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Evidence that CD32a does not mark the HIV-1 latent reservoir

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A recent report by Descours et al.¹ suggests that the cell surface expression of the low affinity Fc receptor CD32a (also known as FcγRIIa) marks the replication-competent HIV-1 reservoir in CD4⁺ T cells from 12 HIV-1-infected participants receiving suppressive anti-retroviral therapy (ART)¹. We have undertaken considerable efforts to replicate these findings using peripheral blood mononuclear cells (PBMCs) from 20 HIV-1-infected, ART-suppressed participants (Extended Data Table 1). We found no evidence to suggest that CD32a marks a CD4⁺ T cell population enriched in either HIV-1 DNA or replication-competent HIV-1 in our study participants. There is a Reply to this Comment by Descours, B. et al. *Nature* **561**, <https://doi.org/10.1038/s41586-s41586-018-0496-1> (2018).

To validate these findings, we adopted the same gating strategy as described by Descours et al.¹ to define CD4⁺ T cell populations (Supplementary Fig. 1a). The CD32 antigen was identified using the same antibody clone (FUN-2) as described by Descours et al.¹. We

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Data availability. All data and reagents are available from the corresponding author upon request.

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observed the same CD4⁺ T cell subsets that stained at a high cell surface density of CD32 (CD4⁺CD32^{high}), an intermediate cell surface density of CD32 (CD4⁺CD32^{int}), and a CD4⁺ T cell subset lacking CD32 expression (CD4⁺CD32^{neg}). We obtained frequencies of CD4⁺CD32^{high} T cells that ranged from 0.002% to 0.026%, with a median value (0.012%) that was identical to that reported by Descours et al.¹ (Extended Data Table 2 and Supplementary Fig. 1a). Notably, we confirmed that this same CD4⁺CD32^{high} population is also present in PBMCs isolated from eight healthy donors and exists at similar frequencies to that in HIV-1-infected samples ($P = 0.971$, Extended Data Fig. 1a).

Next, we assessed the amount of replication-competent HIV-1 isolated from the same 20 participants by measuring the infectious unit per million cells (IUPM) in CD4⁺ T cells (range 0.01–37.5, median 0.46). Participant CD4⁺CD32^{high} T cell populations were colour-coded in descending order, and then divided into quartiles that corresponded to the relative frequency of CD4⁺CD32^{high} cells present in these samples (Fig. 1a).

After cytometric sorting of the various CD4⁺CD32 subsets, we quantified HIV-1 DNA in each population (total CD4⁺, CD4⁺CD32^{neg}, CD4⁺CD32^{int} and CD4⁺CD32^{high}, Fig. 1b) using droplet digital PCR (ddPCR), as described in the Methods. We found no evidence of HIV-1 DNA enrichment in the CD4⁺CD32^{high} fraction. We observed no significant difference in HIV-1 DNA between any populations and the CD4⁺CD32^{high} T cell population ($P = 0.28$). In fact, levels of HIV-1 DNA in the CD4⁺CD32^{high} T cell subsets isolated from nine participants was at the assay limit of detection (Fig. 1b). After correction for cell input in the CD4⁺CD32^{high} fraction, as estimated DNA values, we saw no evidence for HIV-1 DNA enrichment (open symbols in Extended Data Fig. 1b).

We then compared the relative frequency of the CD4⁺CD32^{high} T cell populations and the viral replicative capacity (IUPM values) per participant, but no relationship between the two parameters was observed (Fig. 1c). All values have been tabulated in Extended Data Table 2.

The HIV-1 reservoir largely resides in quiescent CD4⁺ T cells^{2,3}. Therefore, we sought to confirm the activation status of the CD4⁺ T cell populations by measuring the frequency of the activation markers CD69, CD25 and HLA-DR on CD4⁺ T cell subsets from all participants. We found that the CD4⁺CD32^{high} T cells were highly activated compared to the CD4⁺CD32^{neg} T cells ($P < 0.0001$). Notably, among the activation markers, HLA-DR was particularly enriched, marking approximately 75% of all CD4⁺CD32^{high} cells (median 74%), compared to CD4⁺CD32^{neg} cell populations (median 1.4%) (Extended Data Fig. 1c, $P < 0.0001$).

