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CD32 expressing doublets in HIV-infected gut-associated lymphoid tissue are associated with a T follicular helper cell phenotype

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Gut-associated lymphoid tissue (GALT) is a key location for the HIV reservoir. The observation that B-cell–T-cell doublets are enriched for CD32a (a low-affinity IgG receptor) in peripheral blood raises interesting questions, especially as these cells have been associated with HIV DNA in some studies. We sought to determine if similar doublets were present in GALT, the significance of these doublets, and their implications for the HIV reservoir. Given the importance of GALT as a reservoir for HIV, we looked for expression of CD32 on gut CD4 T cells and for evidence of doublets, and any relationship with HIV DNA in HIV + individuals initiated on antiretroviral therapy (ART) during primary HIV infection (PHI). Tonsil tissue was also available for one individual. As previously shown for blood, $CD32^{high}$ CD4 cells were mainly doublets of CD4 T cells and B cells, with T-cell expression of ICOS in tonsil and gut tissue. CD4 T cells associated with CD32 (compared with 'CD32−' CD4 cells) had higher expression of follicular markers CXCR5, PD-1, ICOS, and Bcl-6 consistent with a T follicular helper (TFH) phenotype. There was a significant correlation between rectal HIV DNA levels and CD32 expression on TFH cells. Together, these data suggest that $CD32^{high}$ doublets are primarily composed of TFH cells, a subset known to be preferentially infected by HIV.

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INTRODUCTION

The HIV reservoir is the main barrier to a cure for HIV, and is composed primarily of long lived latently infected CD4 T cells which are unaffected by antiretroviral therapy (ART) and are a source of viral recrudescence on cessation of ART.[1] While much of our knowledge of the HIV reservoir comes from studies in peripheral blood, tissue-resident CD4 T cells found in lymphoid tissue and gut-associated lymphoid tissue (GALT) account for the majority of latently HIV-infected cells in individuals with suppressed plasma viraemia on ART.[2] GALT is preferentially infected during primary HIV infection (PHI) due to the high frequency of activated $CCR5^+$ CD4 T cells.[3] Accordingly, GALT is considered to be one of the most important anatomical reservoirs for HIV proviral DNA.[4]

Initiation of ART during PHI has been used as a strategy to limit the seeding of the HIV reservoir. The total HIV DNA levels are significantly lower in the peripheral blood of individuals treated in the earliest stage of PHI when compared with those treated in chronic-stage HIV infection.[5,6] However, despite early treatment, evidence of incomplete immune recovery and possible on-going viral transcription is evident in lymphoid tissue, even amongst individuals treated during PHI and on ART for over 3 years.[4,7]

Identification of latently infected CD4 T cells in peripheral blood and particularly in tissue sites has been challenging due to the lack of a specific marker on CD4 T cells for latent HIV infection. If identified, such a marker could be used as a target for HIV cure interventions. CD4 T cells in peripheral blood which express the immune checkpoint receptors (ICRs) PD-1, TIGIT and Lag-3 have demonstrated enrichment for HIV DNA,[8] while in rectal tissue PD-1 expression on CD4 T cells may represent a marker of HIV persistence.[9] However, while ICRs may identify cells enriched for HIV DNA, they are not definitive markers of the HIV reservoir. Recent work suggesting that CD32a (a low-affinity IgG receptor not generally expressed on T cells[10]) was a specific marker for latent HIV infection[11] was initially supported by studies showing that CD32 may be found on cells that are transcriptionally active, and was associated with HIV DNA levels in some patients.[12–19] However, the finding that CD32 co-expression with CD4 is predominantly due to T-cell–B-cell doublets[17] likely undermines the argument that CD32 is a definitive marker of latency.

We previously reported partial enrichment for HIV DNA in CD4 T cells associating with CD32 in some individuals,[18] prior to the finding that CD32 + CD4 + flow cytometric events comprised doublets. We therefore wished to explore the phenotype and

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potential physiological—versus artefactual—relevance of T cells engaged in doublet formation, whether they were also present in GALT, and any possible association with the GALT HIV reservoir.

RESULTS

HIV DNA levels measured in gut mucosal mononuclear cells are higher than in peripheral blood mononuclear cells

To confirm enrichment of HIV DNA in gut tissue in our cohort, we initially measured HIV DNA in unstimulated CD4 T cells from peripheral blood mononuclear cells (PBMCs), rectal GALT and terminal ileal GALT from ART-treated individuals who commenced ART during PHI ($n = 18$). Clinical characteristics are shown in Table 1. The median (IQR) duration of ART at time of HIV DNA measurement was 33.5 (19.3–44.3) months. Consistent with published data, we observed significantly higher levels of HIV DNA in both rectal ($P = 0.004$) and terminal ileal ($P < 0.0001$) mucosa compared with matched PBMCs (Fig. 1). Median (IQR) HIV DNA levels in the terminal ileum, rectum and PBMC were 3.49 (3.34–3.87), 3.44 (3.1–3.65) and 3.07 (2.89–3.22) $\log_{10}$ copies per million (CPM) CD4 T cells, respectively.

The frequency of CD32 expression associated with CD4 T cells is similar across anatomical sites, irrespective of HIV status

We next turned to CD32 expression on CD4 T lymphocytes gated both as single cells (or 'singlets') using standard flow-cytometry protocols (Fig. 2a, with gating for CD32 based on an isotype FMO) and as doublets (using imaging flow cytometry).

