

7-29-2017

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APA Citation

Sung, J., Sholtis, K., Kirchherr, J., Kuruc, J., Gay, C., Nordstrom, J., Bollard, C., Archin, N., & Margolis, D. (2017). Vorinostat Renders the Replication-Competent Latent Reservoir of Human Immunodeficiency Virus (HIV) Vulnerable to Clearance by CD8 T Cells. *EBioMedicine*, (). <http://dx.doi.org/10.1016/j.ebiom.2017.07.019>

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Research Paper

Vorinostat Renders the Replication-Competent Latent Reservoir of Human Immunodeficiency Virus (HIV) Vulnerable to Clearance by CD8 T Cells



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ARTICLE INFO

Article history:

Received 12 April 2017

Received in revised form 19 July 2017

Accepted 19 July 2017

Available online 29 July 2017

Keywords:

HIV

Latency

Eradication

Vorinostat

Immune effector

ABSTRACT

Latently human immunodeficiency virus (HIV)-infected cells are transcriptionally quiescent and invisible to clearance by the immune system. To demonstrate that the latency reversing agent vorinostat (VOR) induces a window of vulnerability in the latent HIV reservoir, defined as the triggering of viral antigen production sufficient in quantity and duration to allow for recognition and clearance of persisting infection, we developed a latency clearance assay (LCA). The LCA is a quantitative viral outgrowth assay (QVOA) that includes the addition of immune effectors capable of clearing cells expressing viral antigen. Here we show a reduction in the recovery of replication-competent virus from VOR exposed resting CD4 T cells following addition of immune effectors for a discrete period. Take home message: VOR exposure leads to sufficient production of viral protein on the cell surface, creating a window of vulnerability within this latent reservoir in antiretroviral therapy (ART)-suppressed HIV-infected individuals that allows the clearance of latently infected cells by an array of effector mechanisms.

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1. Introduction

Antiretroviral therapy (ART) is highly successful at suppressing viral replication in HIV-infected individuals, yet viremia returns following the cessation of therapy due to the persistence of replication-competent human immunodeficiency virus (HIV) in a quiescent, latent reservoir (Chun et al., 1997, 2010; Finzi et al., 1999). Currently, one of the most well-developed strategies for eradication of the latent HIV reservoir hinges on first inducing viral antigen expression through exposure to a latency reversing agent, allowing for detection by immune effector cells, followed by subsequent clearance of the now vulnerable infected cells by the immune response or other therapeutics (Archin and Margolis, 2014).

Inhibitors of histone deacetylases (HDACs) target the formation of a repressive chromatin environment that inhibits long terminal repeat (LTR) expression and viral production, and may be useful in eradication strategies. A significant increase in cell-associated ribonucleic acid (RNA) production following in vivo administration of HDAC inhibitors,

including vorinostat (VOR), to ART-suppressed individuals has been shown in initial proof of concept studies (Archin et al., 2014; Sogaard et al., 2015; Elliott et al., 2014; Rasmussen et al., 2014; Archin et al., 2012). Additional studies have shown that VOR leads to induction of genetically diverse cell-associated RNA that is similar to the diversity found in corresponding deoxyribonucleic acid (DNA) sequences, suggesting activation of transcription from a broad representation of persistent HIV-1 proviruses (Barton et al., 2016).

However, measurements of cell-associated HIV RNA expression are limited in several respects. Such RNA measurements following ex vivo stimulation overestimate the frequency of latent but replication-competent virus (Eriksson et al., 2013). The majority of HIV RNA detected in such assays encode mutations or deletions that render them unable to produce infectious virions, and are therefore clinically irrelevant from the standpoint of eradication efforts (Barton et al., 2016; Bruner et al., 2016; Ho et al., 2013). Cell-associated HIV RNA is thus an imperfect surrogate for infectious virion production.

Further, if immunotherapies are to be used to clear persistent infection then effective latency reversal must be defined as the presentation of viral protein or antigen by the latently infected cell in a sufficient quantity and for a sufficient length of time for cells harboring replication competent virus to be recognized and cleared. As direct measurement of

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rare or low-level HIV-1 protein or peptide production following latency reversal is not achievable with currently available assays, we have developed the latency clearance assay (LCA) as an ex vivo assay to validate that this goal, the opening of a “window of vulnerability” in the persistent, latent reservoir of HIV infection, can be achieved by a selected intervention.

