Pure CD32+CD4+ Cells Are Cytotoxic Memory CD4 + T Lymphocytes Highly Enriched for HIV-1 DNA

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Philipp Adams, Ben Berkhout, Alexander O. Pasternak

1 Laboratory of Experimental Virology, Department of Medical Microbiology, Amsterdam UMC, University of Amsterdam, Amsterdam Institute for Infection and Immunity, Amsterdam, the Netherlands

BACKGROUND

- HIV-1 reservoirs in CD4 + T cells are heterogeneous but reshape biased during suppressive ART [1,2]
- Despite promising candidates it appears unlikely that a unique cell surface marker can identify all CD4 + T cell HIV-1 reservoirs [3,4]
- CD32 expressing CD4 + T cells remain ill-defined due to difficulties in purification, although they represent a promising cellular reservoir [5,6]

Objective: Characterize the role of CD32 + CD4 + T cells in health and HIV-1 persistence

METHODS

CD4 + T-cell MACS pre-purification was combined with two consecutive FACS sorts to obtain pure CD32 + CD4 + T cells.

Detailed phenotyping of sorted cells was performed by multiparametric flow cytometry and subsequent unsupervised analysis using tSNE algorithm.

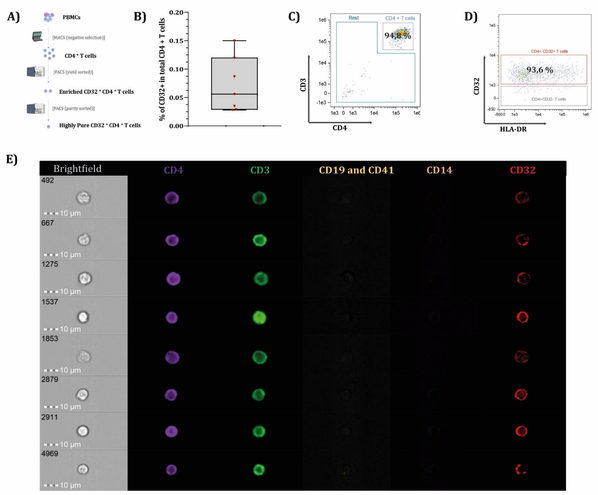
In vitro stimulation assays were executed using primary PBMCs and recombinant human cytokines or anti human antibodies.

Single cell RNA sequencing on sorted cells was performed with 10X genomics and analysed using R studio.

Total HIV-1 DNA in different cellular subsets was measured by qPCR as previously published[7].

RESULTS:

Multistep purification procedure allows isolation of bona fide CD32+ CD4 + T cells



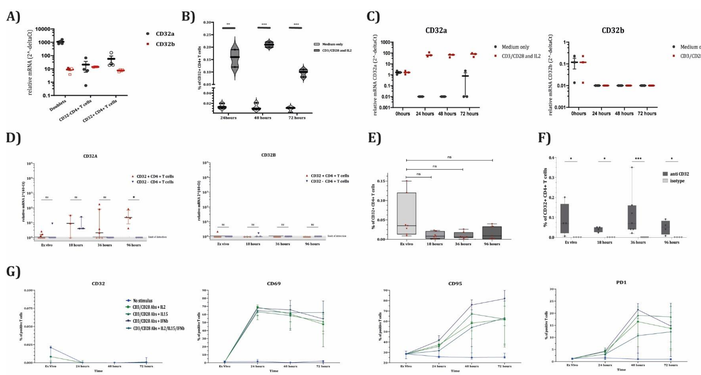
A) Graphical depiction of the developed sorting strategy B) Median frequencies of bona fide, dim CD32+ CD4+ T cells in periphery are 0.053 % of total CD4 + T cells (n=7). C) Post-sorted cells show the phenotype of highly pure CD3 + CD4 + T cells. D) more than 93% of those harbor dim levels of CD32 protein expression.
E) characterization of the staining pattern seen on the highly pure CD32+CD4+ T cells by imaging cytometry.