Using a traditional singlet gate for CD32 + CD4 cells, we compared HIV-infected individuals ($n = 18$) and healthy controls ($n = 5$) (Supplementary Fig. 1). There was no difference in median (IQR) CD32 expression in terminal ileum, rectum or PBMCs in HIV-infected individuals compared with healthy controls: 5.2% (4.2–7.8%) vs. 7.6% (4.7–9.4%) in the terminal ileum, 5.8% (3.8–6.5%) vs. 7.3% (3.4–12.2%) in the rectum and 4.7% (2.7–6.4%) vs. 3.7% (2.5–7.0%) in PBMC. CD32 expression associated with CD4 T cells from the HIV-infected tonsil sample ($n = 1$) was 4.6%. Although we had found HIV DNA to be enriched in GALT compared with PBMCs, these data suggest there is no difference in CD32 expression across anatomical sites in HIV + compared with uninfected participants (Fig. 2b).

We, and others, have previously shown that CD32 + CD4 T cells in peripheral blood exhibit two phenotypic populations, with differential expression of immune checkpoint and activation markers.[18] The gating for $CD32^{low}$ and $CD32^{high}$ populations is based on CD32 expression on CD3-negative lymphocyte populations, which include B cells, and express CD32 at high levels (Fig. 3a). Accordingly, we examined the phenotype and frequency of $CD32^{low}$ and $CD32^{high}$ expression associated with CD4 T cells in GALT and peripheral blood in HIV-infected individuals. No difference in the frequency of $CD32^{low}$ events was noted across anatomical sites, however, $CD32^{high}$ cells were more common in GALT compared with PBMC (Fig. 3b). The median (IQR) frequency of $CD32^{high}$ cells in the terminal ileum, rectum and PBMC was 0.069% (0.026–0.133%), 0.068 (0.024–0.125%) and 0.016% (0.011–0.057%), respectively. The frequency of $CD32^{high}$ cells in tonsil tissue was 0.18%.

$CD32^{high}$ cells are primarily T-cell–B-cell doublets

As we had previously shown that $CD32^{high}$ CD4 T cells from PBMCs expressed non-T-cell lineage markers[18] and others have demonstrated these cells to be doublets,[17] we wanted to explore whether these singlet-gated events were also T-cell–B-cell doublets. We stained PBMCs from healthy donors for CD4 T (CD3 and CD4) and B-cell markers (CD19) as well as CD32 and acquired the samples using imaging flow cytometry, which allows the visualisation of the fluorescence distribution on individual cell events. We found that the CD3 + CD4 + CD14 − $CD32^{high}$ cells were almost entirely T-cell–B-cell doublets. This was shown by co-localisation of CD3 and CD4 on the same cell forming a doublet with a cell expressing both CD19 and CD32 (Fig. 4a; rows 1 and 2). This indicated that the measured CD32 was of B-cell, rather than T-cell, origin. We found no instances of CD32 distributed throughout the surface of a CD4 T cell. Although we found the $CD32^{high}$ population consisted primarily of doublets (Fig. 4a), the $CD32^{low}$ population showed evidence of trogocytosis with regions of punctate CD32 expression on CD4 T-cell singlets, as also reported by Osuna et al.[17] (data not shown).

Tissue-derived CD32+CD4+ cytometric events are also doublets, and stain strongly for ICOS

We next extended our investigations to tissue sites and further probed the phenotype of the T cells comprising these doublets, adding the activation marker HLA-DR and the T follicular helper (TFH) marker ICOS to our imaging flow-cytometry panel. We

Table 1. Participant characteristics

Participant category	Healthy controls ($n = 5$)	ART-treated since PHI ($n = 18$)
Gender		
Male	2	18
Female	3	0
Age at biopsy, years, median (IQR)	71 (56–71)	38 (31–46)
Indication for colonoscopy		
Research only	–	18
Colonic polyps	4	–
Altered bowel habit	1	–
Plasma HIV VL at biopsy, RNA copies per ml, median	NA	<20
Time on ART at biopsy, months, median (IQR)	NA	33.5 (19.3–44.3)
Time from EDI to ART initiation, days, median (IQR)	NA	56 (53–88)
Plasma HIV RNA at HIV diagnosis, $\log_{10}$ RNA copies per ml, median (IQR)	NA	5.6 (4.6–6.1)

IQR interquartile range, *NA* not applicable, *PHI* primary HIV infection, *ART* antiretroviral therapy, *EDI* estimated date of infection, *VL* viral load

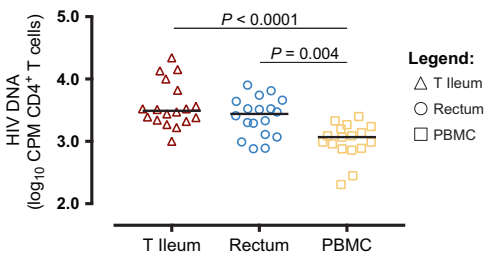

Fig. 1 HIV DNA in gut-associated lymphoid tissue and blood. Levels of HIV DNA (CPM CD4 T cells) in the terminal ileum, rectum and peripheral blood of HIV-infected individuals ($n = 18$). Each symbol represents one individual; the horizontal line through the symbols indicates the median. Comparisons between groups were done using a Kruskal–Wallis test with pairwise comparisons performed using Dunn's test. HIV DNA was quantified using negatively selected CD4 T cells for PBMCs and bulk cells for tissue sites with values corrected for CD4 T-cell frequency using flow-cytometric analysis. CPM copies per million, T Ileum terminal ileum, PBMC peripheral blood mononuclear cells