We have described the LCA in earlier demonstrations of the in-vitro efficacy of various immunotherapeutic approaches (Sung et al., 2015a, b). Previously we have shown that over a prolonged co-culture period of 15 days these interventions led to a reduction in the recovery of replication competent virus, we cannot completely exclude the possibility that inhibition of virus spread contributed to the antiviral effect observed. We therefore modified the LCA to include the addition of antiretrovirals and the removal of CD8 T cells from co-culture following a discrete period of time, and now unequivocally demonstrate that VOR produces a window of vulnerability within the latent HIV reservoir. We used multiple effector mechanisms that detect and clear distinct HIV antigens, including autologous CD8 T cells redirected to infected targets expressing HIV-1 Env on the cell surface via bispecific HIV × CD3 DART® molecules (Sung et al., 2015b, Sloan et al., 2015), and autologous HIV-specific T cells that had been expanded ex vivo (HXTCs) (Sung et al., 2015a; Lam et al., 2015), and target HIV-1 peptides presented by major histocompatibility complexes (MHC) on the cell surface. We show that after VOR induction of viral antigen expression from the autologous resting CD4 T cells of ART suppressed, HIV infected individuals can be detected and cleared by effectors. These approaches are now entering clinical proof-of-concept studies.

2. Materials and Methods

2.1. Participants

This study was reviewed and approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board, and written informed consent obtained from each participant. The study was performed in accordance with the Declaration of Helsinki. Participants were drawn from cohorts of HIV-infected individuals on stable, suppressive ART with undetectable plasma viremia (<50 copies/ml) for at least 24 months.

2.2. Latency Clearance Assay (LCA)

The LCA was performed as previously described (Sung et al., 2015a, b) with the following modifications. Briefly, resting CD4 T-cells were isolated from a leukapheresis product by negative selection and exposed to VOR (335n; gift of Merck Research Laboratories) at a concentration of 5 million/ml in the presence of the antiretrovirals abacavir (4 μM) and raltegravir (1 μM). After 6 h, cells were extensively washed, and effectors added at an effector:target ratio of 1:10. Antiretrovirals were again added, and cells were co-cultured at a concentration of 5 million resting CD4 T cells/ml. After 24 h, cells were extensively washed and depleted of CD8 T cells via three serial magnetic selections (StemCell Technologies, Vancouver, BC). Remaining CD4 T cells were recounted, then plated in 16 to 48 replicate wells at 0.25 to 1 million cells/well (depending on cell availability and reservoir size) and cultured with allogeneic CD8-depleted peripheral blood mononuclear cells (PBMCs) from an HIV-negative donor to amplify any residual virus. The presence of HIV Gag p24 antigen was measured on day 15, and confirmed on day 19 for each well by ELISA (ABL, Rockville, MD). Each LCA was performed at an independent time point.

2.3. Reagents and Generation of HXTCs

HXTCs were generated as previously described (Sung et al., 2015a; Lam et al., 2015). Briefly monocytes were isolated from peripheral blood mononuclear cells (PBMCs) by plastic adherence. Dendritic cells

were generated using standard cytokine cocktails of IL-4, GM-CSF, IL-1β, IL-6, and TNF-α. Non-adherent PBMCs were cryopreserved and thawed upon completion of dendritic cell generation. PBMCs were stimulated with irradiated autologous dendritic cells pulsed with Gag, Pol and Nef peptide pools in the presence of IL7, IL-15, and IL12 for an initial stimulation. Cells were then co-cultured with autologous PHA-treated blasts pulsed with Gag, Pol and Nef peptide pools in the presence of IL-15 and IL-2 for a second and IL-2 for a third round of stimulation. HIV peptide pools comprised 150, 15-mer peptides providing broad coverage of Gag p24, Pol, and Nef across clades (JPT Peptide Technologies). Irradiated K562 cells modified to overexpress the co-stimulatory molecules CD80, CD83, CD86 and 4-1BBL were used in the third stimulation. Cells were cultured in the presence of indinavir and raltegravir (Merck, Kenilworth NJ) to prevent replication of HIV.

