Novel assay reveals a large, inducible, replication-competent HIV-1 reservoir in resting CD4+ T cells

Anwesha Sanyal1, Robbie B Mailliard1, Charles R Rinaldo1,2, Deena Ratner1, Ming Ding1, Yue Chen1, Jennifer M Zerbato3, Nicholas S Giacobbi3, Narasimhan J Venkatachari1, Bruce K Patterson4, Amanda Chargin4, Nicolas Sluis-Cremer3 & Phalguni Gupta1,2

Although antiretroviral therapy can suppress HIV-1 infection to undetectable levels of plasma viremia, integrated latent HIV-1 genomes that encode replication-competent virus persist in resting CD4+ T cells. This latent HIV-1 reservoir represents a major barrier to a cure. Currently, there are substantial efforts to identify therapeutic approaches that will eliminate or reduce the size of this latent HIV-1 reservoir. In this regard, a sensitive assay that can accurately and rapidly quantify inducible, replication-competent latent HIV-1 from resting CD4+ T cells is essential for HIV-1 eradication studies. Here we describe a reporter cell-based assay to quantify inducible, replication-competent latent HIV-1. This assay has several advantages over existing technology in that it (i) is sensitive; (ii) requires only a small blood volume; (iii) is faster, less labor intensive, and less expensive; and (iv) can be readily adapted into a high-throughput format. Using this assay, we show that the size of the inducible latent HIV-1 reservoir in aviremic participants on therapy is approximately 70-fold larger than previous estimates.

The latent HIV-1 reservoir in resting CD4+ (rCD4+) T cells is an obstacle to the eradication of HIV-1 infection. This reservoir is small, consisting of approximately 1–10 infectious units per million cells1–3. Therefore, it is crucial to develop assays that can reproducibly quantify the reservoir size—and changes therein—in participants enrolled in curative intervention strategies. So far, several assays for measuring cell-associated HIV-1 DNA and RNA have been developed4–8. However, their clinical utility is unclear, because the majority of integrated HIV-1 DNA is replication defective9–11, and measurement of viral mRNA expression might not reflect the amount of replication-competent virus. For this reason, the quantitative viral outgrowth assay (Q-VOA)3,12, which quantifies inducible, replication-competent HIV-1 from rCD4+ T cells, is considered to be the gold standard. However, the Q-VOA might provide a minimal estimate of the size of the latent HIV-1 reservoir because it only detects a fraction of the total integrated pool of replication-competent HIV-1, perhaps in part due to stochastic reactivation of the latent reservoir following maximum T cell activation9,10,13. Nevertheless, underestimating the size of the latent reservoir in rCD4+ T cells could result in the misconception that an individual with HIV-1 infection is cured, when in fact they are not. Additionally, the Q-VOA requires a large volume of blood (120–180 ml)13, is labor intensive, time consuming, and expensive. As such, the development of a rapid, high-throughput, sensitive, and validated assay is important for clinical studies that are evaluating cure strategies, and for researchers who are trying to identify new latency-reversing agents and to characterize the latent reservoir ex vivo. In this report, we describe the development of a sensitive assay, termed TZA, to quantify inducible, replication-competent HIV-1. This assay utilizes the TZM-bl cell line, which stably expresses the HIV-1 receptor CD4, and co-receptors CCR5 and CXCR4, and it carries an integrated copy of the β-galactosidase (β-gal) gene under control of an HIV-1 long terminal repeat (LTR) promoter. Using this assay, we show that the size of the inducible latent HIV-1 reservoir in aviremic individuals on antiretroviral therapy (ART) is approximately 70-fold larger than previous estimates.

