

A CD3/CD28 microbead-based HIV-1 viral outgrowth assay

Yury V Kuzmichev¹, Rebecca T Veenhuis², Christopher W Pohlmeier², Caroline C Garliss², Victoria EK Walker-Sperling², and Joel N Blankson^{2*}

¹ Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

² Center for AIDS Research, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Abstract

Aims: Latently infected resting CD4 T cells represent a major barrier to HIV-1 eradication efforts. The standard assays used for measuring this reservoir induce activation of resting CD4 T cells with either phytohaemagglutinin (PHA) with irradiated feeder cells, or with anti-CD3 antibodies. We designed a study to compare the sensitivity of a new assay (based on the stimulation of CD4 T cells with anti-CD3 and anti-CD28 coated microbeads) with that of the traditional PHA- and feeder-based viral outgrowth assay.

Methods: Resting CD4 T cells from 10 HIV-1-infected patients on suppressive combination antiretroviral therapy (cART) regimens were cultured in the traditional PHA/feeders viral outgrowth assay and the new CD3/CD28 bead-based assay. Flow cytometry was used to assess the kinetics of activation of resting CD4 T cells in the two different assays.

Results: There was no significant difference in the sensitivity of the two assays. The median frequency of latently infected cells was 0.83 infectious units per million (IUPM) for the PHA/feeders assay and 0.54 IUPM with the CD3/CD28 bead-based assay. However, while virus was obtained from all 10 patients with the traditional PHA/feeders outgrowth assay, no virus was obtained from two of 10 patients with the novel anti-CD3/CD28 bead-based viral outgrowth assay (IUPM < 0.02).

Conclusion: The new CD3/CD28 bead-based assay has comparable sensitivity to the PHA/feeders assay and does not require the addition of feeders, making it a simpler and less labour-intensive alternative to the standard PHA/feeders-based assay.

Keywords: latency, viral outgrowth assay, HIV-1

Introduction

Combination antiretroviral therapy (cART) controls HIV-1 replication but is not curative due to the presence of a latent reservoir. Resting CD4 T cells are the best characterised cells in this reservoir [1]. Assays that measure this reservoir stimulate replication of latent virus through global activation of CD4 T cells [2]. Methods used to achieve this global activation include treatment with phytohaemagglutinin (PHA) and irradiated allogeneic feeders [3,4] or with immobilised anti-CD3 and anti-CD28 antibodies [5] or a bispecific CD3/CD8 antibody that simultaneously activates CD4 T cells and depletes CD8 T cells [6]. Currently, the PHA/feeders quantitative viral outgrowth assay (QVOA) is considered the gold standard for measuring the size of the latent reservoir [4]. The assay induces activation of resting CD4 T cells by the addition of PHA in the presence of a 10-fold excess of irradiated allogeneic peripheral blood mononuclear cells (PBMCs). After overnight stimulation, the media containing PHA is removed and replaced with fresh media before CD4 lymphoblasts are added to propagate the virus. The need for the addition of feeders followed by a PHA removal step makes this assay costly and labour intensive. The development of alternative assays that are less costly and labour intensive is a major priority for the HIV-1 cure agenda [7].

Antibody-based assays do not require the addition of feeders and are, therefore, less labour intensive. The first antibody-based assays used either immobilised anti-CD3 and anti-CD28 antibodies [5] or a soluble CD3/CD8 bispecific antibody [6]. The two assays were shown to have roughly the same sensitivity in a direct comparison [6]. However, recent studies have shown that anti-CD3/CD28-coated magnetic beads efficiently induce proviral transcription [8], and are more effective at stimulating primary CD4 T cell growth than soluble anti-CD3 antibodies [9]. As such, we hypothesised that the more potent global stimulation and expansion of resting

CD4 T cells induced by anti-CD3/CD28-coated microbeads would lead to better outgrowth of latent virus. We developed a viral outgrowth assay with these beads and performed a direct comparison of the sensitivity of this new assay to that of the standard PHA/feeders assay.

