HIV Proviruses Among CMV-Reactive Cells are Abundant, Defective, and Polyclonal After 4 Years of ART

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461

BACKGROUND

Since clonal expansion plays a major role in the maintenance of HIV-infected CD4+ T cells, antigen exposure and immune reconstitution can affect reservoir dynamics (1). Chronic CMV infection is characterized by percolating antigen expression and progressive inflationary T-cell memory. Previous studies in long-term ART treated PLWH showed how HIV-infected CMV-responding cells can be dominated by large clones (2). However, what factors lead to their selection, and the time frame with which they become dominant, remain unclear.

In this study, we assessed the contribution of immune reconstitution, CMV-related disease, and CMV-DNAemia to HIV persistence, after 4 years following ART introduction.

METHODS

We studied 15 participants living with HIV and CMV, enrolled in two NIAID clinical trials. All had a CD4 nadir <50 cells/uL. CMV-driven immune reconstitution inflammatory syndrome (IRIS) and end-organ disease were present in 3 and 3 participants, respectively. PBMCs from week 192 on ART were CD8-depleted and stimulated with CMV antigens. Reactive cells (positive for CD69 and CD154/CD137) were either plate-sorted at limiting dilution and subjected to whole genome amplification (WGA), or bulk-sorted in tubes (Fig 2). WGA plates were tested for LTR, IPDA, proviral sequencing, and integration site analysis. TCRβ sequencing was performed on bulk-sorted cells reactive to CMV and CD3/CD28.

CD4⁺ T Cells count, HIV Viremia and CMV DNAemia

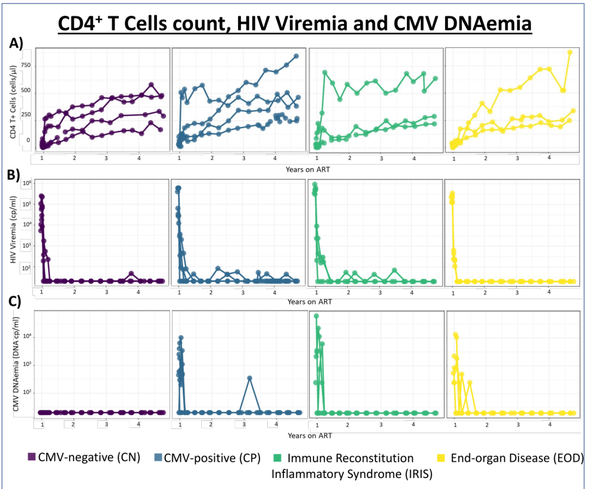

Figure 1. CD4⁺ T cells count, HIV viremia and CMV DNAemia during the study. Individuals were divided into 4 different groups based on the CMV DNAemia at the moment of ART introduction.
A) At nadir, the mean of CD4⁺ T cells count was 34.6±27.8. Despite AIDS-defining condition, we detected a statistically significant recovery in CD4⁺ T cells count at the end of the follow-up (mean 460.5±207.3, p<0.0001), regardless of the group. **B)** Following ART introduction, HIV Viremia decreased below the limit of detection for all the participants. Only few, isolated blips were detected. **C)** No differences in CMV DNAemia before ART introduction were detected among the groups with a detectable CMV viral load. Following ART introduction, CMV DNAemia became undetectable within 6 months, except for a single participant at a single time point.

PLAIN LANGUAGE SUMMARY

- We studied 15 people living with HIV and CMV who started ART with advanced immunodeficiency.
- After 4 years of effective ART, we detected clonally expanded CMV-reactive cells, while proviruses in these cells were diverse and rarely dominated by clones.
- Cells carrying HIV may be older or have a decreased proliferative potential, and may require a longer time to be shaped by antigen-driven proliferation.

