Latently infected CD4+ T cells are a major barrier to HIV eradication [1, 2] but the true size of the inducible HIV reservoir has been challenging to measure [3]. Recent studies have shown that the number of CD4+ T cells with intact proviral DNA far exceeds the frequency of cells that produce infectious virus in the quantitative viral outgrowth assay (QVOA) [4, 5]. One potential reason for this discrepancy is that the activation strategy used in the conventional QVOA does not fully reverse latency. Evidence for this hypothesis comes from studies showing that repeated activation of CD4+ T cells leads to an increase in the number of latently infected CD4+ T cells that produce virus [4, 6]. In this study, we compared different immune activation strategies in order to determine whether these strategies were more effective at reversing latency than the phytohaemagglutinin (PHA) and irradiated feeder combination used in the standard QVOA. We also compared the frequency of cells that produced replication-competent virus with each strategy to the number of copies of intact and total proviral DNA using the newly described intact proviral DNA assay (IPDA) [7]. Our results have implications for HIV cure studies.

METHODS

Patient Cohort
We enrolled HIV-1-infected study subjects on suppressive ART regimens. This study was approved by the Johns Hopkins Institutional Review Board. All patients and HIV-1-negative healthy donors (HD) provided written consent before participating in this study. The clinical characteristics of these patients are shown in Table 1.

Isolation of CD4+ T Cells
Peripheral blood mononuclear cells (PBMCs) from People living with HIV (PLWH) were isolated by Ficoll gradient centrifugation. Bulk CD4+ T cells were isolated from PBMCs by negative selection (Human CD4+ T Cell Isolation Kit, Miltenyi Biotec, Waltham, MA, USA).

PHA/Feeders QVOA
The standard QVOA was performed on CD4+ T cells as described [8, 9]. Cells from each subject were cultured in either a 6-well plate with replicates of 1 × 10^6 cells per well (CP21, CP25, VC20) or a 24-well plate with replicates of 2 × 10^5 cells per well (all other subjects) with a 10-fold excess of irradiated PBMCs (feeders) from a healthy donor and cultured in RPMI 1640 (Thermo Fisher Scientific, Halethorpe, MD, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific), 100 U/mL IL-2 (Chiron, Emeryville, CA, USA), 1% Pen Strept (Thermo Fisher Scientific), 1% T cell growth factor (activation media), and 0.5 μg/mL of PHA (Remel, Thermo Fisher Scientific). PHA was washed off after 16 hours, and 2–4e6
lymphoblasts from healthy donors were added to each 6-well plate well, whereas 0.5–1×10⁶ lymphoblasts were added to each 24-well plate well, as previously described [8, 9]. A second round of lymphoblasts was then added on day 7 after the cultures were split. Supernatants from each well were tested for HIV-1 p24 protein by enzyme-linked immunosorbent assay (ELISA; Perkin Elmer, Waltham, MA, USA) on day 14 and repeated on day 21 of culture. The well size used for each subject was based on prior measurements of the subject’s reservoir size.

**Phorbol 12-Myristate 13-Acetate/Ionomycin QVOA**

CD4+ T-cells from each subject were cultured in a T25 flask at 1–2×10⁶ cells/mL in activation media with 50 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO, USA) and 1 μM ionomycin (Sigma). After 12 hours of stimulation, the cells were washed twice and cultured in either a 12-well plate with replicates of 1×10⁶ cells per well (CP21, CP25, VC20) or a 48-well plate with replicates of 2×10⁵ cells per well (all other subjects); 2–4×10⁶ lymphoblasts from healthy donors were added to each 12-well plate well, and 0.5–1×10⁶ lymphoblasts were added to each 48-well plate well. The second round of lymphoblasts was added on day 7 after the cultures were split. Supernatants from each well were tested for HIV-1 p24 protein by ELISA (Perkin Elmer) on day 14; this was repeated on day 21 of culture. The well size used for each subject was based on prior measurements of the subject’s reservoir size.

**PHA/anti-CD28 QVOA**

CD4+ T-cells from each study subject were cultured in either a 96-well plate with replicates of 2×10⁵ cells per well (CP11, CP70, CP71) or a 48-well plate with replicates of 1×10⁶ cells per well (all other subjects) in activation media containing 1 μg/mL of PHA (Remel) and 5 μg/mL of the superagonistic anti-CD28 monoclonal antibody (Ancell, Bayport, MN, USA clone ANC28.1) [10]. Two days later, the PHA and anti-CD28 were washed off, and the replicates in 48-well plates were transferred into 12-well plates, whereas those from 96-well plates were transferred into 48-well plates. Subsequently, 2–4×10⁶ lymphoblasts from healthy donors were added to each well of a 12-well plate, and 0.5–1×10⁶ lymphoblasts were added to each well of a 48-well plate. The second round of lymphoblasts was added on day 7 after the cultures were split. Supernatants from each well were tested for HIV-1 p24 protein by ELISA (Perkin Elmer) on days 14 and 21 of culture. The well size used for each subject was based on prior measurements of the subject’s reservoir size.