MAIN FINDINGS:

CD32 expression on CD4 + T cells identifies a subset with…

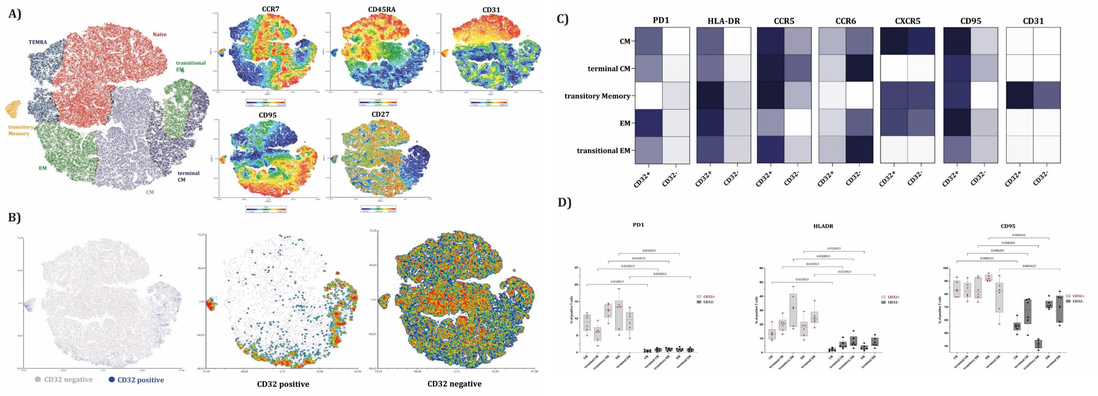
- **effector** and **central memory T cells** phenotype
- increased **expression** of **activation** markers and **immune checkpoints**
- a **cytotoxicity** related **gene** and **protein signature**
- **280 fold enrichment** with **HIV-1 DNA**
- **increased** T cell receptor **clonality**

CD32 expression is not readily inducible on highly pure CD4 + T cells *in vitro*



A) mRNA of freshly isolated, pure CD32+ CD4+ T cells as well as CD32- CD4+ T cells are undetectable for CD32a (in grey) and CD32b transcripts (in red), indicated by non-filled symbols. Control condition, Monocyte-T cell doublets, are as expected clearly detectable for CD32a transcripts. All data is normalized to ribosomal RNA CT values. B) In culture stimulation of the enriched CD4 + T cells induces significant increases in CD32 protein expression from 24 hours on. C) Transcripts of CD32a only do significantly increase when CD4 + T cells are stimulated in vitro. D) Highly pure CD4 + T cells show modest but significant increases in CD32a transcription during activation culture. No increase in CD32b transcription is observed. E,F) Activation of highly pure CD4 + T cells does not show increases of extracellular as well as intracellular CD32 protein expression. G) The stimulation of highly pure CD4 + T cells with different stimuli clearly shows that CD32 is not upregulated upon activation whereas early activation (CD69), intermediate activation (CD95) and later activation/exhaustion marker (PD1) do increase over time. Statistical testing performed using nonparametric unpaired Mann-Whitney method.

CD32+ CD4+ T cells are memory T cells expressing activation and immune checkpoint markers