Characterization of CMV-reactive Cells

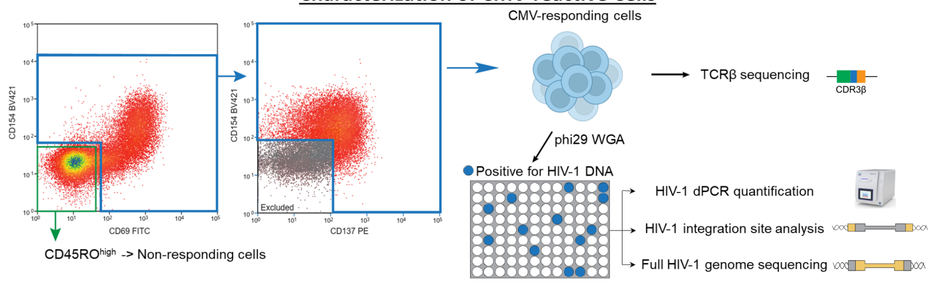

Figure 2. Workflow used to analyze CMV-reactive cells. Following 18h stimulation of CD8-depleted PBMCs with CMV lysate, reactive cells were sorted based on 3 activation markers (CD69, CD154 and CD137). For each participants, a positive (CD3/CD28-stimulated cells), a No-antigen control (No Ag) and the non-responding fraction (CD69⁻ CD154⁻ CD45RO⁺) were sorted and collected. A fraction of CMV-reactive cells were sorted in 96-well plates, while the rest was collected in bulk. 96-well plates were subjected to whole genome amplification (WGA) and screened by dPCR (R-U5 region of the LTR, and IPDA) to detect HIV+ wells. On these, we performed proviral sequencing and integration site analysis. Bulk samples were used to perform TCRβ sequencing.

CMV-reactive cells show high frequency of HIV-infected cells

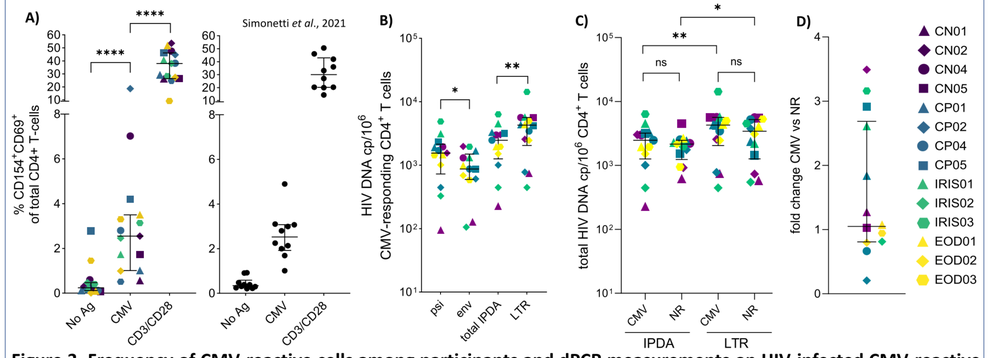

Figure 3. Frequency of CMV-reactive cells among participants and dPCR measurements on HIV-infected CMV-reactive cells. A) CMV-reactive cells were readily detected in all the participants, but one (CP03, excluded from following analyses). Median of CMV-reactive cells was 2.7% [1.2-4.0], higher than No Ag (median 0.2% [0.1-0.5], p<0.001), but lower than CD3/CD28-stimulated cells (median 37.4% [26.6-53.5], p<0.001). Comparison with a previous study (2) showed no differences in the amount of CMV-reactive cells despite the shorter mean time on ART in this study. **B)** We observed a high frequency of HIV-infected CMV-responding cells, with a median of HIV DNA copies/million CD4⁺ T of 4,328 [2,464-5,654] and 2,569 [1,375-3,552] for LTR and IPDA measurements, respectively. We normalized total LTR copies dividing by two, since we estimated that most proviruses retained two LTR copies. Interestingly, total HIV DNA copies by IPDA were significantly lower than HIV DNA copies by LTR (p=0.002), suggesting that a consistent amount of proviruses were either defective or missed by IPDA measurements. **C)** No differences in the frequency of HIV-infected cells were observed between the CMV-responding and the non-responding (NR) fractions. As showed above, HIV DNA copies by LTR quantification were significantly higher than the HIV DNA copies detected by IPDA also in the NR fraction (p=0.03). **D)** Fold change of total HIV LTR cp/10⁶ cells of CMV-responding vs NR fractions.