**Flow Cytometry**

To compare the CD4+ T-cell activation levels between the 3 assays, unfractionated CD4+ T cells were isolated from HLA-A2-positive individuals (3 CP and 7 HD). The cells were either untreated or stimulated with PHA/feeder, PMA/ionomycin, or PHA/anti-CD28 as above. On days 1 through 4, cells were stained with the following panel: CD4-PE (BD, Franklin Lakes, NJ, USA), CD25-FITC (BD), CD69-APC (BD), CD38-PE-Cy7 (BD), HLA-DR-PerCP-Cy5.5 (Biolegend, San Diego, CA, USA), HLA-A2-APC-H7 (BD), AnnexinV-PacBlue (BD). Stained cells were analyzed by flow cytometry on a FACS Canto (BD). Allogeneic feeders were obtained from HLA-A2-negative healthy donors for the PHA/feeder experiment, and we gated on HLA-A2-positive cells in order to distinguish between the subjects’ cells and the allogeneic feeders [11].

**Intact Proviral DNA Assay**

The IPDA measures the HIV-1 reservoir by quantifying both defective and intact proviral DNA. The total provirus is the sum of intact, 5’ defective, and 3’ defective virus detected by the IPDA. Aliquots of 5 million unfractionated CD4+ T cells were assayed at Accelevir Diagnostics as previously described [7].

**Data Analysis**

Flow cytometry data were analyzed using FlowJo, version 10. All statistics were generated in GraphPad Prism 7. The Pearson

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**Table 1. Clinical Characteristics of the Subjects in this Study; Total and Intact DNA Values Were Calculated by the Intact Proviral DNA Assay**

| Subject | Current CD4 T-Cell Count, Cells/μL | HIV-1 RNA, Copies/mL | Time on Suppressive Regimen | Current Regimen | Intact DNA, Copies/Million Cells | Total DNA, Copies/Million Cells |
|---------|-----------------------------------|----------------------|-----------------------------|----------------|----------------------------------|-------------------------------|
| CP11    | 1086                              | <20                  | 8                           | FTC, TAF, EVG/c | 82.82                            | 441.07                        |
| CP21    | 525                               | <20                  | 5                           | FTC, TAF, RPV  | 36.95                            | 602.52                        |
| CP25    | 670                               | <20                  | 1                           | FTC, TAF, RAL  | 23.67                            | 532.48                        |
| CP39    | 829                               | <20                  | 1                           | 3TC, ABC, DTG  | 35.55                            | 708.08                        |
| CP67    | 711                               | <20                  | 6                           | FTC, TAF, EVG/c | 13.66                            | 518.33                        |
| CP68    | 599                               | <20                  | 4                           | FTC, TAF, BTG  | 30.62                            | 546.70                        |
| CP69    | 1395                              | <20                  | 2                           | FTC, TAF, EVG/c | 67.90                            | 1348.72                       |
| CP70    | 805                               | <20                  | 8                           | FTC, TAF, EVG/c | 120.16                           | 1004.87                       |
| CP71    | 561                               | <20                  | 8                           | 3TC, ABC, DTG  | 67.13                            | 738.48                        |
| VC10    | 563                               | <20                  | 3                           | TAF, FTC, DTG  | 3.84                             | 129.18                        |
| VC20    | 575                               | <20                  | 6                           | TAF, FTC, BTG  | 1.82                             | 310.96                        |