HIV DART molecules (provided by MacroGenics) were used as previously described (Sung et al., 2015b; Sloan et al., 2015).

2.4. HIV-specific Responses

IFNγ ELISpot assays were used to assess for the presence of HIV-specific responses in PBMCs from participants. Cryopreserved PBMCs obtained from the same leukapheresis procedure that provided PBMCs for the LCA were thawed and resting overnight for 16–24 h in RPMI with 10% FBS, 5% Pen/Strep. Cells were then harvested, recounted, and plated at 4×10^5 cells/well in triplicate and stimulated with peptide pools at a final concentration of 2 μg/ml for 18–20 h. For each assay, four negative control wells (no peptide, ‘Mock’) and two positive control wells (PHA at a final concentration of 10 μg/ml, Sigma-Aldrich) were included. A threshold of $4 \times$ Mock and >50 SFU/ 10^6 was used to determine positive responses, as previously reported by others (Goonetilleke et al., 2006, 2009).

Clade B consensus peptide pools for Gag, Nef and Pol were obtained from NIH AIDs Reagent program, Division of AIDs, NIAID, NIH.

2.5. Statistical Analysis

Statistical comparisons between groups were analyzed using the Kruskal-Wallis or Friedman nonparametric test with Dunn's posttest correction for multiple comparisons. Fisher's exact test was used for individual participants. $p < 0.05$ was considered significant.

3. Results

3.1. Participants

The 15 participants studied were all suppressed for at least 2 years, with a median duration of suppression of 5.5 years (range 2–8.5) and a wide range of latent reservoir sizes as measured by the standard quantitative viral outgrowth assay following maximal mitogenic stimulation (QVOA; median 0.76, range 0.04 to 2.30 infectious units per million resting CD4 T cells; Table 1), reflecting the spectrum of chronic HIV-1 infection.

3.2. VOR Induces MHC Presentation of HIV Peptides Detectable by CD8 T Cells

An LCA was performed for each individual participant independently, as shown in Fig. 1. Following a 6 h exposure of resting CD4 T cells to 335 nM of VOR, chosen to mimic physiologically achievable levels in vivo following both single and multiple doses of vorinostat, which maintain similar PK parameters (Archin et al., 2012; Archin et al., 2014, 2017), the frequency of cultures in which p24 was detected following VOR exposure is significantly greater than that in cultures treated with only 2 U/ml IL2 (no stimulation; Fig. 2A). As measured by a reduction in the recovery of virus from latently infected cells, VOR induced the expression of HIV to an extent sufficient to allow clearance

Table 1
Demographics of participants and frequency of latent infection.

| Participant | Symbol | Nadir CD4 count | Current CD4 count | Viremia suppression (years) | IUPM ^a | Number cells assayed with VOR/group (million) |
|-------------|--------|-----------------|-------------------|-----------------------------|-------------------|---|
| CP1 | ▲ | 508 | 960 | 3.5 | 0.04 | 34 |
| CP2 | ■ | 480 | 766 | 5.5 | 0.05 | 24 |
| CP3 | ● | 49 | 610 | 8 | 0.10 | 12 |
| CP4 | ◆ | 499 | 706 | 2 | 0.28 | 12 |
| CP5 | ▼ | 277 | 615 | 7.5 | 0.37 | 24 |
| CP6 | ⊙ | N.A. | 704 | 8.5 | 0.37 | 8 |
| CP7 | ◇ | 373 | 391 | 3 | 0.38 | 10 |
| CP8 | ⊖ | 195 | 886 | 8.5 | 0.76 | 30 |
| CP9 | ⊠ | 338 | 857 | 4 | 0.891 | 9 |
| CP10 | ▲ | 81 | 619 | 6.5 | 0.97 | 12 |
| CP11 | ▼ | u/k | 491 | 8 | 1.05 | 12 |
| CP12 | ▽ | 78 | 557 | 8.5 | 1.15 | 9 |
| CP13 | △ | 604 | 1302 | 2 | 1.17 | 24 |
| CP14 | ○ | 243 | 439 | 4.5 | 1.67 | 12 |
| CP15 | □ | 526 | 850 | 2.5 | 2.30 | 18 |

CP = Participant treated in chronic, progressive HIV infection.