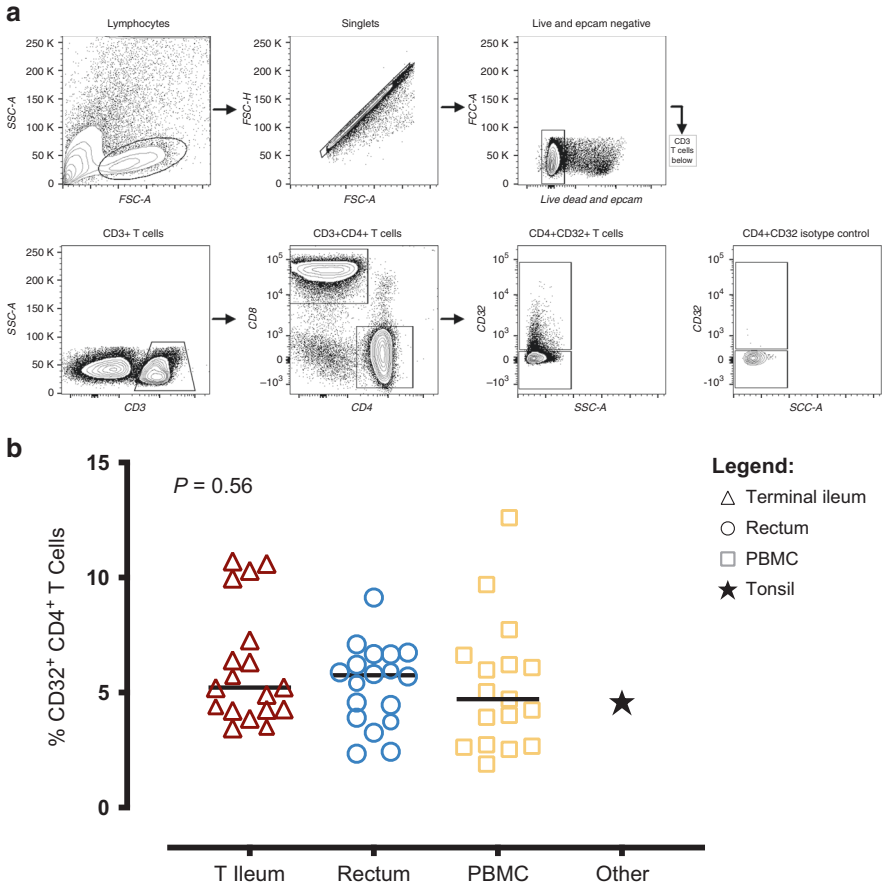

Fig. 2 CD32 expression on CD4 T cells in GALT and PBMCs. **a** Representative CD32 gating strategy; the data shown are gated on mucosal mononuclear cells from terminal ileum GALT. **b** Mucosal mononuclear cells from terminal ileum and rectal mucosa ($n = 18$), and peripheral blood mononuclear cells ($n = 17$) were used to evaluate CD32 expression on CD4 T cells across anatomical sites from HIV-infected individuals. Each symbol represents an individual. The data are shown for HIV-infected individuals only. No statistical difference in the frequency of CD32 expression on CD4 T cells was observed between anatomical sites (rectum, terminal ileum and peripheral blood), $P = 0.56$ by Kruskal–Wallis test. Lines indicate the median. T Ileum terminal ileum, PBMC peripheral blood mononuclear cells

focused our analysis on the CD3 + CD4 + CD14-CD32^{high}CD19 + doublets (referred to as T-cell–B-cell doublets). In the PBMC doublets, only ~16% of the CD4 T cells expressed HLA-DR (10/64 events examined) suggesting the majority of the T cells were not in fact activated (Fig. 4b; rows 3 and 4). We found no ICOS-expressing CD4 T cells in PBMC doublets, consistent with the fact that ICOS is primarily upregulated in activated TFH cells in tissue.[20] We next examined CD32^{high} events in the healthy rectum and terminal ileum (Fig. 4c, d, respectively). As in the periphery, the majority of these comprise T-cell–B-cell doublets. We found no evidence of HLA-DR expression on the CD4 T-cell component of the doublet in either tissue, with HLA-DR expression coming entirely from the B cell (0/7 rectum, 0/20 terminal ileum events examined). Unlike in PBMCs, we observed robust ICOS staining in tissue. The majority of CD32^{high} events were positive for ICOS (6/7 rectum, 14/20 terminal ileum) which always co-localised with CD3 and CD4 rather than CD19 and CD32 (Fig. 4c, d), indicating a TFH phenotype of the T cell within the doublet.

To confirm these findings in HIV-infected tissue—and in the absence of further available HIV + gut biopsies—we stained tonsil from an HIV-infected individual 2 months after ART initiation. Similar to healthy GALT and PBMCs, we again found the CD32^{high} cells primarily consisted of T-cell–B-cell doublets (Fig. 4e). We found that the HLA-DR signal was primarily from the B-cell component of these doublets, with only ~1% HLA-DR expressing T cells (1/81). As in GALT, a substantial proportion of the T cells within the doublets were ICOS positive (59%, 48/81), which was co-localised with CD3 and CD4 rather than with CD19 (Fig. 4e) again indicating a TFH phenotype.

Expression of TFH markers and immune checkpoint receptors is enriched on T cells in CD32^{high} doublets

To explore the T-cell component of the CD32^{high} doublets on a larger scale, we returned to traditional flow cytometry. Based on our imaging flow data, we used ICOS as a TFH marker and examined the T-cells component of the doublet population by gating on ICOS-positive cells only. ICOS expression was significantly higher in the CD32^{high} doublets compared with CD32− cells in both PBMC and rectal tissue, but not in the terminal ileum (Fig. 5a). We then examined double-positive CXCR5 + PD-1^{high} TFH cells (representative gating: Fig. 5b). We found this population was significantly enriched for both Bcl-6 (a TFH transcription factor) and also CD32 when compared to non-TFH in gut tissue (Bcl-6 30.0 vs. 4.3%, $P < 0.0001$; CD32 9.2% and 4.8% $P = 0.0001$), again suggesting an enrichment of TFH in CD32^{high} doublets (Fig. 5c, d). To confirm this signal was not coming from the B cells, we examined the CXCR5 + PD-1^{high} population in bulk cells rather than in CD3 + CD4 + cells, and found these markers almost entirely stained CD3 T cells with minimal background staining of B cells (Supplementary Fig. 2).