Abbreviations: 3TC, lamivudine; ABC, abacavir; BTG, bictegravir; CP, chronic progressor; DTG, dolutegravir; EVG/c, cobicistat boosted elvitegravir; FTC, emtricitabine; RAL, raltegravir; RPV, rilpivirine; TAF, tenofovir alafenamide; VC, viremic controller.
Figure 1. Activation marker profiles induced by different activation strategies. Bulk CD4+ T cells were either not treated (NT) or stimulated with phytohaemagglutinin (PHA)/feeders, PMA/ionomycin, or PHA/anti-CD28. Activation was measured by assessing the upregulation of 4 proteins: CD69, CD25, HLA-DR, and CD38. For each individual, the mean value from duplicates was obtained, and the mean values from 7 HDs (A) and 3 CPs (B) are shown. The error bars represent the standard error of means per treatment group per time point. To determine the differences between PHA/feeder stimulated cells and either treated or untreated cells, a 1-way repeated measures analysis of variance was performed.
test was run for all correlation statistics, and the Kruskal-Wallis test with Dunn’s multiple comparison was performed for the frequency of infectious units per million cells (IUPM) values as well as the IUPM/intact provirus ratio. The IUPMs were based on the percentage of positive replicates and were calculated using the IUPMstats calculator [12]. Cultures that had no positive wells were assigned the median posterior estimate of infection frequency.

RESULTS

Different Stimulation Strategies Result in Different Patterns of Immune Activation

We compared the upregulation of activation markers at different time points after stimulation with PHA/feeders vs PHA and an anti-CD28 monoclonal antibody, PMA and ionomycin in CD4+ T cells from healthy donors and chronic progressors on ART. As shown in Figure 1, PMA/ionomycin stimulation resulted in higher and more sustained expression of the early activation marker CD69 than the other 2 strategies. In contrast, PHA/anti-CD28 stimulation resulted in the most efficient induction of CD25 expression. PHA/feeder stimulation resulted in the highest levels of HLA-DR expression, and stimulation with PHA/feeders and PHA/anti-CD28 was more effective at inducing CD38 expression than PMA/ionomycin stimulation. Interestingly, we found that cells stimulated with PHA/feeders had the highest levels of annexin V expression over the first 2 days of culture (Supplementary Figure 1).

Different Stimulation Strategies Result in Similar Levels of Latency Reversal

To determine how effective each strategy was at latency reversal, we stimulated CD4+ T cells from 11 study subjects on ART including 9 chronic progressors (CPs) and 2 viremic controllers (VCs) (Table 1). We compared the frequency of CD4+ T cells that produced replication-competent virus after stimulation with the 3 different strategies in viral outgrowth assays. Replication-competent virus was obtained from CD4+ T cells from 10, 8, and 9 subjects, after stimulation with PHA/feeders, PMA/ionomycin, and PHA/anti-CD28 strategies (Figure 2) respectively. There were no significant differences in the median frequency of IUPM generated with the different immune activation strategies (Figure 2).

Positive Correlation Between Frequencies of Cells Induced to Produce Replication-Competent Virus by Different Activation Strategies

We asked whether there was any relationship between the frequencies of cells that produced replication-competent virus following stimulation of CD4+ T cells with the 3 different strategies. There was a significantly positive correlation of the IUPMs generated after stimulation with PMA/ionomycin and PHA/feeders (Figure 3A), PHA/anti-CD28 and PMA/ionomycin (Figure 3B), and PHA/anti-CD28 and PHA/feeders (Figure 3C).

Relationship Between Intact DNA and Frequency of Cells Induced to Produce Replication-Competent Virus by Each Strategy

The IPDA is a recently developed digital droplet polymerase chain reaction assay that uses primers developed from near full-length proviral sequences to distinguish intact proviral DNA from most deleted and/or hypermutated proviral DNA [7]. The frequency of intact and total DNA for each study subject is shown in Table 1. As the frequency of intact DNA approximates the size of the inducible reservoir, we asked what fraction of cells with intact DNA produced replication-competent virus. As shown in Figure 4 each activation strategy induced replication-competent virus from only a fraction of the cells with intact DNA.

Finally, we asked whether there was any correlation between intact DNA and IUPM for each of the 3 activation strategies. We found a significant positive correlation between the IUPM generated by the 3 different activation strategies and intact DNA (Figure 5A–C) but not total DNA (Figure 5D–F).

DISCUSSION

Reversal of HIV-1 latency will be required for most HIV cure strategies, but recent studies have suggested that only a small fraction of CD4+ T cells that harbor intact proviral DNA produce replication-competent virus upon activation with PHA and feeders [4, 5]. We asked whether other immune activation strategies would reverse latency more effectively. We used a combination of PMA and ionomycin that is often used to produce maximal T-cell activation. A recent study demonstrated that PHA alone is not as effective at reversing latency as the combination of PHA and allogeneic feeders [13], so for
this study we combined PHA with a superagonistic anti-CD28 antibody that provides a co-stimulatory signal and directly activates CD4+ T cells [10]. The pattern of immune activation produced by these different regimens was quite different with PMA/ionomycin inducing the highest levels of CD69, PHA/anti-CD28 inducing the highest levels of CD25, and PHA and feeders causing the highest expression of the late activation marker HLA-DR. Further studies will be needed to determine whether these differences are due to the activation of different subsets of cells and/or due to different mechanisms of action of the different activation strategies. However, in spite of these different patterns of activation, there was no significant difference in the frequency of cells that produced replication-competent virus when the activated cells were co-cultured with lymphoblasts from healthy donors. Furthermore, there was a positive correlation between the frequency of cells producing virus in the 3 different assays.