RESULTS

Sensitivity of TZM-bl cells to infection by replication-competent HIV-1

To establish the sensitivity of TZM-bl cells to HIV-1 infection, we first serially diluted CD4+ T lymphocytes from uninfected donors—which were acutely infected with the clinical HIV-1 isolate 017—along with uninfected, CD8+ T cell–depleted peripheral blood mononuclear cells (PBMCs), and we added 1 × 10⁶ cells from each serial dilution to 5 × 10⁴ TZM-bl cells in a 96-well plate. β-gal activity was measured by chemiluminescence 48 h later. We found that the TZM-bl cells reproducibly detected infection with a sensitivity down to 0.3 infected cells/10⁶ PBMCs (Fig. 1a). We also treated the latently HIV-1–infected human T cell line ACH-2 (ref. 14) with 100-nM phorbol 12-myristate 13-acetate (PMA), serially diluted ACH-2 cells with uninfected CD8+ T cell–depleted PBMCs, and similarly, added them to TZM-bl cells. We could detect at least 0.1 infectious HIV-1–producing ACH-2 cells/10⁶ CD8+ T cell–depleted PBMCs (Fig. 1b). Next, to determine whether the signal detected by TZM-bl cells was due to replication-competent HIV-1, TZM-bl cells were infected with serially diluted 5′-tropic HIV-1BalLMC2309848

1Department of Infectious Diseases and Microbiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania, USA. 2Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA. 3Division of Infectious Diseases, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA. 4IncellDX, Menlo Park, California, USA. Correspondence should be addressed to P.G. (pgupta1@pitt.edu).

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were infected with HIV-1BaL, and T cell–depleted PBMCs. The HIV-1 RNA data are reported as the mean from uninfected CD8+ T cell–depleted PBMCs for each experiment. The data by chemiluminescence 48 h later. The RLUs for the control were generated performed for each independent experiment.

Cells/106 PBMCs were found to be significantly different from the RLU values determined from rCD4+ T cells derived from participants with HIV-1 infection (from 1.7 \times 10^6 to 4.2 \times 10^6) is indicated in the gray box. The background (control) RLU was subtracted from each data point.

Figure 1. Sensitivity of TZM-bi cells to replication-competent HIV-1. (a) Acutely HIV-1-017-infected CD4+ T lymphocytes (30% infection frequency, as determined by flow cytometry of intracellular p24) were serially diluted with CD8+ T cell–depleted PBMCs maintaining infected cells per million CD8+ T cell–depleted PBMCs at each dilution, as indicated in Figure 1. 1 \times 10^5 cells from each serial dilution were added onto 5 \times 10^6 TZM-bi cells in a 96-well plate. Between four and eight technical replicates were performed for each independent experiment. β-gal activity was measured by chemiluminescence 48 h later. The RLUs for the control were generated from uninfected CD8+ T cell–depleted PBMCs for each experiment. The data are presented as the mean of seven independent experiments. Asterisk (*) indicates significant difference from background, as determined by a paired t-test (P < 0.05). Of note, the β-gal RLU values for 0.3-infected CD4+ T cells/10^6 PBMCs were found to be significantly different from the RLU values for 3.0-infected cells/10^6 PBMCs (paired t-test, P < 0.05); and those for 3.0-infected cells/10^6 PBMCs were significantly different from infection with 30. Data are plotted as mean ± s.e.m. (b) Latently HIV-1-infected ACH-2 cells were treated with 100-nM PMA, serially diluted with uninfected, CD8+ T cell–depleted PBMCs and added to TZM-bi cells in quadruplicate, as described above. The RLUs for the control were generated from uninfected CD8+ T cell–depleted PBMCs for each independent experiment. Data are plotted as mean ± s.e.m. from two independent experiments. (c) Correlation between the TCID_{50} for HIV-1BaL and β-gal activity in the TZM-bi cells. TZM-bi cells were infected with HIV-1BaL, and β-gal activity was measured 48 h later. Data are plotted as mean ± s.e.m. from two independent experiments, each consisting of two technical replicates. The background (control) RLU was subtracted from each data point. The correlation coefficient was determined using the Pearson test. (d) Correlation between the β-gal activity for HIV-1BaL 48 h postinfection and virus production (assessed by extracellular virion-associated HIV-1 RNA) 10 d after infection, following the addition of CD8+ T cell–depleted PBMCs. The HIV-1 RNA data are reported as the mean of two technical replicates. The correlation coefficient was determined using the Pearson test. (e) The relationship between the β-gal RLU signal or extracellular virion-associated HIV-1 RNA and the TCID_{50} for HIV-1BaL representative of three independent experiments. The range of β-gal RLU values determined from rCD4+ T cells derived from participants with HIV-1 infection (from 1.7 \times 10^6 to 4.2 \times 10^6) is indicated in the gray box. The background (control) RLU was subtracted from each data point.