Methods

Patient cohort

Ten HIV-1-infected patients on suppressive cART for more than a year were enrolled in this study. The clinical characteristics of these patients are shown in Table 1. The study was approved by the Johns Hopkins Institutional Review Board. All patients and HIV-negative donors provided written consent before participating in this study.

Isolation of resting CD4 T cells

PBMCs from either HIV-1-infected patients or healthy donors were isolated by Ficoll gradient centrifugation. CD4 T cells were isolated from bulk PBMCs by negative selection (Human CD4 T Cell Isolation Kit, Miltenyi Biotec). Resting CD4 T lymphocytes were further enriched by the depletion of cells expressing HLA-DR, CD25, and CD69 (Anti-HLA-DR Microbeads; CD25 MicroBeads; CD69 MicroBead Kit II; Miltenyi Biotec).

Viral outgrowth assays

Resting CD4 T cells from each patient were divided into two aliquots; one aliquot was used for the CD3/CD28 assay, the other for the PHA/feeders QVOA. We initially started with 25–30 replicates of 2×10^5 resting CD4 T cells. For the PHA/feeders assay 2×10^5 cells were cultured with 2.5×10^6 irradiated PBMCs from a healthy donor (feeders) in each well of a 24-well plate in 2 mL of RPMI 1640 (Thermo Fisher Scientific) containing 10% fetal bovine serum (Thermo Fisher Scientific), 100 U/mL IL-2 (Novartis), 1% Pen Strep (Thermo Fisher Scientific), 1% T cell growth factor (produced in-house, as described previously [4]), and 0.5 µg/mL of PHA (Remel). For the bead-based assay 2×10^5 cells were

*Corresponding author: Joel N. Blankson, Department of Medicine, Johns Hopkins University School of Medicine, 855 N Wolfe Street, Rangos 552, Baltimore, MD 21205, USA
Email: jblankson@jhmi.edu

Table 1. Clinical characteristics of the chronically infected patients on cART studied

Subject	Current CD4 T cell count (cells/ μ L)	HIV-1 RNA (copies/mL)	Nadir CD4 T cell count (cells/ μ L)	Time on suppressive regimen (years)	Current regimen
PT8	424	<50	18	8	3TC, RAL EFV
PT10	1109	<50	NA	12	3TC, ABC, DTG
PT11	1032	<50	177	8	TDF, FTC, DRV/r
PT12	860	<50	494	3	3TC, RAL EFV
PT14	646	<50	12	4	DRV/r, DTG
PT16	921	<50	203	5	3TC, ABC, DTG
PT21	541	<50	388	3	FTC, TDF, RPV
PT40	705	<50	NA	4	FTC, TDF, EFV
PT42	1140	<50	NA	4	MVC, DRV/r, RAL
PT45	402	<50	19	7	DTG, DRV/r

3TC: lamivudine; ABC: abacavir; DRV/c: cobicistat boosted darunavir; DRV/r: ritonavir boosted darunavir; DTG: dolutegravir; EFV: efavirenz; FTC: emtricitabine; MVC: maraviroc; RAL: raltegravir; RPV: rilpivarin; TDF: tenofovir; NA: not available.

cultured in each well of a 96-well plate in 200 μ L of the media described above and 5 μ L of anti-CD3/CD28-coated microbeads (ThermoFisher Scientific) for a bead to cell ratio of 1 : 1 as per the manufacturer's instructions. CD4 T cells were stimulated for 18 hours in both assays. For the PHA/feeders assay the PHA was washed off as previously described and $0.5\text{--}1\times 10^6$ lymphoblasts were added to each well. For the bead-based assay, the cells and beads were re-suspended and transferred to 48-well plate wells with $0.5\text{--}1\times 10^6$ lymphoblast per well. The beads were removed for flow cytometry but were present throughout the culture assay. A second round of $0.5\text{--}1\times 10^6$ blasts were added to each well on day 9 for both assays.