We also examined the immune checkpoint receptors TIGIT and Tim-3 to evaluate T-cell exhaustion, as neither marker is typically expressed on B cells.[21,22] TIGIT was significantly higher in the CD32^{low} population, but not in CD32^{high} doublets when compared with CD32- cells in both rectum and terminal ileum tissues (Fig. 5e).

There was no difference in the expression of TIGIT in any CD32 fraction in PBMC (Fig. 5e). Tim-3, however, was significantly higher in CD32^{high} doublets when compared with CD32- cells in both terminal ileum and PBMC, but not rectal tissue (Fig. 5f). The fact that TIGIT and Tim-3 showed distinct patterns in different tissues and CD32 populations suggests a heterogeneous population of cells.

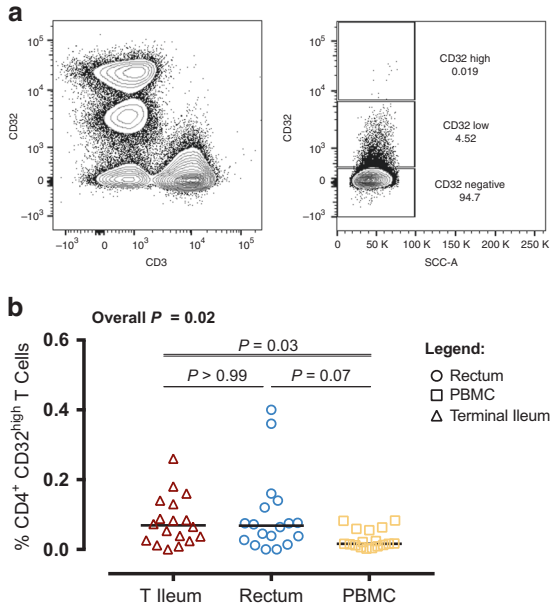

Fig. 3 CD32 high expression on CD4 T cells across anatomical sites. **a** The flow plot on the left shows three distinct CD32 populations in CD3-negative cells; the gating on CD4 T cells are based on these populations. Representative gating of the CD32^{low} and CD32^{high} gating on CD4 T cells is shown on the right. **b** Frequency of CD32^{high} T cells in gut sites and PBMCs in HIV-infected individuals. Comparisons are made using a Kruskal–Wallis test (overall $P = 0.02$), with pairwise comparisons performed using Dunn's test. Lines indicate the median. T Ileum, terminal ileum, PBMC peripheral blood mononuclear cells

Relationship of CD32 expression with HIV DNA

We next wanted to explore the relationship of CD32^{high} doublets with the HIV reservoir. The greatest enrichment of HIV DNA had been reported in relation to a CD32^{high} cell population and—although contested—we had previously seen some HIV enrichment in some HIV-infected individuals in PBMC.[18] Here, we investigated whether there was a correlation between HIV DNA and the frequency of CD32^{high} (Fig. 6a–c) and CD32^{low} (not shown) events across anatomical sites. No relationship was observed in any site. As TFH cells have been shown to be enriched for HIV DNA, we next tested whether the frequency of CD32 + TFH (i.e., TFH cell doublets) correlated with reservoir size. In the rectum (but not terminal ileum; Supplementary Fig. 3), an association was observed between HIV DNA and total CD32 expression associated with TFH cells (with CD32 + events gated as in Fig. 2a) ($r = 0.66$, $P = 0.003$; Fig. 6d). As this was the only positive result in multiple comparisons, we applied the conservative Bonferoni correction accounting for all of the tests run in all sites, and the result remained statistically significant (revised statistical cut-off $P = 0.004$). To confirm this result was not solely attributable to bulk TFH, we tested the relationship between the frequency of TFH in the rectum and terminal ileum (Supplementary Fig. 4) with total HIV DNA and found no significant correlations. Similarly, no relationship was observed between the frequency of $CD3^+CD4^+CD19^+$ cells and HIV DNA (Supplementary Fig. 4). To explore whether the CD32 + TFH correlation with HIV DNA might be present in PBMC and explain some of the disparity in this area, we turned to data from sorted PBMC from ART-treated patients in whom we had previously found some HIV DNA enrichment in some individuals. However, there was no correlation ($P = 0.14$) between HIV DNA enrichment and the level of $CD32^+$ doublets containing TFH cells, suggesting this finding is not expandable to PBMCs (Supplementary Fig. 5).