Sensitivity of TZM-bi cells to infection by replication-defective HIV-1
To ascertain whether replication-defective HIV-1 particles were detected with the TZM-bi assay, we measured β-gal activity following their incubation with three different clones of the latently HIV-1-infected J-Lat Jurkat T cell line15. Each J-Lat clone contains a full-length, integrated HIV-1 genome that expresses green fluorescent protein upon activation, and generates defective virions owing to a frameshift mutation in the HIV-1 env gene. Additionally, each of the J-Lat clones produces a different amount of extracellular virus particles after stimulation with 100-nM PMA (Supplementary Table 1).

Our data show that none of the J-Lat clones produced any signal in the TZM-bi cells (Fig. 2a). We also evaluated the chronically infected T cell line 8E5, which contains a single, integrated copy of proviral X4-tropic HIV-1{LAI} (lymphadenopathy-associated virus) DNA and produces defective virus particles that lack reverse transcriptase16. Our data (Fig. 2b) show that no positive β-gal signal in the TZM-bi cells was detected relative to the control after the addition of up to 4,000 8E5 cells (which produce 8,606 pg/ml of viral p24 protein), although at higher 8E5 cell concentrations, we noted β-gal activity. By contrast, as indicated in Figure 1a,b, we can detect a positive signal by using less than 1 cell infected with replication-competent HIV-1 per 10^6 cells. Finally, we evaluated the ability of a full-length, replication-defective clone of the X4-tropic HIV-1{LAI} laboratory strain that harbors the inactivating L289K mutation in the reverse-transcriptase-encoding gene12 to infect TZM-bi cells. In comparison to wild-type HIV-1, the mutant virus did not induce any β-gal activity in TZM-bi cells, even when 1,000 pg of p24-equivalent virus was added to the cells (Fig. 2c). Collectively, these data demonstrate that TZM-bi cells are insensitive to replication-defective virus particles containing mutations in env or reverse transcriptase.

Quantification of inducible, replication-competent HIV-1 from rCD4+ T cells purified from individuals with HIV-1 infection
We next developed a strategy for quantifying inducible, replication-competent HIV-1 from rCD4+ T cells purified from aviremic individuals with HIV-1 infection who were on suppressive combination antiretroviral therapy (cART). This process involved (i) the induction of latent virus using anti-CD3/CD28 monoclonal antibody (mAb)-coated microbeads; and (ii) quantification of the induced, replication-competent HIV-1 in TZM-bi cells (Fig. 3a). Blood was obtained from 15 participants who were enrolled at the Pittsburgh clinical site.
of the Multicenter AIDS Cohort Study (MACS) (Table 1). rCD4+ T cells were isolated by negative selection from PBMCs to >98% purity (Supplementary Fig. 1) and were comprised of CD45RA+/CCR7+ naïve (36%), CD45RO+/CCR7+ central memory (41%), CD45RO+/CCR7− effector memory (19%), and CD45RA+/CCR7− effector memory RA cells (4%) (data not shown). Purified rCD4+ T cells were exposed

Figure 2  TZM-bl cells are insensitive to infection by replication-defective HIV-1. (a) J-Lat clones 10.3, 9.2, and 8.4 were stimulated with PHA, serially diluted with uninfected CD8+ T cell–depleted PBMCs, and added to TZM-bl cells. Each independent experiment was carried out in quadruplicate. β-gal activity was measured 48 h later. The RLU values for the control were generated from uninfected CD8+ T cell–depleted PBMCs. Data are plotted as the mean from two independent experiments. (b) 8E5 cells were serially diluted with uninfected CD8+ T cell–depleted PBMCs and added to TZM-bl cells. Each independent experiment was carried out in quadruplicate. β-gal activity was measured 48 h later. Data are plotted as the mean from two independent experiments. (c) Different p24 amounts of wild-type (wt) HIV-1 LAI and a mutant virus containing the L289K mutation in reverse transcriptase that renders the enzyme defective were added to TZM-bl cells. β-gal activity was measured 48 h later. RLU values from control TZM-bl cells alone treated with DMSO were used as control. The assay was carried out once, and the data are shown as a mean of three technical replicates.