In patients where virus was not obtained using multiple replicates of 200,000 CD4 T cells, the assay was repeated with 20–30 replicates of 10^6 resting CD4 T cells. For the PHA/feeders assay 1×10^6 cells were cultured with 10×10^6 irradiated feeders in each well of a six-well plate in 8 mL of RPMI containing 10% fetal bovine serum, 100 U/mL IL-2, 1% Pen Strep, 1% T cell growth factor, and 0.5 μ g/mL PHA. For the bead-based assay, 1×10^6 cells were cultured in each well of a 48-well plate in 1 mL of the media described above and 25 μ L of anti-CD3/CD28-coated microbeads. CD4 T cells were stimulated for 18 hours in both assays. For the PHA/feeders assay the PHA was removed and $2\text{--}4\times 10^6$ lymphoblasts were added to each well. For the bead-based assay, the cells and beads were re-suspended and transferred to a 12-well plate with $2\text{--}4\times 10^6$ lymphoblast added per well. A new round of $2\text{--}4\times 10^6$ blasts were added to each well on day 9 for both assays. Lymphoblasts from the same healthy donors were used to amplify virus from the two assays for each patient and the same number of lymphoblasts per well were used for each assay in order to minimise the number of variables in the comparison. Supernatants from each well were tested for HIV-1 p24 protein by ELISA (Perkin Elmer) on day 12 and repeated on day 19 for the negative wells.

Flow cytometric analysis of activated resting CD4 T cells

To compare the activation levels achieved in the two assays, CD4 T cells were obtained from two HLA-A2 positive healthy donors and five chronic progressors. For each time point (Figure 1) 2×10^5 cells were plated in triplicate and stimulated as described above for the bead-based and PHA/feeders assays. The feeders were obtained from a HLA-A2 negative HIV-uninfected donor so that

we could easily distinguish between the feeders and the purified resting CD4 T cells using an antibody specific for HLA-A2. After 18 hours of incubation, the cells from the standard assay were stained with CD3-PacBlue (BD), CD8-APC-H7 (BD), CD4-PE (BD), HLA-A2-PerCP-Cy5.5 (BioLegend), CD25-PE-cy7 (BD), HLA-DR-FITC (BD), CD38-APC (BD) and CD69-BV605 (BD). For the CD3/CD28 assay, the beads were removed prior to staining. Stained cells were analysed by flow cytometry on a LSRFortessa (BD). The procedure was repeated on days 2 and 3.

Data analysis

Flow cytometry data was analysed using FlowJo v10.0.7. All statistical analysis was performed in GraphPad Prism v6.01. A one-way ANOVA adjusted for multiple comparisons was used to assess differences between activation-marker expression at each time point (Figure 1). A Wilcoxon matched-pairs signed-rank test was used for the remaining statistical comparisons (Figure 2). Frequencies of infected cells (IUPM) were based on the percentage of positive replicates and were calculated as previously described [4].

Results

Stimulation with anti-CD3/CD28-coated microbeads results in faster activation of resting CD4 T cells

We first compared the level of immune activation achieved with the traditional PHA/feeders assay to that achieved with anti-CD3/CD28-coated microbeads using resting CD4 T cells from two healthy donors. Analysis of T cell activation by flow cytometry showed that stimulation with microbeads resulted in faster activation of resting CD4 T cells as determined from the percentage of cells expressing very early (CD69) or early (CD25) activation markers (Figure 1A). Interestingly, the late activation markers, HLA-DR and CD38, were present at higher levels on cells stimulated with PHA and feeder. Similar results were obtained when the comparison was performed with resting CD4 T cells from three patients on cART (Figure 1B) and when all five subjects were analysed together, a significantly higher percentage of resting CD4 T cells stimulated with CD3/CD28 microbeads expressed CD69 and CD25 than CD4 T cells that were stimulated with PHA and feeders on each day. In contrast a significantly higher percentage of resting CD4 T cells stimulated with PHA and feeders expressed CD38 and HLA-DR than cells stimulated with CD3/CD28 on each day.