DISCUSSION

In studies of the HIV reservoir, higher HIV DNA levels have been consistently detected in GALT compared with PBMCs.[2,23] However, as yet, there is no specific marker for these latently

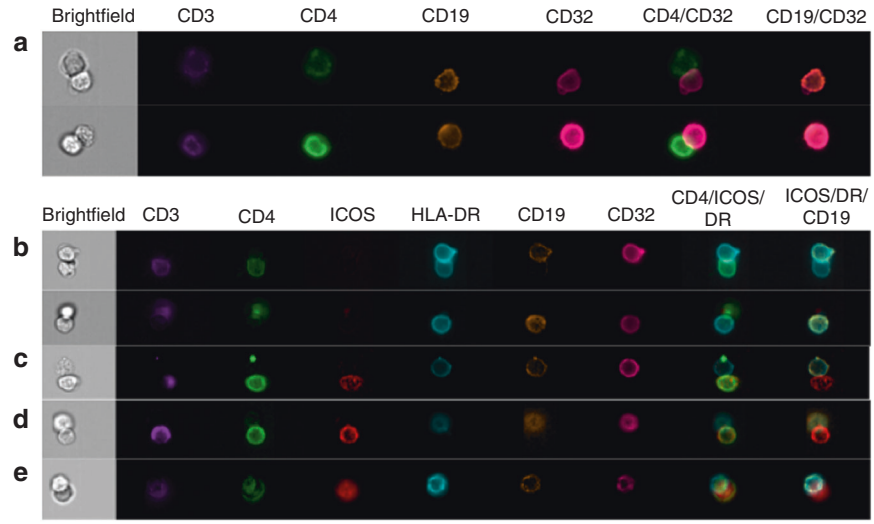

Fig. 4 Expression of ICOS and HLA-DR on CD32^{high} cells across anatomical sites using imaging cytometry. Imaging flow cytometry from PBMCs and tissue sites. For all panels, the representative cells shown are live, singlet, CD3 + CD4 + CD14 − CD19 + CD32^{high}. **a** Rows 1–2; Cells from healthy donor PBMC ($n = 2$) were stained as shown (panel 1: brightfield, CD3, CD4, CD19, CD32, plus CD4/CD32 and CD19/CD32 overlays). For (**b**–**e**) rows 3–7, cells are stained with panel 2 (brightfield, CD3, CD4, ICOS, HLA-DR, CD19, CD32 stains plus CD4/ICOS/HLA-DR (DR) and ICOS/HLA-DR/CD19 overlays). **b** Row 3–4; cells from healthy donor PBMC ($n = 1$) where row 3 shows an HLA-DR + CD4 T cell, while row 4 shows an HLA-DR- CD4 T cell. **c** Row 5; cells from a healthy rectum ($n = 1$). **d** Row 6; cells from a healthy terminal ileum ($n = 1$). **e** Row 7; cells from an infected tonsil ($n = 1$)