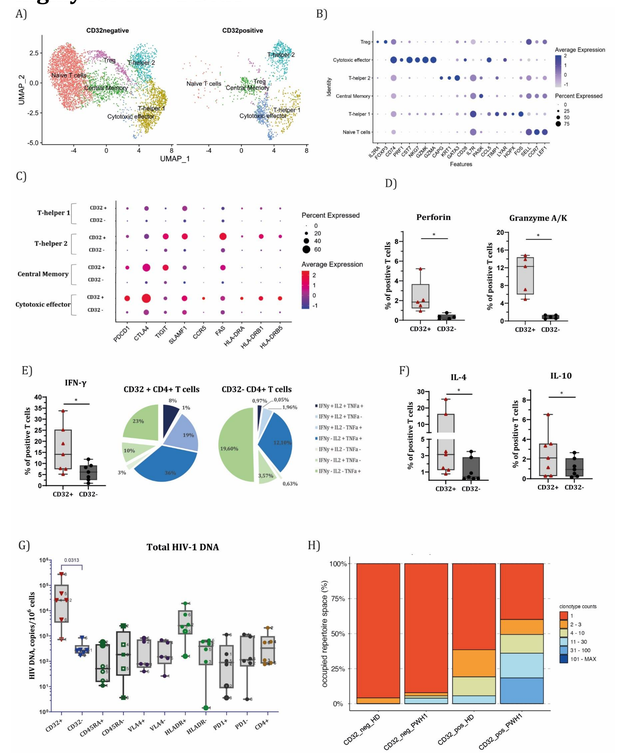


A) Unsupervised tSNE clustering of flow cytometry data files merged from six healthy donors. Main memory populations are mapped as annotated and color coded. Calculation of tSNE was based on the expression levels of the CCR7, CD45RA, CD31, CD95, CD27 for which expression levels are shown on the right side. B) tSNE maps depicting allocation of CD32 + versus CD32 – CD4 + T cells. The graph to the left shows distribution of CD32 + CD4 + T cells in blue and CD32 – CD4 + T cells in grey. Middle graph depicts density distribution of CD32 + CD4 + T cells only, which mostly densly locate in the memory compartment. Graph to the right shows the density distribution of CD32 - CD4 + T cells, which as expected do cluster in all compartments. C) Heatmaps showing median fluorescence intensities convoluted from six healthy donors per marker of interest for CD32 + versus CD32 – CD4 + T cells. Dark blue-grey-white color legends (from high to low values) are normalized to the expression range of each marker. D) Percentage of cells expressing PD1, HLADR or CD95 for CD32 + (red symbols on light grey background) versus CD32 – (black symbols on dark grey background) CD4 + T cells over different memory compartments. Statistical analysis using Wilcoxon rank test showing p values on the graph.

REFERENCES:

1) Einkauf et al., Cell, 2022; 2)Peluso et al., JCI, 2020; 3)Neidleman et al., eLIFE, 2020; 4) Wu et al, Nature Immunology 2022; 5) Darcis et al, Cell reports, 2020; 6) Adams et al., iScience, 2021 7) Malnati et al., PNAS, 2008

CD32+ CD4+ T cells show clear cytotoxic traits and enrich highly for HIV-1 DNA



A) scRNA sequenced transcriptomes allowed identification of six main CD4 + T cell clusters based on the differentially expressed genes between clusters shown in B). C) Relevant HIV reservoir genes differentially expressed between CD32+and CD32- CD4+ T cells over different clusters. D) Intracellular protein staining of Perforin and Granzyme A/K on purified cell populations after 16 hour in vitro reactivation with PMA/Ionomycin. (n=6, non-parametric Wilcoxon rank test with p value cutoff of 0.05). E) Intracellular protein staining of IFN-γ on purified cell populations after 16 hour in vitro reactivation with PMA/Ionomycin. and Relative proportion of combinatorial or multifunctional cytokine production profile of Th1 defining cytokines (n=6, non-parametric Wilcoxon rank test with p value cutoff of 0.05). F) Intracellular protein staining of IL4 and IL10 on purified cell populations after 16 hour in vitro reactivation with PMA/Ionomycin. (n=6, non-parametric Wilcoxon rank test with p value cutoff of 0.05). G) Total HIV-1 DNA on purified subsets of from ART suppressed individuals (n=6). H) TCR clonotype counts in percentages over depicted over the occupied reservoir space in CD32+ CD4+ T cells versus CD32- CD4+ T cells gor a Healthy donor (HD) and a ART suppressed Person Living With HIV-1 (PLWH).

CONCLUSIONS

- A stringent purification procedure allows isolation of bona fide CD32+ CD4+ T cells from PBMCs
- CD32+ CD4+ T cells show a highly skewed memory T cell phenotype
- Transcriptome and protein production unraveled that they make part of cytotoxic effector T cells
- The enrichment for HIV-1 DNA is unparalleled compared to several other cell surface markers

Additional Key information:

Contact: **Philipp Adams**
p.a.adams@amsterdamumc.nl
and/or Alexander O. Pasternak
a.o.pasternak@amsterdamumc.nl