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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. Single-cell analyses are needed to investigate differences in proviral expansion and virus production following latency reversal.

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 $\log_{10}$ 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 determined 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 $\log_{10}$ 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 $\log_{10}$ 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 infrequent 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 $\rho = 0.476$, $P = 0.0338$) (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) ($P = 0.0186$, $P = 0.0075$, respec-

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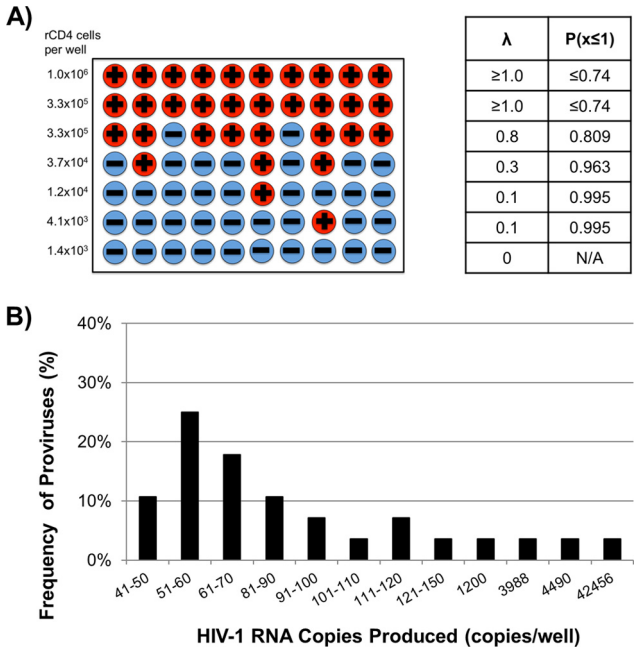

λ	P(x≤1)
≥1.0	≤0.74
≥1.0	≤0.74
0.8	0.809
0.3	0.963
0.1	0.995
0.1	0.995
0	N/A

FIG 1 (A) Schematic of theoretical results using the limiting dilution method and use of Poisson statistics to calculate the probability that a culture with detectable HIV-1 virion production was produced by a single HIV-1 provirus. Wells with detectable HIV-1 virion production are represented by a plus sign (+), and the wells without detectable HIV-1 virion production are represented by a minus sign (−). The Poisson statistics for each row are indicated in the table. λ represents the average rate of success, which is the fraction of wells with detectable HIV-1 virion production at a given dilution. P(x≤1) represents the cumulative probability that a given well contains one or fewer expressing proviruses. (B) Distribution of induced HIV-1 virion production from individual proviruses in resting $CD4^+$ T cells from 14 HIV-infected donors. Purified resting $CD4^+$ T cells were serially diluted and stimulated with anti-CD3/CD28 beads for 7 days. HIV-1 RNA in the supernatants was quantified using Roche Cobas AmpliPrep/TaqMan v2.0, and Poisson statistics were used to identify 19 wells in which single inducible proviruses were present. Only data from wells with detectable HIV-1 RNA are shown.

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).

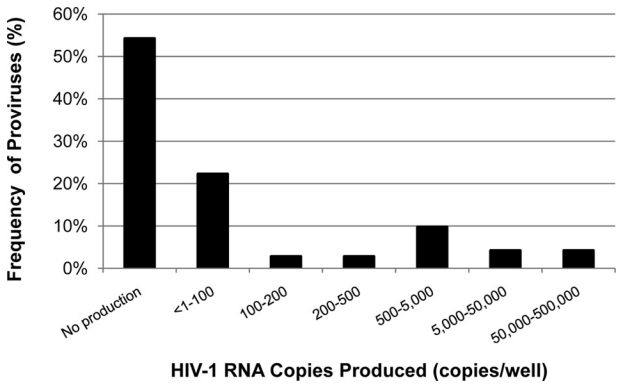

FIG 2 Detailed analysis of HIV-1 virion production from single inducible proviruses. Resting $CD4^+$ T cells from a donor were serially diluted, maximally stimulated with PMA/ionomycin for 7 days, and then assayed for HIV-1 RNA in the supernatant using quantitative reverse transcriptase PCR (qRT-PCR). Data are shown from 6 plate rows (72 wells in total) that were positively screened to contain at least 1 provirus that produces ≥2,000 HIV-1 RNA copies/ml.

