CD32⁺CD4⁺ T cells are enriched in HIV DNA

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Background

CD32 was reported to mark the HIV-1 reservoir harboring replication-competent proviruses, but several recent reports challenged this finding. We aimed to confirm or deny the usefulness of CD32 as a marker of the latent reservoir and to further characterize the phenotype of these CD32⁺ CD4⁺ T-cells, as well as the transcriptional activity of HIV-1 residing in this reservoir.

Methods

CD32 expression and co-expression of HLA-DR, TIGIT, LAG-3 was measured by flow cytometry on PBMCs from ART-suppressed HIV-infected individuals with undetectable plasma viremia (Fig. 1A). Total HIV DNA and unspliced RNA were quantified in bulk PBMC samples and in CD32⁺ and CD32⁻ fractions of CD4⁺ T cells obtained by magnetic sorting (negative selection to isolate CD4⁺ T cells followed by positive selection to isolate CD32⁺CD4⁺ cells) (Fig. 1A).

Results

The median frequency of CD32⁺ cells among CD4⁺ cells was 0.074% (Fig. 1B). Percentages of CD32⁺CD4⁺ T cells positively correlated with total HIV DNA in PBMCs (rho=0.51; p=0.031) (Fig. 1C-D). CD32⁺CD4⁺ T cells demonstrated increased expression of LAG-3 and TIGIT (both p=0.016) and HLA-DR (p< 0.0001) compared with CD32⁻CD4⁺ T cells (Fig. 2). Initially, no enrichment in HIV DNA was observed in CD32⁺CD4⁺ T cells compared with CD32⁻CD4⁺ cells in 18 ART-treated individuals (Fig. 3B). However, the CD32⁺ fraction was found to contain many residual non-T cells, which could have masked the enrichment in HIV DNA (Fig. 3C-F). Indeed, when HIV DNA was normalized to *CD3G* T-cell-specific mRNA, a significant enrichment in HIV DNA in the CD32⁺ fraction was observed (p=0.0003) (Fig. 3G-H). Therefore, we optimized the protocol to isolate a purer fraction of CD32⁺CD4⁺ T cells from additional 23 ART-treated individuals. An extra round of CD4⁺ T-cell purification resulted both in a 22-fold decrease in *CD19* B-cell lineage marker mRNA level in the CD32⁺ fraction (p< 0.0001) and in an 11-fold enrichment in HIV DNA in this fraction (p=0.0003), the latter observed even when HIV DNA was normalized to the total cell numbers (Fig. 4). In a subset of these individuals (n=9), we performed two additional rounds of CD32⁺ positive selection and observed a very high enrichment (mean 292-fold) for HIV DNA in the CD32⁺ fraction (Fig. 5). In contrast, no enrichment for HIV RNA was observed in these cells, yielding a significantly reduced HIV RNA/DNA ratio, which may indicate transcriptional latency (Fig. 6).

Positive correlation between the percentage of CD32⁺ cells among CD4⁺ T cells and HIV DNA load in PBMCs.

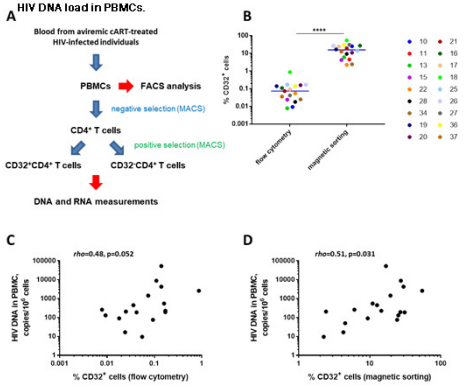

Figure 1. Percentages of CD32⁺CD4⁺ T cells positively correlate with HIV DNA in PBMC. (A) Flowchart of cell isolation. (B) Comparison of percentages of CD32⁺ cells among CD4⁺ cells between flow cytometry- and magnetic sorting-based isolation. Wilcoxon test was used to calculate statistical significance. (C, D) Correlations between percentages of CD32⁺ cells isolated by flow cytometry (C) or magnetic sorting (D) and HIV DNA load in PBMC. Spearman tests were used to calculate statistical significance. ****, p<0.0001.