The sensitivity of the anti-CD3/CD28 bead-based assay is similar to that of the PHA/feeders assay

We originally plated 25–30 replicates of 2×10^5 resting CD4 T cells per well from 10 subjects on cART (Table 1) for both assays. CD4 T cells from two of these patients (PT11 and PT12) were used in the comparative activation assay shown in Figure 1. The median percentage of positive wells was 15% (range 0–40%) for the standard PHA/feeders assay and 10% (range 0–68%) for the bead-based assay. No virus was isolated from the PHA/feeders assay in one of the 10 patients tested (PT40) whereas no virus was obtained with bead-based assay in four out of 10 patients (PT8, PT14, PT21 and PT40). To improve the sensitivity of both assays, we repeated the experiment with more patient cells using

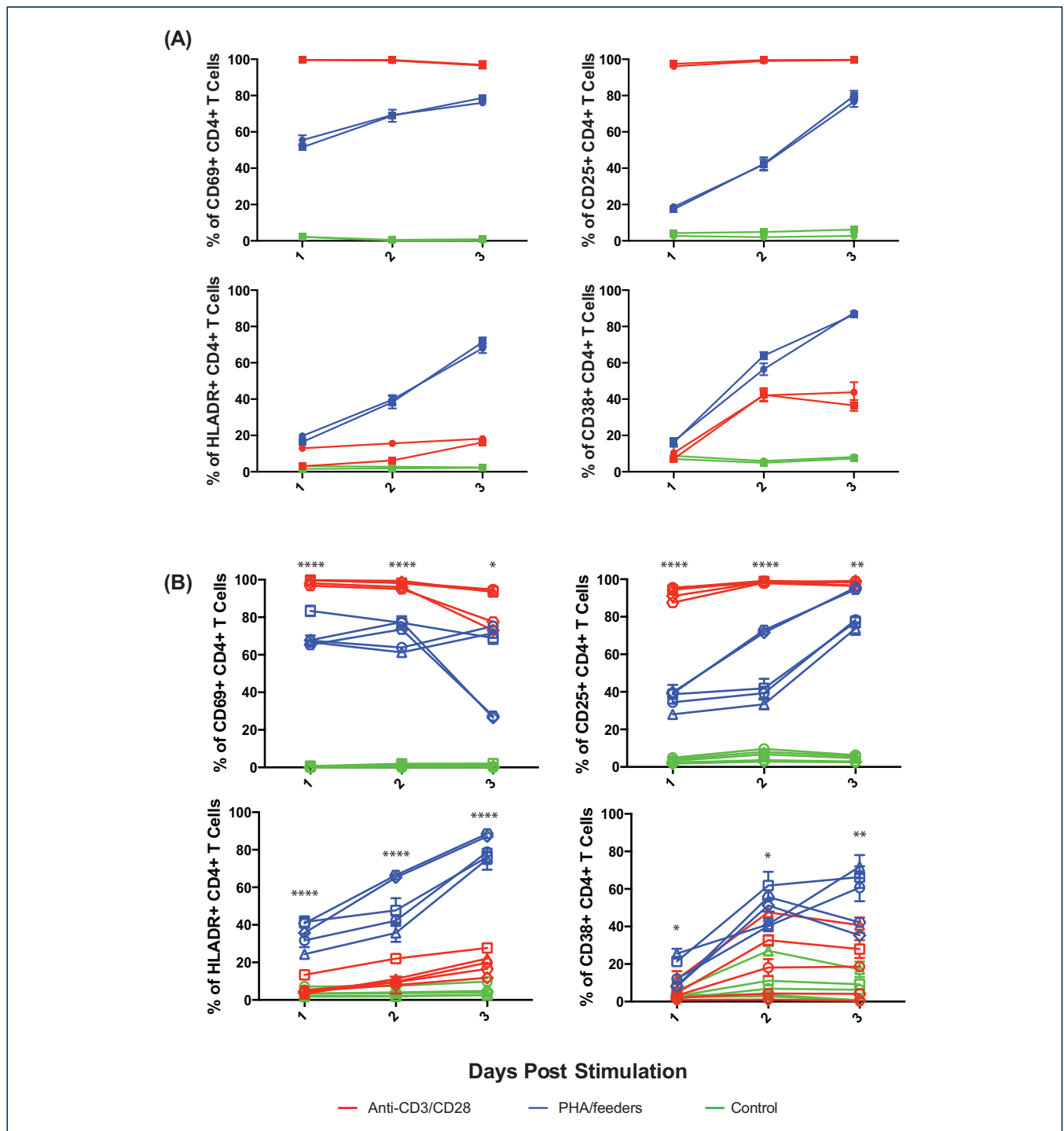


Figure 1. Stimulation of lymphocytes with anti-CD3/CD28 microbeads exhibits faster activation of resting CD4 T cells as compared to the PHA/feeders standard viral outgrowth assay. Resting CD4 T cells from two healthy donors (A) and five patients on suppressive cART regimens (B) were activated with PHA/feeders or with anti-CD3/CD28 microbeads and analysed for the expression of markers of cellular activation by flow cytometry. The amount of activation induced by the two assays are compared with each other. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