We demonstrate that PMA/ionomycin and PHA/anti-CD28 stimulation can be used in place of the PHA/feeders activation step that is used in the standard viral outgrowth assay. Other studies have also shown comparable levels of latency reversal when different immune activation strategies are used in viral outgrowth assays [11, 13–16]. Our results extend these studies by also measuring the level of immune activation induced by each strategy. The PMA/ionomycin and PHA/anti-CD28 methods of immune activation do not require a 10:1 ratio of allogeneic feeders to patient CD4+ T cells and thus are less labor-intensive and less expensive. These methods will also be easier to use for the repeated stimulation of CD4+ T cells needed to trigger replication of non-induced viral clones that are refractory to a single round of T-cell activation [4, 6]. A recent study showed lower levels of HIV transcriptional initiation in infected rectal CD4+ T cells compared with infected peripheral CD4+ T cells in patients on ART, suggesting that cells in this compartment may be in a deeper state of latency [17]. Thus, it will be important to compare latency reversal in CD4+ T cells from different tissues with these alternate activation strategies. We also determined the frequency of CD4+ T cells that harbored intact proviral DNA and showed that there was a positive correlation between the frequency of intact proviral DNA and the IUPM induced with each activation method. A comparison of the 2 parameters suggested that <10% of CD4+ T cells with intact proviral DNA produced replication-competent virus with each activation method. This is consistent with a prior study that found that only a very small fraction of CD4+ T cells with proviral DNA could be induced to produce replication-competent virus following stimulation with antibodies to CD3 and CD28 [18]. These data suggest that different strategies that produce global immune activation

![Figure 3. Correlation of quantitative viral outgrowth assay infectious units per million generated by the different activation strategies. Comparisons of phorbol 12-myristate 13-acetate (PMA)/ionomycin vs phytohaemagglutinin (PHA)/feeders (A), PHA/anti-CD28 vs PMA/ionomycin (B), and PHA/anti-CD28 vs PHA/feeders (C) are shown with the Pearson's correlation coefficients. Values that were below the limit of detection were not included in the analysis.](image)

![Figure 4. Ratio of quantitative viral outgrowth assay infectious units per million (IUPM)/intact proviral DNA for each activation strategy. The open symbols indicate assays where there were no positive wells, and the maximum possible IUPM value was divided by the intact proviral DNA frequency.](image)
are inefficient at reversing latency. The Simoa ultrasensitive p24 assay is 1000-fold more sensitive than the standard ELISA we used for this study [19]; thus it is possible that we are underestimating the frequency of cells that produce replication-competent virus. However, in some studies, viral isolates that were positive by the ultrasensitive p24 assay alone were not always capable of replication in subsequent cultures, suggesting that some of these isolates may not be replication-competent [20, 21]. RNA induction assays are also more sensitive than QVOAs [22], but a recent study showed that a large percentage of transcription-competent virions produced after CD4+ T-cell activation are not replication-competent [23]. Further studies will therefore be required to determine what percentage of CD4+ T cells with intact DNA produce replication-competent viruses that are capable of initiating subsequent rounds of replication vs defective or attenuated viruses and/or transcription-competent viruses that are not replication-competent. In summary, our data suggest that different immune activation strategies induce different patterns of activation marker expression on CD4+ T cells and produce replication-competent virus from similar frequencies of latently infected CD4+ T cells. Novel latency-reversal strategies or repeated stimulation may be needed to fully reverse latency in viral outgrowth assays.

**Supplementary Data**

Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copy-edited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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**Figure 5.** Correlations between quantitative viral outgrowth assay infectious units per million (IUPM) from each activation strategy and proviral DNA. IUPM derived from stimulation with phytohaemagglutinin (PHA)/feeders (A, D) phorbol 12-myristate 13-acetate (PMA)/ionomycin (B, E), and PHA/anti-CD28 (C, F) are plotted against intact (A–C) and total (D–F) proviruses per million CD4+ T cells. Pearson’s correlation coefficients are shown.
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