Figure 3  A TZA to quantify inducible, replication-competent HIV-1 from rCD4+ T cells. (a) Schematic overview of the TZA. (b) Statistical comparison of the IUPM values determined by the TZA or the Q-VOA was assessed using the two-sample t-test (P = 0.006). (c) Statistical comparison of the pFVE was assessed by using the ratio paired t-test (P = 0.001). For b and c, the control included uninfected donor CD4+ T cells that were added to TZM-bl cells. The RLU values from the control were subtracted from each assay sample. (d) Correlation between TZA and total HIV-1 DNA in rCD4+ T cells. The correlation coefficient r and the P value were determined using the Pearson test. (e) Correlation between the Q-VOA and total HIV-1 DNA in rCD4+ T cells. (f) Correlation between TZA and intracellular HIV-1 gag-pol mRNA-positive cells/10^6 rCD4+ T cells. (g) Correlation between TZA and Q-VOA.
to anti-CD3/CD28 mAb–coated microbeads for 5 d to induce latent HIV-1 expression (i.e., bulk stimulation). Efavirenz (300 nM), a non-nucleoside reverse-transcriptase inhibitor, was included in the culture medium to prevent the spread of de novo HIV-1 infection. Treatment of rCD4+ T cells with anti-CD3/CD28 mAbs resulted in a ~1.6-fold increase in CD4+ T cell proliferation, but approximately a 0.8-fold decrease in the frequency of HIV-1 DNA/10^6 cells (Supplementary Table 2). The mechanisms responsible for the observed decrease in the frequency of HIV-1 DNA/10^6 cells after T cell activation are unclear, but they might be due to the cytopathic effect of the virus produced by infected, activated CD4+ T cells. Next, we added serially diluted rCD4+ T cells to 3–6 × 10^6 TZM-bl cells in a 96-well plate, and after 48 h of co-culture, we quantified β-gal expression. As a control, anti-CD3/CD28 mAb–treated rCD4+ T cells from healthy donors without HIV-1 infection were similarly co-incubated with TZM-bl cells, and expression of β-gal was measured. Infectious units per million (IUPM) cells ranged from 1.2 to 141.7 (mean 46.9; n = 13) for the TZA (Fig. 3b and Table 1). The IUPM values calculated for rCD4+ T cells from the same participants using the Q-VOA ranged from 0.26 to 2.37 (mean 0.70; n = 12) (Fig. 3b and Table 1), which is almost 70-fold lower (P = 0.006). In this study, the IUPM values calculated from the Q-VOA (0.28–2.37) were similar to previously reported values (0.03–3.00)³. In a subset of nine participants, we also determined the fractional provirus expression (fPVE) that could be reactivated to produce infectious virus in both assays, as described previously¹¹. The fPVE provides an estimate of the size of the replication-competent viral reservoir. The fPVE calculated from the TZA (mean 4.03%; range 0.12–13.93%) was 28-fold higher (P = 0.001) than that from the Q-VOA (mean 0.14%; range 0.02–0.9%) (Fig. 3c). We found a weak correlation that was not significant between total HIV-1 DNA/10^6 rCD4+ T cells and the IUPM calculated from the TZA (r = 0.7; P = 0.06) (Fig. 3d). In our data set, there was no correlation between HIV-1 DNA/10^6 rCD4+ T cells, HIV-1 gag-pol RNA-positive CD4+ T cells/10^6 rCD4+ T cells, and the IUPM calculated from the Q-VOA, or between the TZA and Q-VOA assays (Fig. 3e–g).