Two CD32 isoforms (CD32a and CD32b) are known to be expressed among all antigen presenting cells (APCs), but not typically on T cells. Therefore, we sought to exclude any APCs as potential contaminants of flow cytometry sorting. We evaluated the co-expression of lineage markers for all major CD32-bearing cells including monocytes, B cells, dendritic cells, granulocytes and natural killer cells. As expected, all CD32⁺ T cells expressed high amounts of CD3 and CD4 (Fig. 1d). However, we found that most CD4⁺CD32^{high} T cells from HIV-1⁺ patients, and also from healthy donors, co-expressed several B cell markers including CD19, CD40 and HLA-DR (Fig. 1d, e, Extended Data Fig. 2a). Notably, the B cell

antigens found on CD4⁺CD32^{high} T cells were present at similar cell-surface densities as detected on bona fide B cells (Fig. 1d, e, Extended Data Fig. 2a).

The demonstration that the CD4⁺CD32^{high} fraction seen in HIV-1⁺ patients was marked with several B cell antigens and was similarly present in naive donors led us to investigate the origin of these B cell markers on a CD4⁺ T cell. Several reports have shown that B cells exclusively express the CD32b isoform⁴. The FUN-2 antibody clone used by Descours et al.¹ cannot distinguish between the CD32a and CD32b isoforms. Therefore, we used the monoclonal antibody clone 2B6 that has been reported to exclusively bind to CD32b^{4,5}. After co-staining PBMCs from HIV-1⁺ and HIV-1⁻ individuals with both the FUN-2 and the 2B6 antibodies, we found that all CD4⁺CD32^{high} T cells were marked only by the CD32b isoform and not by CD32a (Fig. 1d, e, Extended Data Fig. 2b), indicating that B cells are the origin of the CD32b antigen that marks the CD4⁺CD32^{high} T cells.

We sought to confirm this by determining whether *CD32A* (also known as *FCGR2A*) or *CD32B* (*FCGR2B*) mRNA was endogenously produced in the CD4⁺CD32^{high} subsets. After isolating total cellular RNA from various sorted T cell subsets, we used established reverse transcription PCR (RT-PCR) primers and probes that are specific to the CD32a and CD32b isoforms, as described in the Methods. We found that sorted CD4⁺CD32^{high} T cells from four HIV-1-infected participants did not contain detectable levels of the *CD32A* isoform. However, the *CD32B* mRNA isoform was readily detected in CD4⁺CD32^{high} T cells isolated from two out of four HIV-1⁺ patients (Extended Data Fig. 2c). By additional RT-PCR analysis, we detected both *CD3G* and *CD19* transcripts in the same CD4⁺CD32^{high} T population, indicating that the CD32b marking the CD4⁺CD32^{high} T cells may be from B cells expressing cognate CD32b (Extended Data Fig. 2d).

Because this may require cell-to-cell interaction, we performed a back-gating analysis of our flow cytometry data and confirmed that all CD4⁺CD32^{high} populations were identified within single-cell gates (Supplementary Fig. 1b). However, post-hoc analysis comparing the forward and side scatter light pulse area (FSC-A and SSC-A, respectively) values between CD4⁺CD32^{neg} and CD4⁺CD32^{high} T cells showed that the CD4⁺CD32^{high} populations had both a significantly higher FSC-A ($P < 0.0001$) and SSC-A ($P < 0.0001$), suggesting that the CD4⁺CD32^{high} population may consist largely of cell doublets (Fig. 1f, g).

