the described histological score, obtaining values between 0 and 9, that were found to be similar in caecum and terminal ileum (3.0 [1.7–6.0] (n = 26) and 2.5 [1.0–5.7] (n = 24), respectively, $p = 0.4$). In fact, damage values from both locations correlated with each other (r = 0.645; $p = 0.001$; n = 22). A strong association between caecum OX40+CD4 T-cells and caecum damage (r = 0.606; $p = 0.008$; n = 18), but a trend to correlation between ileum OX40+CD4 T-cells and the ileum damage (r = 0.441; $p = 0.076$; n = 17) were observed (Fig. 2).

Mucosal damage, mainly at the caecum, was tightly related to clinical parameters

Both mucosal damage values correlated negatively with CD4 counts (r = −0.705; $p < 0.001$; n = 26 and r = −0.589; $p = 0.002$; n = 24 for caecum and ileum, respectively); and less intensely to nadir CD4 (r = −0.468; $p = 0.016$; n = 26 and r = −0.441; $p = 0.031$; n = 24, for caecum and ileum, respectively). However, only caecum damage, but not ileum, strongly correlated with CD4/CD8 ratio (r = −0.637; $p < 0.001$; n = 26 and r = −0.343; $p = 0.101$; n = 24, for



	CAECUM (C)	T. ILEUM (I)	PBMC (P)
% OX40+CD4+	18.8 [11.2-27.1]	10.1 [6.6-16.2]	3.8 [1.7-7.1]
% KI67+CD4+	3.1 [2.2-5.2]	2.6 [1.5-4.4]	3.3 [2.8-4.0]

Figure 1. Frequency of OX40+CD4 and Ki67+CD4 T-cells in the gut mucosal sites and the peripheral blood. A) Frequency of OX40+ or Ki67+ among CD4 T-cells in both locations from the gut mucosal and peripheral blood. Comparisons were tested two by two by Wilcoxon paired tests. Dotted-lines represent comparisons between different cellular subsets at the same locations, whereas filled-lines represent comparisons between the same cellular subsets at different locations. B) Correlations between frequencies of OX40+ or Ki67+CD4 among CD4 T-cells from different gut locations and peripheral blood. Correlations were assessed using Spearman rank test and r coefficient is represented in grayscale (all observed correlations were positive). Statistical significance was indicated in the corresponding legends for *p* values and for adjusted *p*-values (*q*-values), by using the Benjamini–Hochberg method to control the false discovery rate (FDR). *ns* means statistically non-significant ($p > 0.05$). C, Caecum; I, Terminal Ileum; P, PBMC.

caecum and ileum damage, respectively). Ileum damage, but no caecum, did correlate with caspase-3 expression ($r = 0.748$; $p = 0.013$; $n = 10$) and mucin-2 expression ($r = -0.691$; $p = 0.016$; $n = 11$). Also ileum damage, but not caecum, correlated with NLR ($r = 0.429$; $p = 0.036$; $n = 24$).

OX40+CD4 T-cells from the caecum negatively correlated with mucosal Th22, but positively with Th17

We also analyzed different mucosal T-cell subsets known to be involved in mucosal immune regulation, inflammation and integrity such as Treg, Th17 and Th22, as well as CD8 and TCR$\gamma\delta$ T-cells producing IL17 or IL22. The frequencies of caecum Treg, Th17 and Th22 were higher than those at terminal ileum, whereas the rest of subsets showed similar frequencies between gut sites (Supplementary Table 6). Caecum OX40+CD4 T-cells showed a strong negative correlation with caecum Th22 ($r = -0.635$; $p = 0.002$; $n = 21$), as well as with caecum Th22 ratios, both regarding Treg ($r = -0.639$; $p = 0.002$; $n = 20$) and Th17 ($r = -0.685$; $p = 0.001$; $n = 21$) (Fig. 3A). Inversely, caecum OX40+CD4 T-cells positively correlated with caecum Th17 ($r = 0.474$; $p = 0.03$; $n = 21$). No correlations were observed with ileum OX40+CD4 T-cells (Fig. 3B).