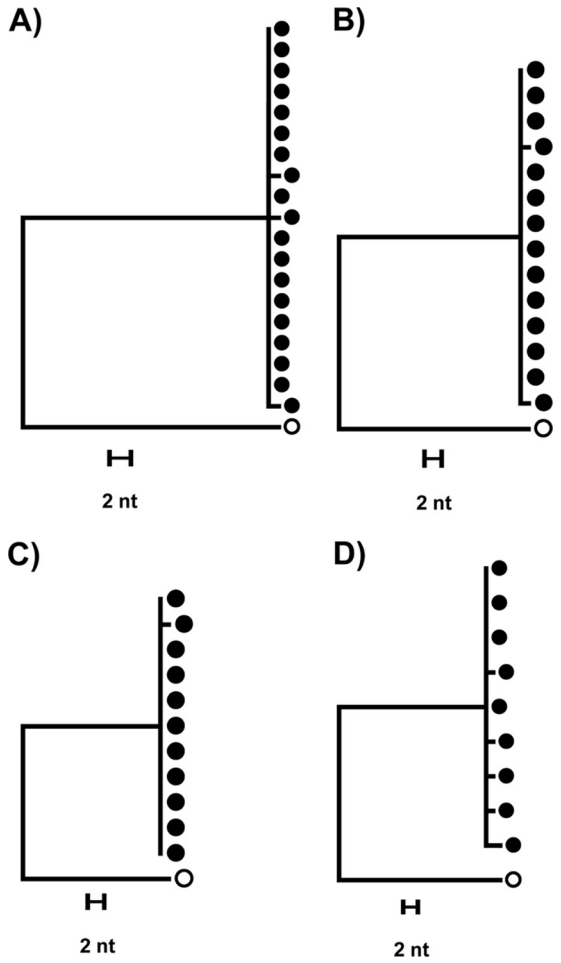

FIG 3 Single-genome sequence and phylogenetic analysis of virions induced from proviruses at the limiting dilution endpoint. Closed symbols represent supernatant RNA sequences, and open symbols represent the consensus HIV-1 subtype B sequence to which each tree was rooted. The single nucleotide (nt) differences of the sequences in panels A to C are within the expected error rate of SGS ($\sim 1.1 \times 10^{-4}$ errors per base sequenced or ~1 to 2 errors per 10 sequences) and are thus consistent with virus production from single proviruses. The sequences in panel D show multiple nucleotide differences and therefore were likely derived from two or more reactivated proviruses in that culture.

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-

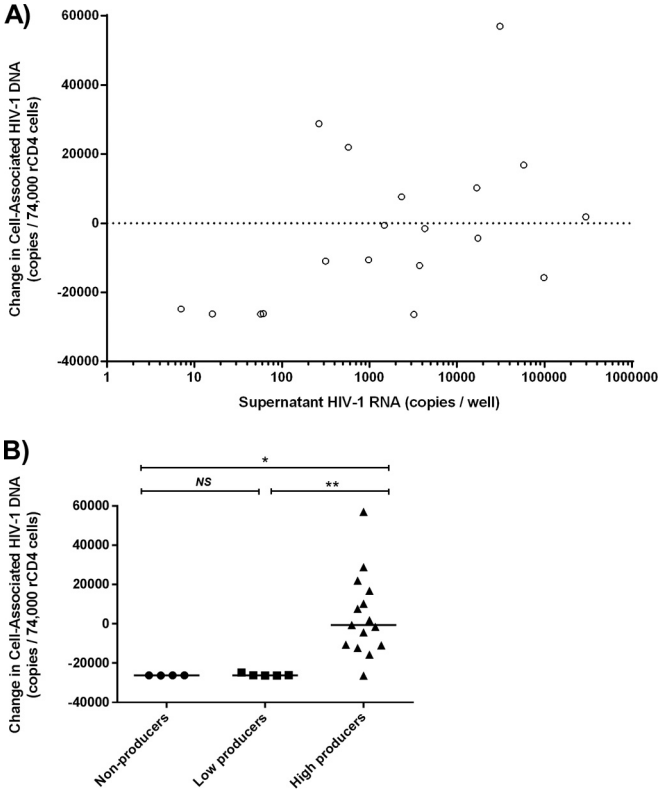

FIG 4 Higher virion production is associated with greater levels of cell-associated HIV-1 DNA (CA-DNA) after activation of resting $CD4^+$ T cells. Changes in CA-DNA levels after activation are shown relative to the levels seen before activation. (A) Spearman correlation between HIV-1 virion production and change in CA-DNA levels in wells with detectable HIV-1 RNA ($\rho = 0.476$; P value = 0.03). (B) Change in CA-DNA levels for nonproducer wells (<1 copy HIV-1 RNA), low-producer wells (≤200 copies HIV-1 RNA), and high-producer wells (>200 copies HIV-1 RNA); horizontal lines reflect the median; statistical significance was tested with the Mann-Whitney U test with a Bonferroni correction. *NS*, = not significant; *, $P < 0.05$; **, $P < 0.01$.