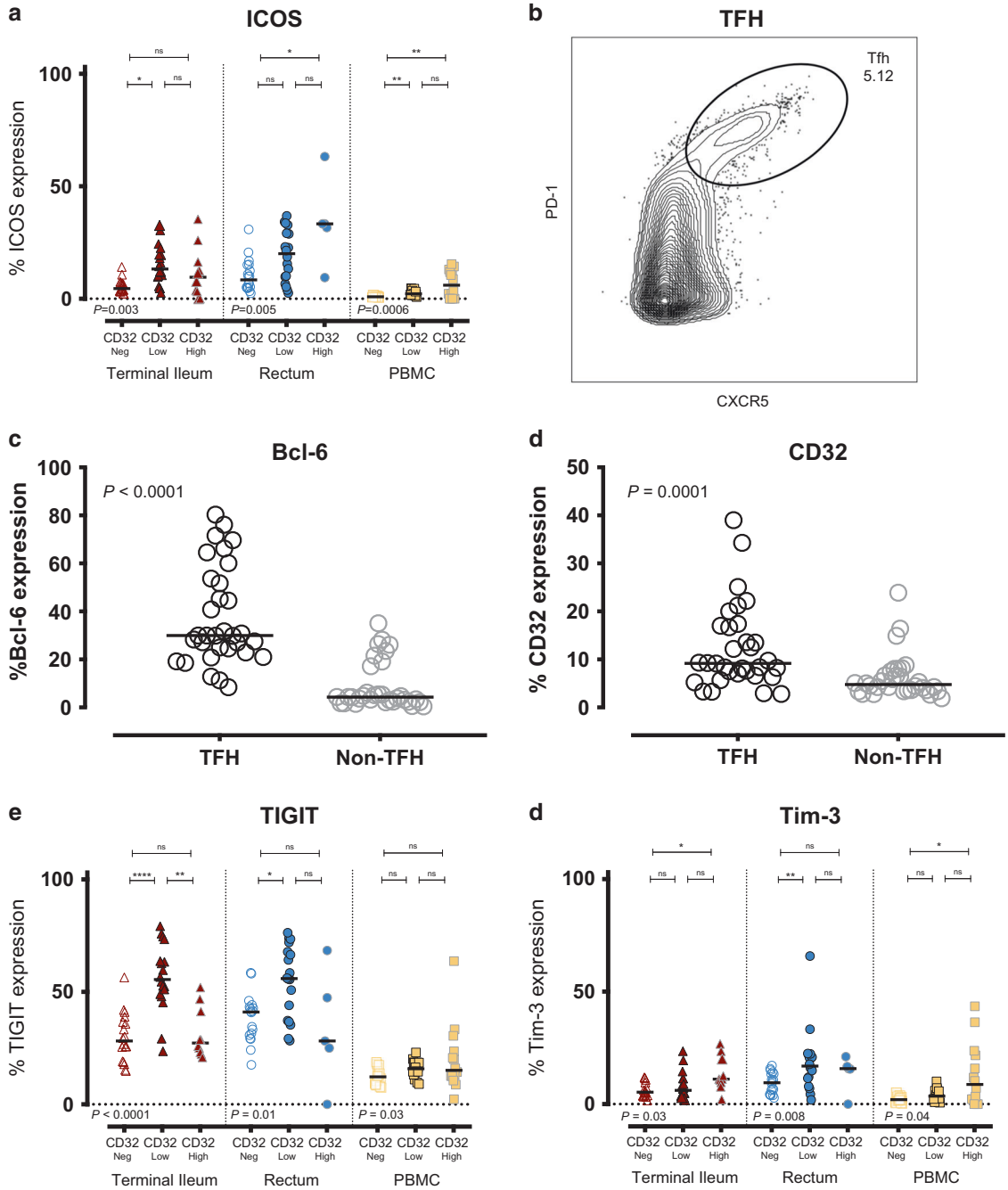

Fig. 5 TFH marker expression on CD32-, CD32^{low} & CD32^{high} CD4 T cells across anatomical sites. **a** ICOS on CD32 negative, CD32^{low} & CD32^{high} CD4 T-cell populations in terminal ileum, rectum and PBMC. Three groups were compared using a Kruskal–Wallis test (overall *P*-values shown at the bottom of each graph), with pairwise comparisons performed using Dunn's test. Statistical significance levels after adjustment for multiple comparisons (by Dunn's test) are indicated by $*P < 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$, lines indicate the median. **b** Representative plot of mucosal mononuclear cells from the terminal ileum, illustrating the GALT TFH population based on CXCR5 + and PD-1^{high} expression (gated on live, singlet CD3 + CD4 T cells). **c** Bcl-6 and (**d**) CD32 expression from GALT mucosal mononuclear cells (combined terminal ileum and rectum; $n = 32$, on TFH and non-TFH CD4 T cells). Lines represent the median. *P*-values calculated using a Wilcoxon test. TFH T follicular helper cells. **e** TIGIT and (**f**) Tim-3 expression on CD32 negative, CD32^{low} & CD32^{high} CD4 T cells populations in the terminal ileum, rectum and PBMC. Three groups were compared using a Kruskal–Wallis test (overall *P*-values shown at the bottom of each graph), with pairwise comparisons performed using Dunn's test. Statistical significance levels are indicated by $*P < 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$, lines indicate the median. Neg negative

infected cells. Highly-publicised work investigating the role of CD32 as a marker of the HIV reservoir was focused on peripheral blood sampling[11] and has been contested, especially in view of the unusual finding that the capturing of T-cell–B-cell doublets with 'singlet' gates in flow cytometry might explain the confusion. However, even then, the reported degrees of association between HIV DNA levels and CD4 T cells 'associated' with CD32 has varied from zero to partial enrichment in different studies. We were interested in the significance of these doublets, with which T cells were being captured within them and whether

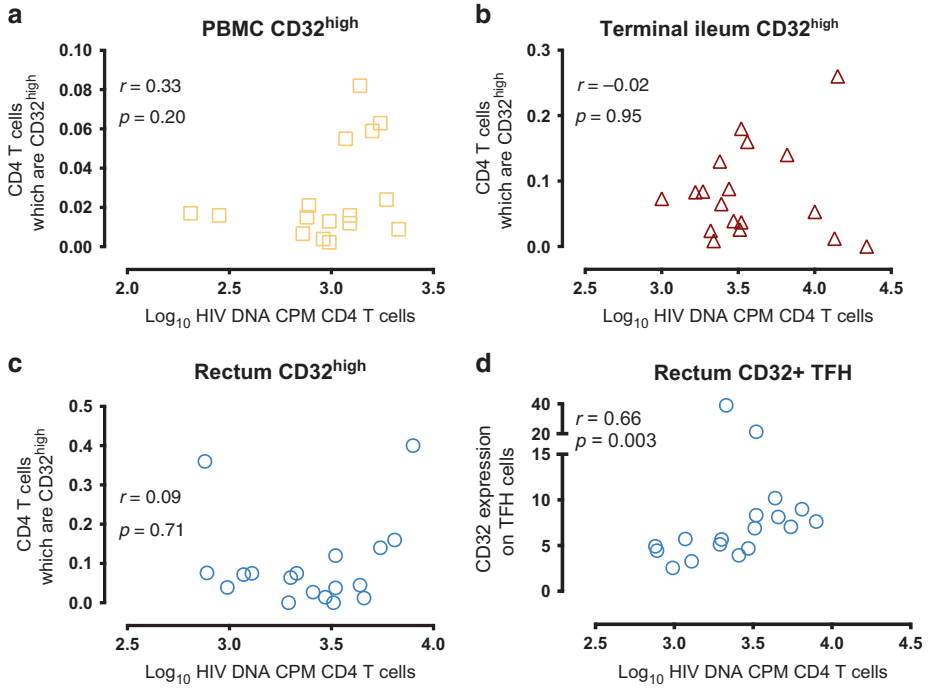

Fig. 6 Correlation between HIV DNA and CD32 expression on CD4 T cells. Spearman's correlations of HIV DNA (Log_{10} copies per 10^6 CD4 T cells) and $CD32^{high}$ expression on CD4 T cells in (**a**) PBMCs, (**b**) terminal ileum and (**c**) rectum. **d** Correlation of HIV DNA and CD32 expression (CD32 +) on TFH cells in the rectum. (Note: the *P*-value remains strongly significant on exclusion of the two outlying data points). CPM copies per million, PBMC peripheral blood mononuclear cells

this finding was also relevant to GALT, the predominant HIV reservoir.

Having previously proposed that T-cell–B-cell conjugates may be the source of CD32 CD4 co-expression,[18] our imaging cytometry data confirmed that $CD32^{high}$ cells in both PBMC and tissue are primarily T-cell–B-cell doublets, with no evidence of CD4 T cells expressing CD32. Some CD32 was observed on certain CD4 T cells, however, this was localised to a small region of the cellular membrane, suggesting that this CD32 expression was acquired through trogocytosis, supporting recent findings in blood by Osuna et al.[17] A previous study found CD32a RNA staining co-localising with HIV RNA, suggesting that CD4 T cells were expressing CD32. However, it is difficult to definitively exclude the possibility of doublets contributing to this signal. It is, of course, also possible that the CD4 T cells expressed CD32a RNA without expressing surface CD32.