We next used Amnis imaging flow cytometry to visualize the sorted CD4⁺CD32^{neg}, CD4⁺CD32^{int} and CD4⁺CD32^{high} cell populations directly. As expected, the CD4⁺CD32^{neg} and the CD4⁺CD32^{int} cell populations each consisted of more than 99% single cells. However, the CD4⁺CD32^{high} fraction contained a high frequency of cell doublets (mean value 94%) (Extended Data Fig. 3). Of these ‘doublets, approximately 70% seemed to be coincident doublets, and 30% were conjugates of T and B cells (Fig. 2a and Extended Data Fig. 3b).

We observed no examples in which CD32 staining on T cells was distributed throughout the cell membrane, supporting the idea that the CD32 found in the CD4⁺CD32^{high} population is not the result of endogenous expression from CD4⁺ T cells. Of the instances in which CD32 was detected on a T cell in the CD4⁺CD32^{high} population, the staining was punctate and

often co-localized with punctate CD19 staining (Fig. 2b), suggesting that CD32 was acquired via contact between B and T cells. We noted that the frequency of T cells with punctate CD32 staining was substantially higher in the sorted CD32^{int} population. Thus, sorting for CD4⁺ T cells with a 'high' surface density of CD32 results in the selective enrichment of contaminating T–B cell doublets. As shown in Supplementary Fig. 1, these doublets cannot be discerned by routine cytometric FSC and SSC singlet gating strategies.

In summary, using samples from 20 HIV-1-infected, ART-suppressed participants, our data contradict the assertion that CD32a is a marker of the replication-competent viral reservoir. Although we did detect similar frequencies of CD4⁺CD32^{high} populations to Descours et al.¹, we found no difference in the total HIV-1 DNA content between CD4⁺ T cell populations including or excluding the CD32^{high} fractions (Fig. 1b).

Notably, the CD4⁺CD32^{high} population was highly activated. Previous studies that have evaluated CD32 expression on T cells suggest that it may be detected after activation^{6,7} and led us to believe that this population may be atypical compared to a quiescent population harbouring the HIV-1 reservoir^{2,3}.

Our additional findings are incongruent with CD32a marking the replication-competent reservoir in CD4⁺ T cells; our phenotyping and RT–PCR experiments indicate that it is the CD32b isoform that marks the CD4⁺CD32^{high} cells (Fig. 1d, e, Extended Data Fig. 2b–d). This finding, combined with the demonstration that this cell population is found in uninfected individuals, conflicts with the assertion of Descours et al.¹ that CD32a is upregulated after the establishment of viral latency. Recent reports have corroborated the absence of CD32a transcripts in reactivated, clonal HIV-1-infected CD4⁺ T cells⁸.

The surface density of CD32b (and other B cell markers) on the CD4⁺CD32^{high} population was observed at similar densities to that on B cells. These data, combined with the post-hoc analysis, suggests that this population may be largely comprised of doublets. Direct interrogation of the CD4⁺CD32^{high} population via Amnis imaging confirmed that this population consisted largely of contaminating doublets; either co-incident events or cell-to-cell conjugates (Fig. 2a).

We demonstrate that the mechanism by which the CD32b isoform labels the CD4⁺CD32^{high} populations is through the direct interaction of CD4⁺ T and B cells, and possible trogocytotic transfer of B cell antigens to T cells, as observed in the CD4⁺CD32^{int} population (Fig. 2b). This may explain the transfer or membrane painting of antigens such as CD32b, CD40 and HLA-DR, among other markers^{9–11}. Not only have cell-to-cell membrane transfers been shown to occur commonly in vivo during viral infections, but such transfers largely occur on activated cells¹². Membrane-bound Fcγ receptors, including CD32b, are known to be extracted from APCs and then transferred to T cells, and serve as a surrogate of recent T cell and APC interactions¹³. Our demonstration of T–B cell conjugates in the CD4⁺CD32^{high} population and high levels of single cells in the CD4⁺CD32^{int} population support this notion (Fig. 2a, b).