20–30 replicates of 1×10^6 resting CD4 T cells from the four patients from whom no virus was obtained in one or both assays (PT8, PT14, PT21 and PT40). While virus was amplified in all four patients with the PHA/feeders assay, no virus was amplified from PT21 and PT40 with the bead-based assay (Figure 2B). The median percentage of positive wells was 19% (range 13–40%) for the PHA/feeders assay and 5% (range 0–10%) for the bead-based assay. IUPMs were calculated using the 2×10^5 cell replicates for the six patients for whom virus was amplified with both assays. The 1×10^6 cell replicates were used to determine the IUPMs for the other four patients. No significant difference was observed between the frequency of latently infected resting CD4 T cells as measured by the PHA/feeders assay compared to the

bead-based assay (Figure 2C, Wilcoxon matched-pairs signed-rank test, $P = 0.5469$). Moreover, there was a significant correlation between the frequency of latently infected cells measured in the two different assays (Figure 2D, Pearson's correlation coefficient, $r = 0.8520$, $P = 0.0017$).

Discussion

The CD3/CD28 bead-based assay represents a less labour intensive way of culturing replication-competent virus. We compared the kinetics of T cell activation and efficiency of viral outgrowth in the two assays. While the bead-based assay induces more rapid stimulation of resting CD4 T cells than the PHA/feeders assay,