Further characterization of the TZA

In control experiments, we exposed TZM-bl cells to efavirenz before adding activated rCD4+ T cells, which would block active HIV-1 replication while enabling us to ascertain whether cytokines released from the activated rCD4+ T cells increased TZM-bl cell β-gal activity. In rCD4+ T cells from two participants with HIV-1 infection, we observed that the IUPM values decreased from 81 and 26, respectively, in the absence of inhibitor to undetectable levels in the presence of inhibitor. This finding suggests that the higher IUPM values determined using the TZA (as compared to the Q-VOA) are not due to cytokines released after T cell activation with the anti-CD3/CD28 mAbs. We also compared our bulk rCD4+ T cell stimulation approach (Fig. 3a) to one in which we first serially diluted the rCD4+ T cells before adding anti-CD3/CD28 mAbs. The latter approach is used in the Q-VOA. We found no notable difference in the IUPM values when using these two different approaches with rCD4+ T cells isolated from three different participants with HIV-1 infection (Supplementary Fig. 2). Finally, we evaluated whether a positive IUPM in the TZA reflected productive HIV-1 replication. As discussed above in Sensitivity of TZM-bl cells to infection by replication-competent HIV-1’ (Fig. 1e), the β-gal activity for the detection of HIV-1 replication in TZA-bl cells was sensitive down to a TCID₅₀ of 10⁻², whereas virus production measured 10 d postinfection was sensitive only down to a TCID₅₀ of 1. The β-gal relative light unit (RLU) values determined from the rCD4+ T cells from the 15 participants with HIV-1 infection included in this study ranged from 1.7 × 10⁴ to 4.2 × 10⁶ (Fig. 1e). In this regard, if we evaluate rCD4+ T cells from participants with HIV-1 infection who have a low RLU value, we cannot reproducibly detect extracellular virion-associated RNA following 10 d of co-culture of the TZM-bl cells with freshly added CD8+ T cell–depleted PBMCs. However, we can readily detect virus production at day 3 and day 10 after infection from rCD4+ T cells that were derived from participants with HIV-1 infection and yielded higher RLU values (Supplementary Table 3).

DISCUSSION

There is a critical need for the development of sensitive assays that can accurately quantify inducible, replication-competent latent HIV-1. In this study, we developed a reporter cell-based assay, TZA, to quantify inducible, replication-competent latent HIV-1 in blood. In this regard, control experiments revealed that the TZA is insensitive to virus particles defective in env or reverse transcriptase, and that β-gal...
activity is not altered by cytokine release from activated CD4+ T cells. Using the TZA, we show that the size of the inducible, latent HIV-1 reservoir in aviremic participants on therapy is approximately 70-fold larger than previous estimates. The larger IUPM (46.9) and fPVE (4.03%) values determined for the TZA are consistent with the fPVE of 1.5% obtained by Cillo et al. through the quantitation of extracellular virion-associated HIV-1 RNA, and with the number of multiple spliced HIV-1 RNA cells/million CD4+ T cells (mean, 24), as reported by Procopio et al. using the tat/rev-induced limiting dilution assay. Furthermore, our IUPM values are in line with the observation that the Q-VOA underestimates the amount of replication-competent virus by 60-fold, as compared to the level of intact proviruses estimated by sequence analysis. There are, however, several differences between the TZA and Q-VOA that might contribute toward some of the differences in IUPM values noted in this study. Specifically, the Q-VOA and TZA use different measures of replication-competent HIV-1: the Q-VOA employs multiple rounds of infection and quantification of extracellular virion-associated HIV-1 RNA, whereas the TZA is based on a single round of infection and quantitation of tat-induced activation of β-gal activity. There might be some virus particles that can efficiently undergo a single cycle of replication but are not capable of undergoing multiple rounds of infection. In this regard, full-length genome sequencing of virus particles produced in the TZM-bl cells may provide insight into the frequency of detecting partially attenuated replication-competent viruses in the TZA.