Collectively, our findings confirm that selectively sorting for T cells with a high surface density of CD32 results in the enrichment of T–B cell doublet contaminants, which cannot

be discerned by routine gating strategies. The true isoform, CD32b, that marks the CD4⁺CD32^{high} population is probably indicative of dynamic CD4⁺ T cell interaction with B cells, rather than a marker of the HIV-1 reservoir^{14,15}.

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Methods

HIV-1⁺ participants were recruited through: The Maple Leaf Medical clinic in Toronto, Canada; The HIV Eradication and Latency (HEAL) cohort of Brigham and Women's and Massachusetts General Hospital; The Whitmann Walker Clinic in Washington, DC; or the Hospital of the University of Pennsylvania. The study was approved by the University of Toronto, The University of Pennsylvania and George Washington University ethics committees and according to the protocol approved by the Partners Human Research Committee and Institutional Review Board (IRB). Written informed consent was obtained from each participant.

The percentage of CD32⁺ (clone FUN-2) CD4⁺ T cells was measured in samples from study participants. Both CD32⁺ and CD32^{neg} CD4⁺ T cells were sorted and viral DNA was measured using ddPCR. The analysis of cell lineage markers by flow cytometry and RT-PCR was also conducted. Flow cytometry sorts from PBMCs used in HIV-1 DNA analyses were performed on cell subsets and assessed using Amnis imaging flow cytometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Extended Data

Extended Data Fig. 1 |. Frequency and activation status of CD32-expressing CD4⁺ T cells and their HIV-1 DNA content.

a, The frequency of CD32^{high} CD4⁺ T cells was measured by flow cytometry in PBMCs from ART-suppressed, HIV-1⁺ ($n = 20$) and HIV-1⁻ ($n = 8$) donors. Bars denote median values. P values were determined by a Mann–Whitney test. **b**, DNA copies per cell in sorted subsets of total CD4⁺, CD4⁺CD32^{neg}, CD4⁺CD32^{int} and CD4⁺CD32^{high} T cells are shown with median values and the IQR. The results are shown as either the actual HIV-1 DNA copies per million cells (filled symbols) or as estimated values calculated using the LOD and

applied to the number of cells when the DNA input did not reach the threshold (open symbols). *P* values were determined by a Kruskal–Wallis test. **c**, The percentage of CD69, CD25 and HLA-DR expression was measured by flow cytometry on CD32^{neg} and CD32^{high} (FUN-2) CD4⁺ T cells from PBMCs from HIV-1⁺ participants (*n* = 20). Error bars show the median and IQR. *P* values were determined by Wilcoxon matched-pairs signed rank tests.

Extended Data Fig. 2 |. Detection of B cell proteins and mRNA in CD32-expressing CD4⁺ T cells.

a, CD32^{neg} and CD32^{Wgh} (FUN-2) CD4⁺ T cells from human PBMCs were assessed by flow cytometry for the expression of CD19, CD40 and HLA-DR, and compared to B cells (CD3⁻ CD14⁻ CD19⁺ lymphocytes). Representative flow cytometry results of per cell antigen levels on B cells (top, blue histograms) and CD32^{neg} and CD32^{high} CD4⁺ T cells (bottom, grey and red histograms, respectively) from an HIV-1⁻ donor. **b**, Representative CD32b staining of PBMCs from an HIV-1⁺, ART-suppressed participant. PBMCs were stained with an optimized concentration of the 2B6 monoclonal anti-CD32b antibody,

followed by an antibody cocktail that included the FUN-2 monoclonal pan-CD32 antibody, as described in the Methods. Shown are the 2B6 and FUN-2 fluorescence minus one (FMO) antibody cocktail-stained samples and a sample co-stained with 2B6 and FUN-2. **c, d**, *CD32* mRNA expression levels in $CD4^+CD32^+$ subsets. **c**, The relative expression of *CD32A* and *CD32B* mRNA isoforms in sorted $CD4^+CD32^{int}$ and $CD4^+CD32^{high}$ subsets from HIV-1⁺, ART-suppressed participants ($n = 4$). **d**, mRNA expression of *CD32A* and *CD32B* from patient G07W1610. **e**, T and B cell lineage-specific mRNA transcripts in sorted $CD4^+CD32^+$ subsets from participant G07W1610. Relative mRNA expression of target genes was normalized to *ATCB* using the comparative C_t method. Results are mean $\pm$ s.d. of each value from each participant ($n = 4$; **c**), or from values generated from two separate experiments using samples from the same patient (**d**).