Caecum HIV reservoir correlated with clinical parameters and tended to correlate with caecum OX40+CD4 T-cells

Total amounts of HIV DNA copies/million cells were similar in caecum and ileum (45.95 [13.83–106.3] *vs.* 72.1 [28.9–110.9], respectively, $p = 0.182$] and strongly correlated ($r = 0.707$, $p < 0.001$; $n = 21$) (Fig. 4A). Very few data was available from immunohistochemistry, so we could not normalize the reservoir raw data with mucosal CD4 T-cell numbers. As an alternative approach, we used circulating CD4 T-cell counts. Despite limited data, trends for positive associations between adjusted caecum HIV reservoir and caecum OX40+CD4 or activated CD4 T-cells at periphery were observed ($r = 0.547$; $p = 0.069$; $n = 12$ and $r = 0.503$; $p = 0.082$; $n = 13$, respectively) (Fig. 4B and C). Remarkably, adjusted caecum HIV reservoir correlated with nadir CD4 ($r = -0.512$; $p = 0.016$; $n = 21$), CD4 counts ($r = -0.622$; $p = 0.003$; $n = 21$) and NLR ($r = 0.556$; $p = 0.011$; $n = 20$) (Fig. 4D–F).

Discussion

We have explored for the first time the gut mucosal OX40+CD4 T-cell subsets in the HIV context. We found a high frequency of mucosal OX40+CD4T-cells, mainly at

