HIV-1 Virion Production from Single Inducible Proviruses following T-Cell Activation Ex Vivo

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Quantifying induced virion production from single proviruses is important for assessing the effects of HIV-1 latency reversal agents. Limiting dilution ex vivo cultures of resting CD4+ T cells from 14 HIV-positive volunteers revealed that virion production after T-cell activation from individual proviruses varies by 10,000-fold to 100,000-fold. High-producing proviruses were associated with increases in cell-associated HIV-1 DNA levels, suggesting that reactivated proviruses proliferate.

HIV-1 virion production from resting CD4+ T cells (rCD4) is commonly measured to assess the size of the latent reservoir and the effectiveness of latency reversal agents (1–4). Prior in vitro and in vivo studies have estimated the average viral burst size, defined as the total number of virions produced by an HIV-1-producing cell over its lifetime, to be 3 to 4 log10 virions/cell (5–9). However, most of these values were derived using parameters estimated from bulk proviral populations. No studies have quantified the distribution of virion production ex vivo from individual reactivated proviruses.

To better understand latency reversal at the single-provirus level, we isolated peripheral blood rCD4 from 14 HIV-1-infected participants on suppressive antiretroviral therapy (ART) for ≥2 years by negative selection as described previously (1). The study was approved by the University of Pittsburgh Institutional Review Board, and all blood donors gave written informed consent. The rCD4 were serially diluted and stimulated for 7 days with anti-CD3/CD28 beads (Life Technologies) at 3 beads/cell in the presence of 300 nM efavirenz and 100 nM raltegravir, which block viral replication as defined by single-genome sequencing (SGS) analysis of supernatant HIV-1 RNA (data not shown). HIV-1 virion production was measured using Roche Cobas AmpliPrep/TaqMan v2.0 (1). Preliminary experiments demonstrated that virion production peaked after 7 days of stimulation, with high cellular viability (data not shown). Using Poisson’s distribution, we identified 19 wells with a ≥96% chance of containing only 1 reactivated provirus. The levels of virus production by these single proviruses differed by ~4 log10 HIV-1 RNA copies/provirus (range, 42 to 42,456) (Fig. 1).

To perform a more detailed analysis of single reactivated proviruses, rCD4 were isolated from a participant and cultured in 352 wells of 96-well plates at 74,000 cells/well (a concentration empirically determined to identify individual reactivated proviruses). The rCD4 were stimulated for 7 days with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml of ionomycin (PMA/ionomycin) in the presence of 300 nM efavirenz and 100 nM raltegravir.

To characterize the upper limit of virion production by individual reactivated proviruses, a screening method was developed to identify wells with high virion production. Aliquots of supernatant from wells in each plate row were pooled (12 wells/row, 32 rows total), and HIV-1 RNA was quantified using an integrase single-copy assay (iSCA) (10) that was modified to include initial centrifugation of supernatants at 21,000 × g for 1 h at 4°C. We identified six rows containing at least 1 provirus each producing ≥2,000 HIV-1 RNA copies/well. For these six rows, the remaining supernatant from each well was extracted and HIV-1 RNA was quantified using the modified iSCA to identify wells containing single reactivated proviruses (Fig. 2). Virion production among individual reactivated proviruses from this donor spanned ~5 log10 HIV-1 RNA copies/provirus (range, 1 to 296,759).

Approximately 47% of the wells in the positively screened rows had detectable HIV-1 virion production following stimulation. According to Poisson’s distribution, ~80% of wells with detectable HIV-1 RNA were expected to contain a single expressing HIV-1 provirus. Single-genome sequencing (SGS) (11, 12) revealed that three of four wells had monotypic sequences with frequent single-base-pair differences within the known error rate of SGS (~1.1 × 10^-4 errors/nucleotide) (Fig. 3)( 11), confirming that most wells with detectable HIV-1 RNA contained a single expressing provirus. The diversity in the fourth well (Fig. 3D) was likely a result of the presence of >1 reactivated proviruses rather than viral replication, which is blocked by 300 nM efavirenz and 100 nM raltegravir as described above.

We next quantified cell-associated HIV-1 DNA (CA-DNA) (1, 13) in 14 culture wells with >200 HIV-1 RNA copies/well and in 9 wells that produced <200 HIV-1 RNA copies/well. A statistically significant, positive correlation was found between the increase in CA-DNA levels in wells and HIV-1 RNA production (Spearman ρ = 0.476, P = 0.0338) (Fig. 4A). Grouped by the number of virions produced, wells with higher virion production (≥200 HIV-1 RNA copies/well) had greater CA-DNA levels than the lower producers (<200 HIV-1 RNA copies/well) and nonproducers (<1 HIV-1 RNA copy/well) (P = 0.0186, P = 0.0073, respec-
tively [Mann-Whitney U test with Bonferroni adjustment]), suggesting that expansion and survival of virus-producing cells may contribute to the higher virion production observed for some proviruses (Fig. 4B).

Interpretation of changes in CA-DNA levels is complicated, however, because wells with single induced proviruses contained multiple noninduced proviruses. Specifically, an average of 144 HIV-1 DNA copies were seeded per well. Although the majority (up to 98.5%) of proviruses are not inducible (1, 14), levels of both induced and noninduced proviruses can expand following activation. Hence, the association between CA-DNA level increases and higher virion production may arise from (i) selective proliferation of induced proviruses, (ii) proliferation of noninduced proviruses, or (iii) proliferation of both induced and noninduced proviruses. Because CA-DNA increases were observed only in wells with high virion production (Fig. 4B), proliferation of induced proviruses could well have played a role.

Variable expansion of provirus levels may be attributable to differences in proviral integration sites and in infected T-cell subsets. Certain integration sites may promote survival and cellular proliferation, as observed in vivo (15, 16). In addition, HIV-1 pro-
viruses can be found across many T-cell subsets (17, 18), which vary in proliferative and apoptotic potential (19). Virion production may also vary as a result of differences in proviral transcription. Integrations in inducible genes could contribute to higher levels of virion production, but could also lead to lower virion production from transcriptional interference (15, 20, 21). Epigenetic modifications that promote or inhibit HIV-1 transcription (22) may differ between individual infected cells. Cells may also possess variable amounts of key transcription factors (e.g., P-TEFB, NF-κB) (23). Finally, differential expression of inhibitory receptors (24) and cytokines (25) may limit T-cell activation and HIV-1 transcription. Detailed single-cell analyses are required to differentiate among these mechanisms of cell proliferation and virion production.

In summary, analysis of single inducible proviruses reveals that levels of induced virion production can vary by 100,000-fold. Given the wide range of virion production following latency reversal, results obtained from bulk cell cultures should be interpreted with caution. Detailed single-cell analyses are needed to investigate the mechanisms that contribute to the wide variation in virion production and cellular proliferation following activation.

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REFERENCES
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