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

1. **Cillo AR, Sobolewski MD, Bosch RJ, Fyne E, Piatak M, Jr, Coffin JM, Mellors JW.** 2014. Quantification of HIV-1 latency reversal in resting $CD4^+$ T cells from patients on suppressive antiretroviral therapy. Proc Natl Acad Sci U S A **111:**7078–7083. http://dx.doi.org/10.1073/pnas.1402873111.
2. **Wei DG, Chiang V, Fyne E, Balakrishnan M, Barnes T, Graupe M, Hesselgesser J, Irrinki A, Murry JP, Stepan G, Stray KM, Tsai A, Yu H, Spindler J, Kearney M, Spina CA, McMahon D, Lalezari J, Sloan D, Mellors J, Geleziunas R, Cihlar T.** 2014. Histone deacetylase inhibitor romidepsin induces HIV expression in CD4 T cells from patients on suppressive antiretroviral therapy at concentrations achieved by clinical dosing. PLoS Pathog **10:**e1004071. http://dx.doi.org/10.1371/journal.ppat.1004071.
3. **Laird GM, Eisele EE, Rabi SA, Lai J, Chioma S, Blankson JN, Siliciano JD, Siliciano RF.** 2013. Rapid quantification of the latent reservoir for HIV-1 using a viral outgrowth assay. PLoS Pathog **9:**e1003398. http://dx.doi.org/10.1371/journal.ppat.1003398.
4. **Bruner KM, Hosmane NN, Siliciano RF.** 2015. Towards an HIV-1 cure: measuring the latent reservoir. Trends Microbiol **23:**192–203. http://dx.doi.org/10.1016/j.tim.2015.01.013.
5. **Althaus CL, Joos B, Perelson AS, Gunthard HF.** 2014. Quantifying the turnover of transcriptional subclasses of HIV-1-infected cells. PLoS Comput Biol **10:**e1003871. http://dx.doi.org/10.1371/journal.pcbi.1003871.
6. **O'Connell KA, Rabi SA, Siliciano RF, Blankson JN.** 2011. $CD4^+$ T cells from elite suppressors are more susceptible to HIV-1 but produce fewer virions than cells from chronic progressors. Proc Natl Acad Sci U S A **108:**E689–E698. http://dx.doi.org/10.1073/pnas.1108866108.
7. **Chen HY, Di Mascio M, Perelson AS, Ho DD, Zhang L.** 2007. Determination of virus burst size in vivo using a single-cycle SIV in rhesus macaques. Proc Natl Acad Sci U S A **104:**19079–19084. http://dx.doi.org/10.1073/pnas.0707449104.
8. **Dimitrov DS, Willey RL, Sato H, Chang LJ, Blumenthal R, Martin MA.** 1993. Quantitation of human immunodeficiency virus type 1 infection kinetics. J Virol **67:**2182–2190.
9. **Tsai WP, Conley SR, Kung HF, Garrity RR, Nara PL.** 1996. Preliminary in vitro growth cycle and transmission studies of HIV-1 in an autologous primary cell assay of blood-derived macrophages and peripheral blood mononuclear cells. Virology **226:**205–216. http://dx.doi.org/10.1006/viro.1996.0648.
10. **Cillo AR, Vagratian D, Bedison MA, Anderson EM, Kearney MF, Fyne E, Koontz D, Coffin JM, Piatak M, Jr, Mellors JW.** 2014. Improved single-copy assays for quantification of persistent HIV-1 viremia in patients on suppressive antiretroviral therapy. J Clin Microbiol **52:**3944–3951. http://dx.doi.org/10.1128/JCM.02060-14.
11. **Palmer S, Kearney M, Maldarelli F, Halvas EK, Bixby CJ, Bazmi H, Rock D, Falloon J, Davey RT, Jr, Dewar RL, Metcalf JA, Hammer S, Mellors JW, Coffin JM.** 2005. Multiple, linked human immunodeficiency virus type 1 drug resistance mutations in treatment-experienced patients are missed by standard genotype analysis. J Clin Microbiol **43:**406–413. http://dx.doi.org/10.1128/JCM.43.1.406-413.2005.
12. **Kearney M, Palmer S, Maldarelli F, Shao W, Polis MA, Mican J, Rock-Kress D, Margolick JB, Coffin JA, Mellors JW.** 2008. Frequent polymorphism at drug resistance sites in HIV-1 protease and reverse transcriptase. AIDS **22:**497–501. http://dx.doi.org/10.1097/QAD.0b013e3282f29478.
13. **Cillo AR, Krishnan A, Mitsuyasu RT, McMahon DK, Li S, Rossi JJ, Zaia JA, Mellors JW.** 2013. Plasma viremia and cellular HIV-1 DNA persist despite autologous hematopoietic stem cell transplantation for HIV-related lymphoma. J Acquir Immune Defic Syndr **63:**438–441. http://dx.doi.org/10.1097/QAI.0b013e31828e6163.
14. **Ho YC, Shan L, Hosmane NN, Wang J, Laskey SB, Rosenbloom DI, Lai**