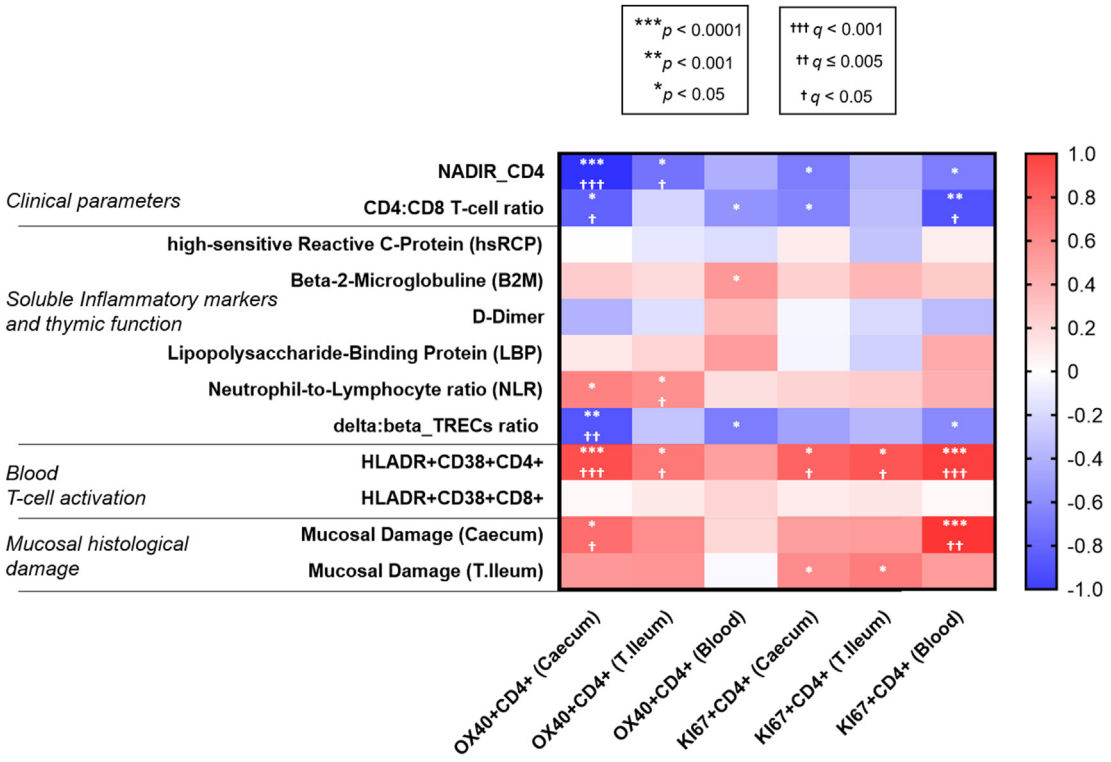


Figure 2. Correlations between the CD4 T-cell subsets from different locations and key markers of disease. We explored potential associations of several markers of disease with OX40+ and Ki67+ among CD4 T-cells in gut mucosal and circulating cells. HLA-DR+CD38+CD4/CD8 refers to HLA-DR+ CD38+ among CD4/CD8 T-cells. Correlations were assessed using Spearman rank test and r coefficient is represented in a double-colored scale. Statistical significance was indicated in the corresponding legends for *p* values and for adjusted *p*-values (*q*-values), by using the Benjamini–Hochberg method to control the false discovery rate (FDR).

caecum (up to 30% among CD4 T-cells), in chronically-treated PWH, strongly correlating with relevant clinical parameters, local mucosal damage, inflammatory cell ratios, thymic output and blood CD4 T-cell activation.

Previous reports focused on the equivalent blood cellular subset had related it not only to HIV viral reservoir and the susceptibility of *de novo* infections[2–6,8] but also to inflammation in different clinical scenarios.[10–12] We still lack cell markers faithfully reflecting the current paradigm of chronically-treated HIV-infection, that is the persistence of viral reservoirs, immune damage and systemic inflammation. Gut-associated lymphoid tissue plays a key role in global immunity. We were able to study gut biopsies, focusing on two gut locations because of the heterogeneity in microbiota diversity and immune tolerance.[13] The integrity of gut-associated mucosa is pivotal to contain the microbial translocation and the activation of immune cells,[14] but it is early altered during HIV-infection[15,16] and remains harmed despite successful treatment, unless initiated very early.[17] Although no previous study has analyzed OX40+CD4 T-cells at human gut tissues, it has been shown that HIV-infection increases the expression of OX40 and its signaling in human jejunal primary cultures,[18] a canonical survival pathway, also described for memory CD4 T-cells from mice gut lamina propria.[19]

Importantly, the frequency of OX40+CD4 T-cells was associated with the mucosal histological damage, as well as with clinical markers of disease. Outstandingly, barrier integrity-related proteins, such as mucin-2 and caspase-3, correlated with histological mucosal damage only at ileum, although limited data could have prevented us to find similar correlations at caecum. Moreover, cellular subsets known to be involved in the regulation of mucosal integrity and inflammation, were associated with local OX40+CD4 T-cells at caecum, negatively in the case of Th22, whereas positively for Th17. These two cellular subsets differ in their tissue pro-inflammatory or regenerative potential, in different autoimmune and inflammatory pathologies, as well as in cancer.[20] Regarding HIV-infection, evidence suggest that Th22 cells play more important protective role than that of Th17 cells.[21] Furthermore, a recent work using the HIV-Flow technique for detecting translation-competent viral reservoirs, showed a majority of Th17 cells within the pool of HIV-1-p24+CD4 T-cells.[22]

A striking result is the strong inverse relation-ship between OX40+CD4 T-cells in the caecum and thymic output. OX40 is upregulated after T-cell receptor (TCR) engagement[4] and is related to clonal expansion.[2,3] Since the thymic output constitutes the supply of new T-cells in depleted settings, it is reasonable that OX40+CD4 T-cells can be enriched because of compensatory T-cell homeostatic proliferation.[23] In this line, the fast homeostatic proliferation in mouse models of adoptive transfer in lymphopenic hosts is specifically linked to the expression of