Extended Data Fig. 3 | Doublet composition of the sorted CD4⁺CD32^{high} T cells. Sorted B cells and CD4⁺CD32^{neg}, CD4⁺CD32^{int} and CD4⁺CD32^{high} T cells from an HIV-1⁺, ART-suppressed participant were analysed using an Amnis imaging cytometer. Singlets and doublets were quantified using the aspect ratio and nuclear staining. **a**, The proportion of total singlet and doublet events among total nucleated cells detected on the Amnis cytometer in each sorted population was determined, and is shown as individual composite bar graphs for two patients (G07W1610 and OM5365). **b**, A composite bar graph

of the proportion of conjugates, doublets and trogocytotic events that comprised the sorted CD4⁺CD32^{high} population ($n = 2$).

Extended Data Table 1 |

Viral suppression of 20 HIV-1-infected participants on ART

Cohort	Participant ID	Date of Initial Suppression (MM/YY)	Length of suppression (yrs)
HEAL	HEAL-009	3/14	3
	HEAL-019	8/09	8
	HEAL-020	3/08	9.3
	HEAL-034	11/05	11.5
	HEAL-053	11/16	1
	HEAL-055	11/00	17
Maple Leaf	CIRC0024	6/98	17.0
	CIRC0133	7/08	7.0
	CIRC0196	4/14	1.2
	OM5011	11/08	6.6
	OM5148	1/08	7.5
	OM5162	9/04	10.8
	OM5203	3/12	3.3
	OM5334	7/14	0.9
	OM5365	3/08	7.3
WWH	WWH-B001	7/11	6.4
	WWH-B005	12/17	0.3
	WWH-B008	11/14	3.1
	WWH-B011	11/11	6
UPenn	G07W1610	10/05	11.8

Extended Data Table 2 |

CD4⁺CD32^{high} subset proportions and HIV-1 DNA compared to total CD4⁺ and CD32^{neg} CD4⁺ T cells

Participant ID	CD32 ^{High}			HIV-DNA enrichment		
	% in total CD4	Absolute cell count	HIV-DNA ₁ copies/cell	CD32 ^{High} / ₂ CD4 total	CD32 ^{High} / ₂ CD32 ^{Neg}	CD32 ^{Neg} / ₂ CD4 total
HEAL-009	0.007	11,427	>0.000002	0.010	0.008	1.236
HEAL-019	0.002	4,911	>0.000002	0.002	0.001	1.500
HEAL-020	0.011	8,482	>0.000002	0.008	0.008	1.036
HEAL-034	0.022	8,238	0.000426	0.199	0.212	0.937
HEAL-053	0.004	9,544	>0.000002	0.017	0.015	1.161