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CD32⁺CD4⁺ T cells co-express HLA-DR, TIGIT and LAG-3.

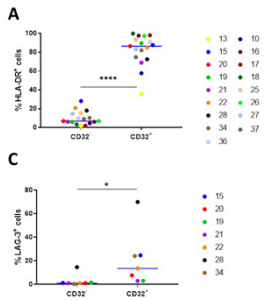

Figure 2. CD32⁺CD4⁺ T cells are enriched for HLA-DR, TIGIT and LAG-3 expression. Expression of HLA-DR (n=17) (A), TIGIT (n=7) (B), and LAG-3 (n=7) (C) was measured by multi-parametric flow cytometry on CD32⁻CD4⁺ and CD32⁺CD4⁺ T cells isolated from HIV-infected individuals. Wilcoxon tests were used to calculate statistical significance. ****, p<0.0001; *, 0.01<p<0.05.

Lack of HIV DNA enrichment in the CD32⁺CD4⁺ fraction is due to the presence of residual non-T cells.

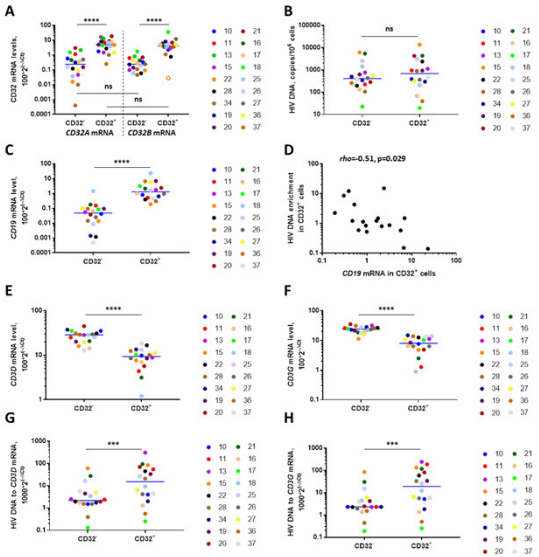

Figure 3. Lack of enrichment for HIV DNA in CD32⁺CD4⁺ fraction is due to the presence of residual non-T cells in this fraction. (A) *CD32A* and *CD32B* mRNA levels in CD32⁺ and CD32⁻ fractions of CD4⁺ T cells. The open circle depicts an undetectable value, censored to the detection limit. (B) HIV DNA levels, normalized to the total cellular DNA, in CD32⁺ and CD32⁻ fractions. (C) *CD19* mRNA levels in CD32⁺ and CD32⁻ fractions. (D) Correlation between the levels of *CD19* mRNA in CD32⁺ fraction and HIV DNA enrichment in this fraction. (E, F) *CD3D* (E) and *CD3G* (F) mRNA levels in CD32⁺ and CD32⁻ fractions. All mRNA levels were normalized to *GAPDH* mRNA. (G, H) HIV DNA levels, normalized to *CD3D* (G) and *CD3G* (H) mRNA, in CD32⁺ and CD32⁻ fractions. Wilcoxon tests (all panels except D), or Spearman tests (panel D) were used to calculate statistical significance. ****, p<0.0001; ***, 0.0001<p<0.001; ns, not significant.

An additional round of CD4⁺ T-cell purification leads to a significant HIV DNA enrichment in the CD32⁺CD4⁺ fraction.