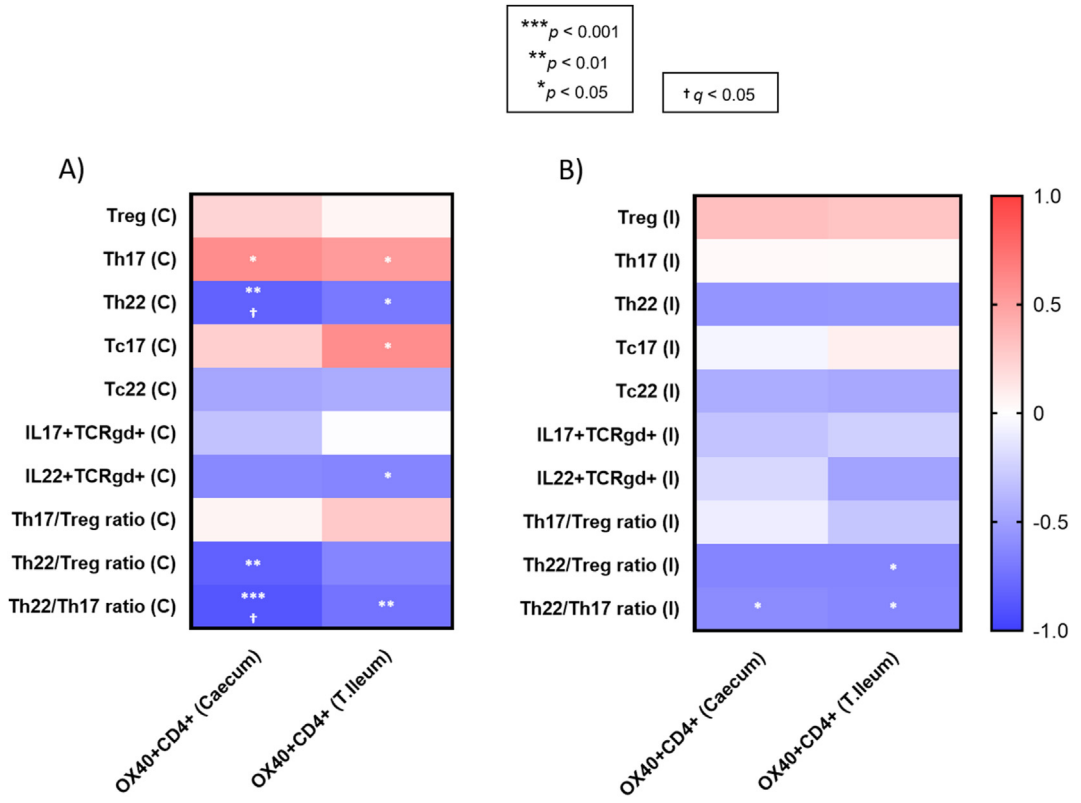


Figure 3. Correlations between mucosal OX40+CD4 T-cells and different mucosal T-cell subsets in gut sites. Different mucosal T-cell subsets known to be involved in mucosal immune regulation, inflammation and integrity such as Treg, Th17 and Th22, as well as CD8 and TCR$\gamma\delta$ T-cells producing IL17 or IL22 were assessed at caecum (A) and ileum (B) and their frequencies correlated with the frequencies of mucosal OX40+CD4 T-cells both, at caecum and terminal ileum. Correlations were assessed using Spearman rank test and r coefficient is represented according to a double-colored scale. Statistical significance was indicated in the corresponding legends for *p*-values and for adjusted *p*-values (*q*-values), by using the Benjamini–Hochberg method to control the false discovery rate (FDR).

OX40[24] and also to the generation of Th17 cells.[25] Consistently, we found OX40 specifically upregulated during the fast homeostatic proliferation of human naïve CD4 T-cells, in an *in vitro* model.[26] Importantly, suboptimal T-cell activation during homeostatic proliferation, mediated or not by OX40 signaling, induces low-level NF-κB preserving HIV latency.[27] Besides, homeostatically expanded T-cells could be less efficient at maintaining gut mucosal integrity than those naturally generated by the thymus due to both, the constriction of the TCR diversity[28] and the probably accumulation of senescent properties, that could limit their effector functions.[29] In this line, several immune T-cell types, essential to maintain gut-mucosal integrity and homeostasis, seem to be directly derived from thymus, as intraepithelial lymphocytes,[30] thymic-derived Th17[31] and thymic-derived Treg cells, which have been reported to play a key role mediating tolerance to commensal microbiota.[32] Furthermore, it was reported that the ablation of thymus in mice was associated with an increase of soluble Lipopolysaccharide-Binding Protein (LBP).[33] All these evidences point to the importance of a continued replenishment with newly generated cells from the thymus to maintain gut mucosal integrity. Moreover, thymic output could also be relevant for the maintenance of the HIV reservoir, since homeostatic proliferation is one of the major drivers of clonal expansion of HIV latently-infected cells.[34,35]