The confirmation of $CD32^{high}$ T-cell–B-cell doublets led us to examine whether there was anything unique about these T cells. Using imaging flow cytometry, we found ICOS was localised to the T-cell portion of the doublet indicating the GALT T cells were primarily TFH cells, while HLA-DR was mostly found on B cells, suggesting the T cells may not be activated. We confirmed this TFH finding by flow cytometry, demonstrating an enrichment of CXCR5 + $PD\text{-}1^{high}$ events expressing CD32. Importantly, the vast majority of CXCR5 + $PD\text{-}1^{high}$ cells were CD3 +, confirming that B cells were likely not responsible for the signal. To examine T-cell exhaustion, we measured TIGIT and Tim-3 expression and found differing patterns between these two ICRs; TIGIT showed no increase on any $CD32^{high}$ cells while Tim-3 was higher on $CD32^{high}$ cells in the terminal ileum and PBMC. While we did not measure Tim-3 and TIGIT expression using imaging cytometry (due to a limitation in the number of antibodies that could be used in a single panel) the fact that these markers are not typically expressed by B cells,[21,22] suggests these results pertain to the T-cell portion of the doublet.

Some groups have shown an enrichment of HIV DNA or RNA expression in this $CD32^{high}$ population.[11,12,18,19] Even though we have shown that these are largely doublets, this finding could still be of further interest if these doublets were of physiological interest or provided information about the HIV reservoir. We found $CD32^{high}$ cells at a higher frequency in GALT sites compared with blood with an enrichment for markers of TFH in the T-cell portion of the doublets. While our previous sort data in PBMC suggested that these cells were not universally enriched for HIV DNA,[18] we found a statistically robust correlation between the frequency of TFH cells in CD32 + doublets and total HIV DNA in the rectum. Interestingly, we found no correlation with the frequency of TFH and total HIV DNA, suggesting this correlation was not due to overall higher levels of TFH. This correlation was observed in the rectum but not the terminal ileum, mirroring the pattern of ICOS enrichment in $CD32^{high}$ cells. The correlation in the rectum but not the terminal ileum could also be due to the larger frequency of lymphocyte aggregates found in the rectum, which would be more likely to contain these TFH-B-cell doublets.[24,25] One cannot definitively exclude that these doublets are artefacts of flow cytometry—and more detailed studies would be need to explore this—but the clear enrichment for certain T-cell phenotypes suggests this is not random. In questioning, the possible physiological relevance of these doublets one might argue, for example, that the correlation with HIV DNA could reflect an association with rectal lymphocyte aggregates, which in turn could be marked by higher levels of $CD32^{high}$ TFH doublets.

Interestingly, CD32 levels and the frequency of the TFH doublet population did not depend on HIV status. This agrees with our data showing that the doublets were not enriched for activated cells. If they were, we would expect HIV infection to substantially increase their frequency. As we did not find a correlation with TFH doublets in PBMC and HIV DNA, what then could explain this correlation in the rectum alone? First, peripheral TFH are not the same as tissue-resident TFH, and it is possible that any enrichment

is exclusive to tissue. Second, it is possible—in fact, likely—that TFH-B-cell doublets are not directly involved in HIV reservoir formation, but represent a surrogate marker of another host process, such as lymphocyte aggregate formation in GALT.

Our data confirm the role of tissue and peripheral T-cell–B-cell doublets contributing to the finding of CD32 on CD4 T cells, extend these findings to tissue and suggest that TFH cells are the predominant T cell involved. It remains to be seen whether these are artefactual or represent a physiological process which may originate within lymphoid tissue. Our data also reveal the potential for labelling doublets when identifying markers of the HIV reservoir, particularly with markers typically expressed on non-T cells. Adding a 'dump' channel for non-T-cell lineage markers would reduce the likelihood of identifying cell doublets. Of course, if doublets are functionally relevant, they would be missed using this strategy. Whether the enrichment within the doublets for T follicular helper cells bound to B cells has a bearing on the HIV reservoir or potential therapeutic interventions requires further investigation.

METHODS

The HEATHER cohort

HEATHER (HIV Reservoir targeting with Early Antiretroviral Therapy) is an observational cohort of treated PHI.[18,26] For inclusion in the cohort, participants with identified PHI commenced ART within 3 months of diagnosis and did not have co-infection with Hepatitis B or C. Methods for diagnosing PHI and estimating date of seroconversion are described elsewhere.[18] Terminal ileal and rectal samples were obtained from a subset of participants in HEATHER. Tonsil tissue was obtained from one individual with PHI who was undergoing routine tonsillectomy (after 2 months of ART).

Terminal ileal and rectal samples from healthy controls were obtained from individuals undergoing routine endoscopy from the Translational Gastroenterology Oxford Tissue Biobank.

Ethics statement

This HEATHER gut study was approved by the West Midlands—South Birmingham Research Ethics Committee (reference 14/WM/1104). The Translation Gastroenterology Tissues biobank was approved by the Sheffield Research Ethics Committee (reference 11/YH/0020).

All participants have given written informed consent for their participation in these studies.

Processing of tissue samples

Tonsillar tissue from an elective tonsillectomy for prevention of recurrent infections was dissected and mechanically digested, prior to cryopreservation of the cellular suspension.