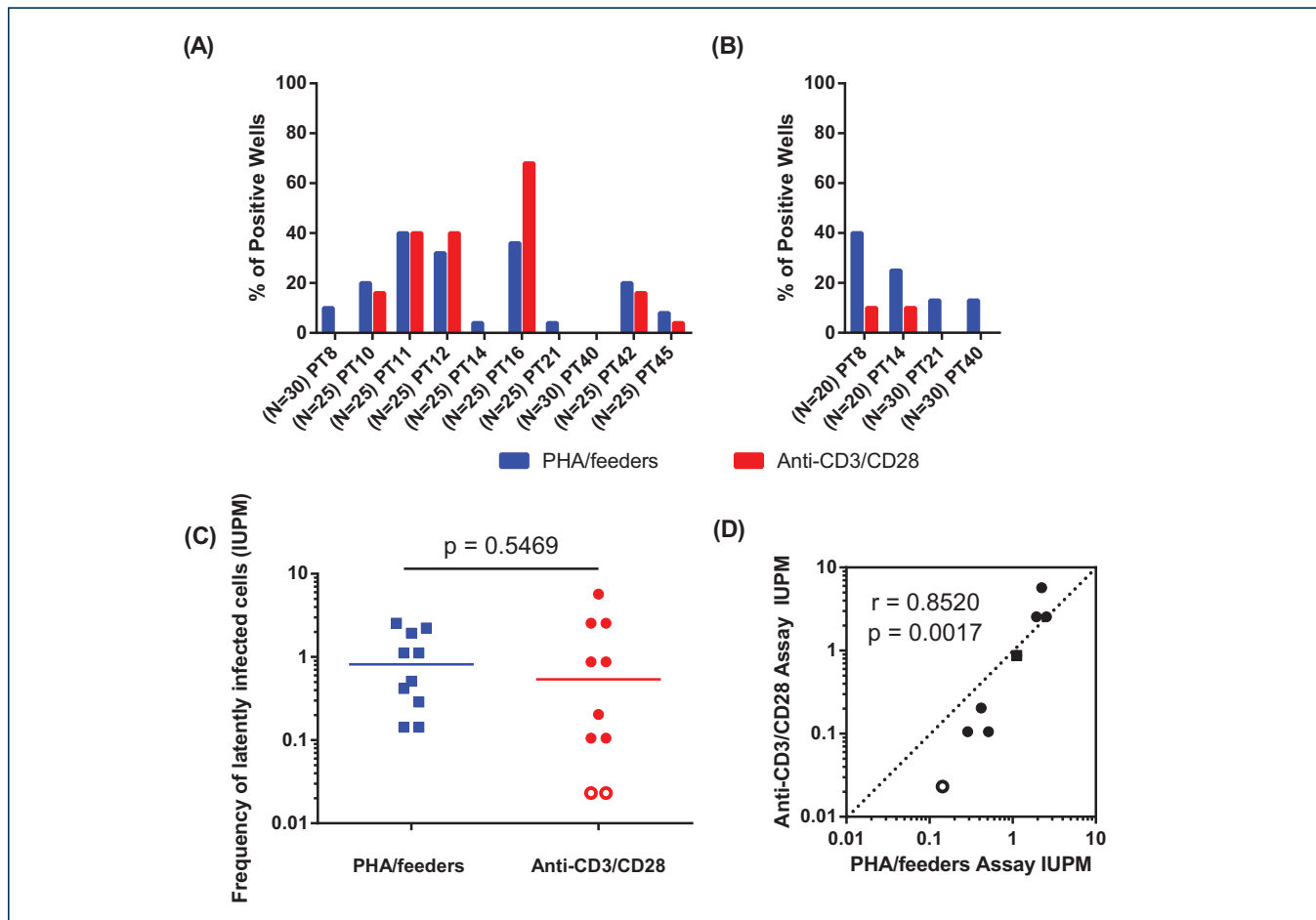


Figure 2. The standard PHA/feeder and anti-CD3/CD28-beads-based viral outgrowth assays yield comparable results. (A) For both assays, 2×10^5 cells were plated side-by-side in appropriate multiwell culture plates. The percentage of wells positive for the p24 antigen is shown. N = number of wells tested for each assay. (B) For both assays, 1×10^6 cells were plated side by side in appropriate multiwell culture plates. N = number of well total for each assay. The percentage of wells found positive for the p24 antigen is shown. N = number of well tested for each assay. (C) Statistical comparison of the infectious units per million cells (IUPM) values using the two assays by Wilcoxon matched pairs signed rank test. Virus was not detected in two patients with the anti-CD3/CD28 bead based assay (PT21 and PT40) and the upper limit of the IUPM for both patients is shown in open circles. (D) The correlation of infectious units per million cells (IUPM) values using the standard PHA/feeder and anti-CD3/CD28 assays (Pearson's correlation coefficient, r). PT21 and PT40 are shown in an open circle. PT42 and PT10 are shown in a square.

the latter assay was more efficient at amplifying virus when replicates of 2×10^5 resting CD4 T cells were tested. The mechanisms responsible for the improved activation kinetics with the antibody coated microbeads compared to PHA and feeders are unclear and it is interesting that faster immune activation did not translate into better latency reversal. Of note, the three patients for whom virus was selectively not amplified with the bead-based assay had relatively low frequencies of latently infected cells in the PHA/feeder assay: just one out of 25 wells was positive for PT14 and PT21 and three out of 30 wells were positive for PT8. When higher numbers of cells were tested, virus was amplified from all four patients using the PHA/feeder assay and two patients with the bead-based assay. Again the percentage of positive wells in PHA/feeder assay in these two patients (PT21 and PT40) was relatively low (four out of 30 wells). Thus, while there was not a statistically significant difference in the sensitivity of the two assays, the PHA/feeder assay appeared to be more effective at viral amplification in some patients. This advantage must be balanced against the fact that the need for feeders and a PHA washout step makes it much more labour intensive and costly than the bead-based assay. The bead-based assay would be particularly convenient when very large numbers of patient CD4 T cells are tested for residual replication-competent virus given the 10 to 1 ratio of feeders to patient CD4 T cells used in the PHA/feeder assay.