OX40 is also emerging as a relevant immune checkpoint regulator in non-infectious, inflammatory and autoimmune diseases.[10–12] The signaling through OX40-OX40L induces the production of proinflammatory cytokines by dendritic cells,[36] and the blockade of the OX40-OX40L axis reduces inflammation.[10] In our setting, mucosal OX40+CD4+ T-cells, mainly from caecum, positively correlated with peripheral CD4 T-cell activation and with systemic neutrophil-to-lymphocyte ratio. Taken that into consideration, together with its relationships with mucosal damage and tissue integrity modulating T-cell subsets, it is not surprising that caecum OX40+CD4 T-cells also showed strong inverse relation-ships with nadir CD4 counts and CD4/CD8 T-cell ratio, two key markers of inflammation-driven clinical progression.

Finally, blood OX40+CD4 T-cells from chronic PWH have been pointed as relevant contributors of HIV reservoir.[2–6] We observed a trend for an association between the frequency of OX40+CD4 T-cells in the caecum and the HIV reservoir measured at this site. As expected, we found consistent associations between the caecum HIV reservoir and several parameters related to inflammation and CD4 count measures, including nadir CD4.[37] Unfortunately, we

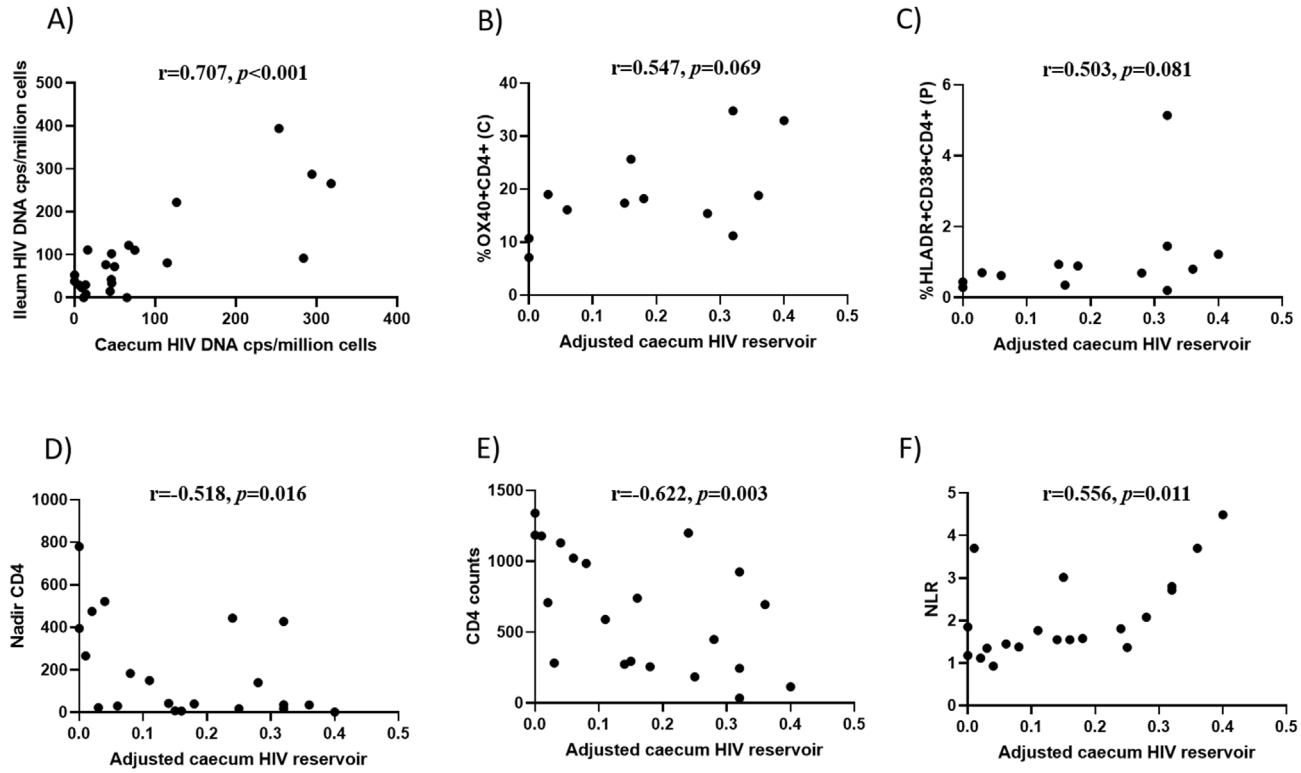


Figure 4. Correlations between adjusted HIV reservoir in the caecum and different immunological and clinical markers. A) Correlation between total HIV DNA (copies/million cells) in the caecum and the ileum is represented. As an approach to normalize the HIV reservoir in the caecum, raw data was adjusted by peripheral CD4 counts (at the same time-point for sampling than biopsies) and then potential associations were explored. Several correlations came up with the frequency of caecum OX40+CD4 (B) or peripheral HLADR+CD38+CD4 (C) T-cells, as well as with nadir CD4 (D), CD4 counts (E) and NLR (F). Correlations were assessed using Spearman rank test and both, r coefficient and p value are represented. Of note, an outlier value of adjusted caecum HIV reservoir (value = 1) was identified by the ROUT method (Q value = 1%) and excluded from all correlation analysis. C, Caecum; P, PBMC.

were unable to explore whether the OX40+CD4 T-cell subset might have higher proviral levels compared to other CD4 T-cell subsets.