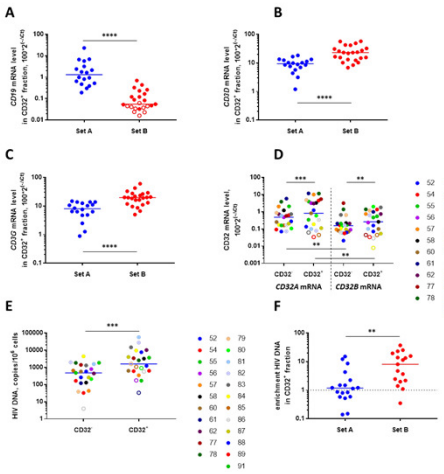

Figure 4. An extra round of CD4⁺ T-cell purification leads both to a reduction in residual non-T cell contribution to CD32⁺ fraction and to a significant enrichment for HIV DNA in this fraction. (A-C) Comparison of *CD19* (A), *CD3D* (B), and *CD3G* (C) mRNA levels in the CD32⁺ fraction between Set A and Set B. (D) *CD32A* and *CD32B* mRNA levels in CD32⁺ and CD32⁻ fractions of CD4⁺ T cells in Set B. All mRNA levels were normalized to *GAPDH* mRNA. (E) HIV DNA levels, normalized to the total cellular DNA, in the CD32⁺ and CD32⁻ fractions, Set B. Open circles depict undetectable values, censored to the assay detection limits. The latter depended on the amounts of input cellular DNA or RNA and therefore differed between samples. (F) Comparison of HIV DNA enrichment in the CD32⁺ fraction between Set A and Set B. Only detectable values were included in the enrichment calculation. Mann-Whitney tests (panels A-C, F) or Wilcoxon tests (panels D, E) were used to calculate statistical significance. ****, p<0.0001; ***, 0.0001<p<0.001; **, 0.001<p<0.01

One or two extra rounds of CD32 positive selection lead to further progressive enrichment for HIV DNA in the CD32+ fraction

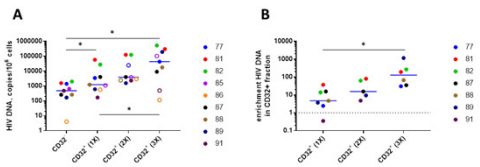

Figure 5. One or two extra rounds of CD32 positive selection lead to further progressive enrichment for HIV DNA in the CD32⁺ fraction. (A) Comparison of HIV DNA levels, normalized to the total cellular DNA, between the CD32⁻ fraction and the CD32⁺ fraction obtained after one, two, or three consecutive rounds of CD32 positive selection. Open circles depict undetectable values, censored to the assay detection limits. The latter depended on the amounts of input cellular DNA and therefore differed between samples. (B) Comparison of HIV DNA enrichment between the CD32⁺ fractions obtained after one, two, or three consecutive rounds of CD32 positive selection. Only detectable values were included in the enrichment calculation. Wilcoxon tests were used to calculate statistical significance. *, 0.01<p<0.05.

HIV proviruses are more transcriptionally silent in the CD32⁺ fraction.

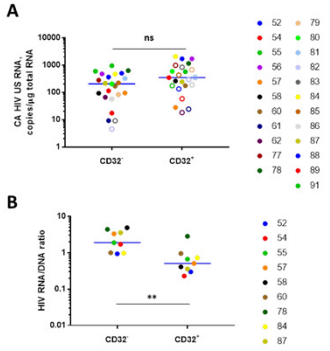

Figure 6. HIV proviruses are significantly more transcriptionally silent in the CD32⁺ fraction. (A) Comparison of cell-associated (CA) HIV unspliced (US) RNA levels, normalized to the total cellular RNA, between the CD32⁻ and CD32⁺ fractions. Open circles depict undetectable values, censored to the assay detection limits. The latter depended on the amounts of input cellular RNA and therefore differed between samples. (B) Comparison of HIV RNA/DNA ratios between the CD32⁻ and CD32⁺ fractions. Only samples where both HIV DNA and RNA were detectable were included in the calculation. Wilcoxon tests were used to calculate statistical significance. **, 0.001<p<0.01; ns, not significant.

Conclusions

- CD32⁺CD4⁺ T cells are significantly enriched for HIV latent proviruses
- The HIV DNA enrichment observed in the CD32⁺CD4⁺ T cells is highly dependent on the purity of the isolated CD32⁺ T cell fraction.
- A positive correlation was observed between the percentage of CD32⁺ cells among CD4⁺ T cells and HIV DNA load in peripheral blood.
- CD32⁺CD4⁺ T cells demonstrate increased expression of HLA-DR and immune checkpoint molecules.

Our results indicate that CD32 remains a promising candidate marker of the HIV reservoir.

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