J, Blankson JN, Siliciano JD, Siliciano RF. 2013. Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. Cell **155:**540–551. http://dx.doi.org/10.1016/j.cell.2013.09.020.

15. **Maldarelli F, Wu X, Su L, Simonetti FR, Shao W, Hill S, Spindler J, Ferris AL, Mellors JW, Kearney MF, Coffin JM, Hughes SH.** 2014. HIV latency. Specific HIV integration sites are linked to clonal expansion and persistence of infected cells. Science **345:**179–183.
16. **Wagner TA, McLaughlin S, Garg K, Cheung CY, Larsen BB, Styrchak S, Huang HC, Edlefsen PT, Mullins JI, Frenkel LM.** 2014. HIV latency. Proliferation of cells with HIV integrated into cancer genes contributes to persistent infection. Science **345:**570–573.
17. **Brenchley JM, Hill BJ, Ambrozak DR, Price DA, Guenaga FJ, Casazza JP, Kuruppu J, Yazdani J, Migueles SA, Connors M, Roederer M, Douek DC, Koup RA.** 2004. T-cell subsets that harbor human immunodeficiency virus (HIV) in vivo: implications for HIV pathogenesis. J Virol **78:**1160–1168. http://dx.doi.org/10.1128/JVI.78.3.1160-1168.2004.
18. **Buzon MJ, Sun H, Li C, Shaw A, Seiss K, Ouyang Z, Martin-Gayo E, Leng J, Henrich TJ, Li JZ, Pereyra F, Zurakowski R, Walker BD, Rosenberg ES, Yu XG, Lichterfeld M.** 2014. HIV-1 persistence in $CD4^+$ T cells with stem cell-like properties. Nat Med **20:**139–142. http://dx.doi.org/10.1038/nm.3445.
19. **Mueller SN, Gebhardt T, Carbone FR, Heath WR.** 2013. Memory T cell subsets, migration patterns, and tissue residence. Annu Rev Immunol **31:**137–161. http://dx.doi.org/10.1146/annurev-immunol-032712-095954.
20. **Shan L, Yang HC, Rabi SA, Bravo HC, Shroff NS, Irizarry RA, Zhang H, Margolick JB, Siliciano JD, Siliciano RF.** 2011. Influence of host gene transcription level and orientation on HIV-1 latency in a primary-cell model. J Virol **85:**5384–5393. http://dx.doi.org/10.1128/JVI.02536-10.
21. **Lenasi T, Contreras X, Peterlin BM.** 2008. Transcriptional interference antagonizes proviral gene expression to promote HIV latency. Cell Host Microbe **4:**123–133. http://dx.doi.org/10.1016/j.chom.2008.05.016.
22. **Mbonye U, Karn J.** 2014. Transcriptional control of HIV latency: cellular signaling pathways, epigenetics, happenstance and the hope for a cure. Virology **454–455:**328–339.
23. **Chakraborty AK, Das J.** 2010. Pairing computation with experimentation: a powerful coupling for understanding T cell signalling. Nat Rev Immunol **10:**59–71. http://dx.doi.org/10.1038/nri2688.
24. **Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, Shin T, Tsuchiya H, Pardoll DM, Okumura K, Azuma M, Yagita H.** 2002. Expression of programmed death 1 ligands by murine T cells and APC. J Immunol **169:**5538–5545. http://dx.doi.org/10.4049/jimmunol.169.10.5538.
25. **Santa-Marta M, de Brito PM, Godinho-Santos A, Goncalves J.** 2013. Host factors and HIV-1 replication: clinical evidence and potential therapeutic approaches. Front Immunol **4:**343.