Participant ID	CD32 ^{High}			HIV-DNA enrichment		
	% in total CD4	Absolute cell count	HIV-DNA ¹ copies/cell	CD32 ^{High} / ₂ CD4 total	CD32 ^{High} / ₂ CD32 ^{Neg} / ₂	CD32 ^{Neg} / ₂ CD4 total
HEAL-055	0.011	21,806	0.00037	0.604	0.555	1.088
CIRC0024	0.015	26,200	>0.000002	0.023	0.029	0.782
CIRC0133	0.017	14,602	>0.000002	0.005	0.010	0.517
CIRC0196	0.006	5,935	0.000694	1.722	1.066	1.615
OM5011	0.008	8,862	0.001942	1.871	2.187	0.855
OM5148	0.004	8,788	0.001191	1.043	1.049	0.994
OM5162	0.016	7,133	0.000923	0.842	0.842	1.000
OM5203	0.026	12,254	>0.000002	0.021	0.011	1.911
OM5334	0.016	6,275	0.000959	0.579	0.499	1.160
OM5365	0.006	11,027	>0.000002	0.003	0.003	0.803
WWH-B001	0.020	10,922	>0.000002	0.007	0.006	1.076
WWH-B005	0.013	5,964	0.00547	0.650	0.550	1.182
WWH-B008	0.023	6,464	0.000805	0.696	0.595	1.169
WWH-B011	0.004	5,953	0.001653	1.154	1.016	1.135
G07W1610	0.012	7,984	0.002482	1.452	1.411	1.029
Median	0.012	8,635	0.000398	0.389	0.356	1.082

¹Values below the LOD (2 copies per 10⁶ cells) are shaded in grey.

²To calculate HIV-1 enrichment, 0.000002 was used for all values below the LOD.

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Fig. 1 |. CD32-expressing CD4⁺ T cells are not enriched in HIV-1 DNA and express markers of B cell origin.

a–c, CD4⁺CD32^{int} and CD4⁺CD32^{high} T cells from PBMCs of ART-suppressed, HIV-1-infected patients ($n = 20$) were sorted, and HIV-1 DNA was measured by ddPCR. **a**, Dividing the frequency (in percentage) of CD4⁺CD32^{high} T cells from all participants into quartiles, the values are shown as below or above the median. **b**, DNA copies per cell in sorted subsets of total CD4⁺, CD4⁺CD32^{neg}, CD4⁺CD32^{int} and CD4⁺CD32^{high} T cells are shown, with median and interquartile range (IQR). P value determined by Kruskal–Wallis test. LOD, limit of detection. **c**, IUPM in CD4⁺ T cells of each participant is shown in the colour corresponding to its frequency of CD4⁺CD32^{high} cells in panel **a**. **d**, **e**, CD32^{neg} and CD32^{high} (identified using FUN-2) CD4⁺ T cells from human PBMCs were assessed by flow cytometry for the expression of CD32b (2B6 antibody), CD19, CD40 and HLA-DR and compared to B cells (CD3⁺CD14⁺CD19⁺ lymphocytes). **d**, Representative flow cytometry results per cell antigen levels on B cells (top, blue histograms) and on CD32^{neg} and CD32^{high} CD4⁺ T cells (bottom, grey and red histograms, respectively) from PBMCs from an HIV-1⁺ participant. **e**, Frequency of CD4⁺CD32^{high} T cells staining positive for CD32b (2B6), CD19, CD40 or HLA-DR from HIV-1⁺ ($n = 5$) and HIV-1⁻ ($n = 5–8$) human donor PBMC samples. Bars denote median values. **f**, Representative histograms of the FSC-A and SSC-A of B cells and CD32^{neg} and CD32^{high} CD4⁺ T cells from PBMCs of an HIV-1⁺, ART-suppressed participant sorted on a BD FACSaria II. **g**, Comparisons of the median FSC-A and SSC-A values between CD32^{neg} and CD32^{high} CD4⁺ T cell subsets from HIV-1⁺, ART-suppressed participants ($n = 20$). P values were determined using a paired t -test.

Fig. 2 |. Flow cytometry imaging of sorted CD32-expressing T cells.

a, Representative bright-field and pseudo-colour fluorescence images of T-B cell conjugates found in the CD32^{high} CD4⁺ T cell population sorted from PBMCs from HIV-1⁺, ART-suppressed participants, and imaged using Amnis technology. **b**, Representative images of punctate CD32 staining found on single T cells in the CD32^{high} and CD32^{int} population sorted from HIV-1⁺, ART-suppressed participant PBMCs.