An additional measure for simplifying the bead-based assay would be to use a cell line such as MOLT-4/CCR5 cells [10], or SupT1-CCR5 cells [11] instead of lymphoblasts from healthy donors for amplifying virus as previously described for the PHA/feeder assay. This would completely eliminate the need for any healthy donor cells in the culture. It will be interesting to compare the two assays using qPCR [10] or ultrasensitive p24 assays [12] for the detection step instead of the standard p24 assay to determine whether the bead-based assay leads to the production of virus at a level that is simply below the limit of detection of the standard assay. It will also be interesting to determine whether the bead-based assay results in less long-term CD4 T cell death than the PHA/feeder assay. This would make it a better assay to use for the repeated stimulation of CD4 T cells that is needed to trigger replication of non-induced viral clones that are refractory to a single round of T cell activation [13].

In summary, the anti-CD3/CD28 bead-based assay represents an assay that is less labour intensive, but perhaps also slightly less sensitive than the PHA/feeder assay that can be used to measure the latent reservoir in clinical trials of latency-reversal agents.

Acknowledgements

This work was supported by the Johns Hopkins University Center for AIDS Research (P30AI0941890) and the National Institute of

Allergy and Infectious Diseases (2R56AI080328-05A1 and 1R01AI120024-01 JNB).

References

- Eisele E, Siliciano RF. Redefining the viral reservoirs that prevent HIV-1 eradication. *Immunity* 2012; **37**: 377–388.
- Bruner KM, Hosmane NN, Siliciano RF. Towards an HIV-1 cure: measuring the latent reservoir. *Trends microbiol* 2015; **23**: 192–203.
- Finzi D, Hermankova M, Pierson T *et al*. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* 1997; **278**: 1295–1300.
- Siliciano JD, Siliciano RF. Enhanced culture assay for detection and quantitation of latently infected, resting CD4+ T-cells carrying replication-competent virus in HIV-1-infected individuals. *Methods Mol Biol* 2005; **305**: 3–15.
- Wong JK, Hezareh M, Günthard HF *et al*. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science* 1997; **278**: 1291–1295.
- Tremblay CL, Giguel F, Merrill DP *et al*. Marked differences in quantity of infectious human immunodeficiency virus type 1 detected in persons with controlled plasma viremia by a simple enhanced culture method. *J Clin Microbiol* 2000; **38**: 4246–4248.
- Deeks SG, Autran B, Berkhout B *et al*. Towards an HIV cure: a global scientific strategy. *Nat Rev Immunol* 2012; **12**: 607–614.
- Cillo AR, Sobolewski MD, Bosch RJ *et al*. Quantification of HIV-1 latency reversal in resting CD4+ T cells from patients on suppressive antiretroviral therapy. *Proc Natl Acad Sci U S A* 2014; **111**: 7078–7083.
- Li Y and Kurlander RJ. Comparison of anti-CD3 and anti-CD28-coated beads with soluble anti-CD3 for expanding human T cells: differing impact on CD8 T cell phenotype and responsiveness to restimulation. *J Transl Med* 2010; **8**: 104.
- Laird GM, Eisele EE, Rabi SA *et al*. Rapid quantification of the latent reservoir for HIV-1 using a viral outgrowth assay. *PLoS Pathog* 2013; **9**: e1003398.
- Fun A, Mok HP, Wills MR, Lever AM. A highly reproducible quantitative viral outgrowth assay for the measurement of the replication-competent latent HIV-1 reservoir. *Sci Rep* 2017; **7**: 43231.
- Passaes CP, Bruel T, Decalf J *et al*. Ultrasensitive HIV-1 p24 assay detects single infected cells and differences in reservoir induction by latency reversal agents. *J Virol* 2017; **91**: e02296.
- Ho YC, Shan L, Hosmane NN *et al*. Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. *Cell* 2013; **155**: 540–551.