Rectal and terminal ileum biopsies (up to 12 from each site) were collected at endoscopy and immediately placed in complete media (RPMI-1640 media with 5% heat-inactivated foetal bovine serum (FBS), 0.04 mg/mL gentamicin, 100 IU/mL penicillin, 0.1 mg/mL streptomycin and 2 mM L-glutamine). Biopsies were processed within 3 h of sampling. Briefly, samples were washed in 1 mM dithiothreitol (DTT) solution and then with PGA solution (Hanks' balance salt solution with 0.04 mg/mL gentamicin, 100 IU/mL penicillin and 0.1 mg/mL streptomycin with or without 250 µL/mL amphotericin B). Biopsy samples subsequently underwent collagenase and mechanical digestion using Collagenase D (1 mg/mL) for 30 min and a gentleMACS dissociator (Miltenyi Biotec), respectively. The resulting cell suspension was then strained using a 70 µM filter, washed with PGA solution and then used for staining.

Up to four biopsies from the terminal ileum and rectum were put directly from biopsy into RNA later and stored at −80 °C for subsequent DNA extraction.

Flow cytometry and cell sorting

The single-cell suspension of mucosal mononuclear cells was washed in complete media and stained fresh. Cryopreserved PBMCs or tonsillar tissue were thawed in the RPMI-1640 medium supplemented with 10% FBS, L-glutamine, penicillin and streptomycin as above (R10) containing 2.7 Kunitz units/mL of DNAse (Qiagen).

Mucosal and peripheral blood mononuclear cells were stained in BD Horizon Brilliant Stain Buffer (BD) with LiveDead Near IR (Life Technologies) and the following antibodies anti-CD3 BV570 (UCHT1), anti-CXCR5 Pacific Blue (J252D4), anti-CD32 PE (FUN-2) [all BioLegend], anti-CD4 BV605 (RPA-T4), anti-CD8 BV650 (SK-1), anti-PD-1 PE-Cy7 (EH12.1), anti-ICOS/CD278 AlexaFluor647 (DX29), anti-TIGIT PerCP-eFluor710 (MBSA43), anti-Tim-3 PE-CF594 (7D3) and anti-HLA-DR AlexaFluor700 (MAB) [all BD]. EPCAM APC-Vio770 (HEA-125) [Miltenyi Biotec] was also used to allow for the exclusion of epithelial cells. Isotype controls for CD32 were used for gating purpose, and were prepared using an irrelevant IgG2bκ antibody (MPC-11) [BioLegend].

Staining was performed for 20 min at room temperature, followed by fixation and permeabilisation using the Human FoxP3 Buffer Set (BD Pharmingen) as per the manufacturer's protocol. This was followed by staining for Bcl-6 AlexaFluor488 (K122-91) [BD] for 30 min.

All samples were acquired on a LSR II (BD). The same machine was used for all experiments with daily calibration with Rainbow Calibration Particles (Biolegend) to maximise comparability between days. The data were analysed using FlowJo Version 10.4.1 (Treestar).

Imaging-flow cytometry

Imaging-flow cytometry was performed on healthy PBMC, rectum, terminal ileum and tonsil from an HIV-infected individual. Cells from the gut and tonsillar tissue were isolated as above. Cells were stained using one of two panels. Panel 1: CD14 VioBlue (TUK4), CD19 PE (LT19) [Miltenyi], CD3 FITC (UCHT1), CD4 APC (RPA-T4), CD32 PE-Cy7 (FUN-2) [Biolegend], LiveDead Near IR [Life Technologies].

Panel 2: CD3 VioGreen (BW264) [Miltenyi Biotec], HLA-DR PE (L243), CD4 eFluor 450 (OKT4) [eBioscience], CD32 PE-Cy7 (FUN-2), CD14 FITC (HCD14) [Biolegend], ICOS AlexaFluor647 (DX29) [BD Bioscience], CD19 ECD (J3-119) [Beckman Coulter], LiveDead Near IR [Life Technologies].

Cells were stained at 4 °C for 30 min, washed twice and resuspended in 2% formaldehyde at a concentration of 15 million cells/mL and run using an Amnis ImageStream Mark II and analysed using IDEAS software v6.2.

Measurement of HIV DNA

Tissue samples stored in RNA later were homogenised using a MagNA Lyser (Roche), DNA was then extracted using a Qiagen AllPrep DNA/RNA Mini Kit as per the manufacturer's specifications.

PBMC samples were CD4 enriched by negative selection (Dynabeads, Invitrogen or EasySep Human CD4 Enrichment Kit, StemCell). CD4 T-cell DNA was extracted (Qiagen AllPrep DNA/RNA Mini Kit) and used as input DNA for PCR. HIV DNA levels from GALT tissue were measured in bulk tissue and then normalised to copies per million CD4 T cells using flow cytometry data. Cell-copy number and total HIV DNA levels were quantified both in triplicate using previously published assays.[27]

Statistical analysis

$CD32^{high}$ events are rare in CD4 T-cell populations and for this analysis $CD32^{high}$ events were only analysed when there were greater than five events in the $CD32^{high}$ gate. Numbers of $CD32^{high}$ events are shown in Supplementary Table 1. Non-parametric tests were used to compare continuous variables throughout. Comparisons between CD32+ and CD32− populations were performed

using the Wilcoxon matched-pairs signed rank test. Where three groups were compared, a Kruskal–Wallis test (unpaired data) was used. Correlative analyses were performed using Spearman's rank correlation. For all tests, *P*-values < 0.05 were considered statistically significant. Analyses were performed using GraphPad Prism (GraphPad Software) version 7.0b.

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ADDITIONAL INFORMATION

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