The TZA has several advantages in that it (i) is sensitive; (ii) requires only a small blood volume (2 × 10^6 rCD4+ cells); (iii) is fast (7 d, as compared to 14 d for the Q-VOA), less labor intensive, and less expensive ($350 per assay relative to more than $1,000 for the Q-VOA). These features may allow the TZA to be used for in vitro screening to identify latency-reversing agents, as well as in clinical HIV-1-eradication studies, although the latter will necessitate further validation of the assay. Because of its low cell requirement, the TZA may also be useful for the quantification of replication-competent HIV-1 in the pediatric population, as well as in tissue.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

P.G., A.S., N.S.-C., R.B.M., B.K.P., C.R.R., N.I.V., and Y.C. designed the study, analyzed data, and wrote the manuscript. D.R., M.D., A.S., A.C., J.M.Z., and N.S.G. performed the experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

**Cells and viruses.** TZM-bl cells (catalog number 8129), ACH-2 (catalog number 349), J-Lat clone 10.6 (catalog number 9849), J-Lat clone 9.2 (catalog number 9848), J-Lat clone 8.4 (catalog number 9847), and BES cells (catalog number 95) were obtained from the NIH AIDS Reagent Program. TZM-bl cells are positive for murine leukemia virus. The clinical HIV-1 isolate 017 was isolated from a participant with chronic HIV-1 infection from the MACS and expanded in PHA-stimulated lymphoblasts. HIV-1LAI (catalog number 11414) was obtained from the NIH AIDS Reagent Program. The L289K mutation in the reverse-transcriptase-encoding gene of HIV-1LAI was constructed by site-directed mutagenesis using the QuickChange Lightning mutagenesis kit (Agilent Technologies, Santa Clara, CA). Viral-infectivity titer (TCID50) of HIV-1LAI was measured using the end-point dilution method by infecting PHA-stimulated CD8-depleted PBMCs by serially diluted virus and incubating for 7 days. The culture supernatants were monitored after 7 days for viral growth by HIV-1 p24 using an enzyme-linked immunosorbent assay (ELISA). The TCID50 was determined by using the Reed Munch method of quantification.

**Isolation of rCD4+ T cells from participants with HIV-1 infection on ART.** PBMCs were isolated from 200 ml of heparinized blood from participants in the MACS by ficoll-hypaque density-gradient centrifugation. All study participants provided written consent, which was approved by the University of Pittsburgh Institutional Review Board. The study population is predominantly participants with HIV-1 infection was considered to be positive if the chemoluminescence signal was above the mean + two s.d. of the signal obtained from a control sample (healthy donor cells) well.

**Q-VOA.** The Q-VOA was carried out as described previously.22, Viral outgrowth was assessed at day 14 by quantification of the extracellular virion-associated HIV-1 RNA using a qPCR for HIV-1 gag RNA, with a sensitivity/cutoff of 10 copies/ml. A well with HIV-1 gag RNA of ≥10 copies/ml was considered to be positive.

**IUPM calculations.** The maximum likelihood estimate was applied to determine the infectious unit per million (IUPM) for both TZA and Q-VOA by using online software, available at http://silicianolab.johnshopkins.edu/, developed by Rosenbloom et al.25.

**fPVE calculations.** The fPVE was calculated by dividing IUPM values by the total HIV-1 DNA copies/million cells, as described by Cillo et al.21.

**Statistical analyses.** A ratio paired test or equal variance two-sample t-test was used to calculate the significance while comparing sets of data whenever necessary. All statistical analysis was performed using PRISM software. A P value of less or equal to 0.05 was considered to be significant in each of the statistical analyses performed. Correlations between two independent sets of data were performed using the Pearson correlation coefficient.

**Data-availability statement.** All primary data used in generating the tables and figures are attached as supplementary material as data files. Source data files for Figures 1–3 are available online.

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Corrigendum: Novel assay reveals a large, inducible, replication-competent HIV-1 reservoir in resting CD4+ T cells

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In the version of this article initially published online, the description of the fPVE calculation was incorrect. The correct description is as follows: “The fPVE was calculated by dividing IUPM values by the total HIV-1 DNA copies/million cells, as described by Cillo et al.18.” The error has been corrected in the print, PDF and HTML versions of this article.