The correlation between peripheral Ki67+CD4 T-cells and caecum OX40+CD4 T-cells deserves mention, since it could be reasoned as a more accessible, potential biomarker for the assessment of mucosal damage. However, such peripheral subset did not show as strong relationships with key clinical parameters of disease, systemic inflammation or thymic output. As a possible explanation of the correlation between these two subsets, caecum OX40+CD4 T-cells strongly correlated with peripheral activated T-cells and Ki67 is, indeed, a marker of T-cell activation and cell cycling.

Summing up, despite limited data for certain parameters, our study relies on a multidimensional approach with human gut biopsies. Our results point to the presence of high frequencies of caecum OX40+CD4 T-cells in people with HIV (PWH) but successful viral control. Interestingly, this cellular subset reflects key markers of disease and peripheral T-cell activation, as well as HIV-driven mucosal damage. OX40+CD4 T-cells deserve further investigation since they could expand because of T-cell homeostatic proliferation and relate to the Th22/Th17 gut mucosal ratio.

Ethic statement

The study was approved by the Comité de Ética de la Investigación de los Hospitales Universitarios Virgen Macarena-Virgen del Rocío. Informed consents were obtained from all participants.

Consent for publication

Not applicable.

Availability of data and materials

Main datasets supporting the conclusions of this article are included within the article and its additional files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

I. R-S contributed to the design, optimized protocols for biopsy experimentation, performed experiments, analyzed data and contributed to the data interpretation and to the writing of the manuscript. I. H-F performed experiments and contributed to data analysis. S. S performed the biopsies collection. AE. C, R.M. de P and R. R performed all the histology, immunofluorescence and immunohistochemistry. L T-D performed thymic output determinations. MC. G-G, MC. P and J. M-P performed HIV reservoir analyses. M. L and M. G. recruited study subjects. M.L and Y.M. P conceived the study. Y.M. P designed the study, contributed to data analyses and interpretation and to the writing of the manuscript. All the authors critically reviewed the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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Abbreviations

ART	Antiretroviral treatment
HIV	Human Immunodeficiency Virus
MMC	Mucosal Mononuclear Cells
NLR	Neutrophil to Lymphocyte ratio
PBMC	Peripheral Blood Mononuclear Cells
PWH	People living With HIV
Treg	Regulatory T-cells
TRECs	T-cell Receptor Excision Circles

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2023.08.011.