Proviruses within CMV-reactive cells are heterogeneous, and the few clonal ones are defective

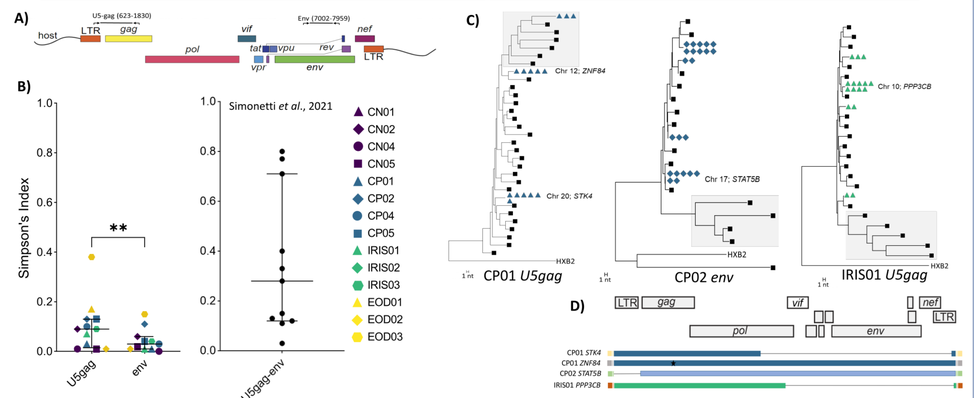

Figure 4. Single genome sequencing of HIV-infected CMV-reactive cells. A) Two specific regions were chosen to perform SGS. **B)** Using SGS data, we calculated the Simpson's Index to define HIV clonality. A higher number of identical sequences were detected for the *U5gag* region, compared to *env* region (p=0.006). Overall HIV clonality was lower than that observed in a previous study (2). **C)** Representative phylogenetic trees for 3 participants (*U5gag* for CP01 and IRIS01, *env* for CP02). Identical sequences are collapsed onto the same branch and showed with the respective symbol of the participant. Black squares are used to show unique sequences. Where recovered, integration sites of the clones are indicated. Grey shaded areas represent hypermutated proviruses. **D)** Full length sequences of the clonal proviruses. All the proviruses detected were defective. Of note, CPO1 *STK4*, CP02 *STAT5B* and IRIS01 *PP3CB* proviruses would have been missed by standard FLIP-seq assay due to their structure.

CMV-reactive cells are characterized by higher clonality

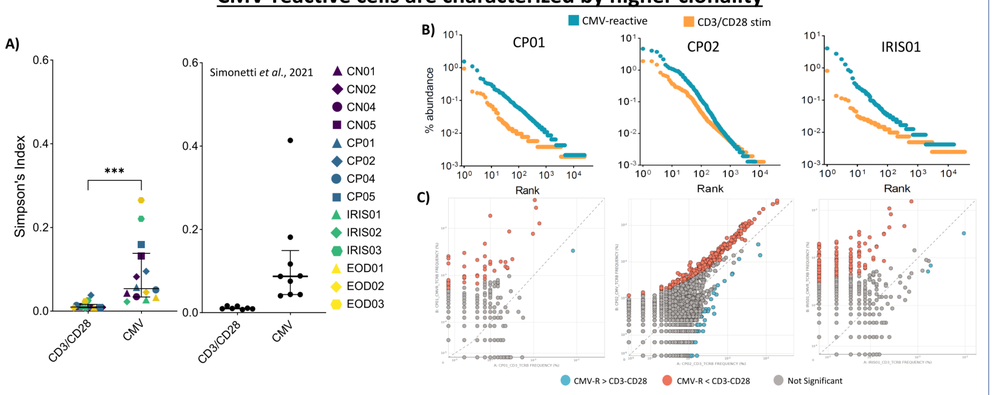

Figure 5. TCRβ sequencing of CMV-reactive cells. A) TCR sequencing revealed marked clonality of CMV-reactive cells compared to those activated by polyclonal CD3/CD28 stimulation (p<0.0001). Results were comparable to those of a previous study (2). **B)** Log-ranked abundance and **C)** Scatter Plot of 3 participants showing enriched clonality for CMV-reactive cells.

CONCLUSIONS

In this study, we observed high infection frequency among CMV-reactive cells, regardless of immune reconstitution and CMV DNAemia. The discrepancy in clonality between total CMV-responding cells and those infected by HIV suggests a preferential expansion of uninfected cells, likely selected during ART, while older infected clones may require longer time to be substantially shaped by proliferation. We are recalling some of the study participants for a 10 year follow up, to investigate whether clonal dominance takes place later during ART, as suggested by a recent study on long-term survivors who have been on therapy for more than 20 years (3).

ACKNOWLEDGMENTS

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