^a Infectious units per million resting CD4 cells (IUPM) determined by Quantitative Outgrowth Assay (QVOA (Lam and Bollard, 2013)).

by ex vivo autologous CD8 + T cells. Addition of ex vivo autologous CD8 + T cells at an effector to target ratio of 1:1 led to a significant reduction in the percentage of p24 + wells recovered, with complete ablation of viral recovery in 7 of the 9 participants studied at this ratio (Fig. 2A, $p = 0.001$ by Kruskal-Wallis test with Dunn's posttest correction for multiple comparisons). This effect was dependent on CD8 T cell frequency; while there was a reduction in the percentage of p24 + wells recovered with the addition of autologous CD8 T cells at an effector:target (E:T) ratio of 1:10, this did not reach significance (Fig. 2A).

The QVOA is designed to detect the presence of absence of HIV-1 p24 within each well as a qualitative measure, rather than as a quantitative measure within each well (Archin et al., 2008). Similar to what we have seen with the traditional QVOA when PHA or VOR are used as the stimulus, in which the presence of reactivated virus in a well leads to robust viral replication and spread over the two week period that exceeds the quantifiable limits of the assay, positive wells in the LCA were also above

the limit of quantification of the assay. This was true for all conditions tested; although the frequency of positive wells differed, the ability of HIV to spread within wells that were positive, as reflected by the high quantity of p24 produced, was unaffected. This is what would be expected from an effect that takes place after virus reactivation but before virus spread.

A potential approach to increasing the frequency of effective HIV-1 specific T cell immune responses is through the use of adoptive T cell therapy with ex-vivo expanded HIV-1 specific T cells (Lam et al., 2015; Sung et al., 2015a; Lam and Bollard, 2013). HIV-specific ex vivo expanded T cells (HXTCs; (Sung et al., 2015a)) are currently under clinical evaluation in ART suppressed, HIV-infected individuals (clinicaltrials.gov NCT registration number pending). HXTCs were used as effectors in the LCA, to illustrate the relevance of VOR activity in the context of a more potent antiviral response (Fig. 2B). In three participants with an incomplete reduction in virus recovery with CD8 T-cells at an E:T ratio of 1:10 (participants CP1, CP6, and CP9), the addition

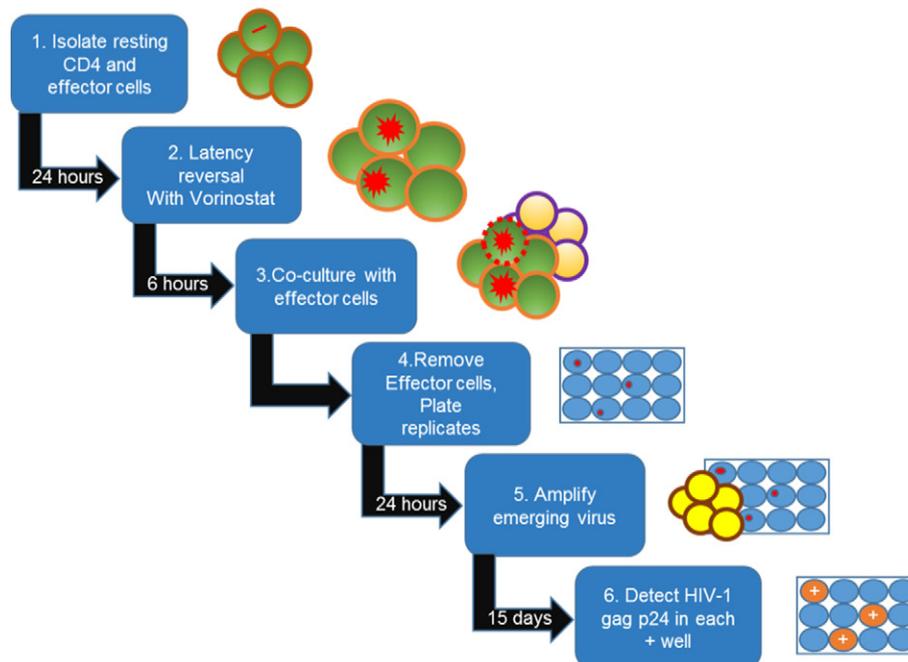


Fig. 1. Latency clearance assay overview.

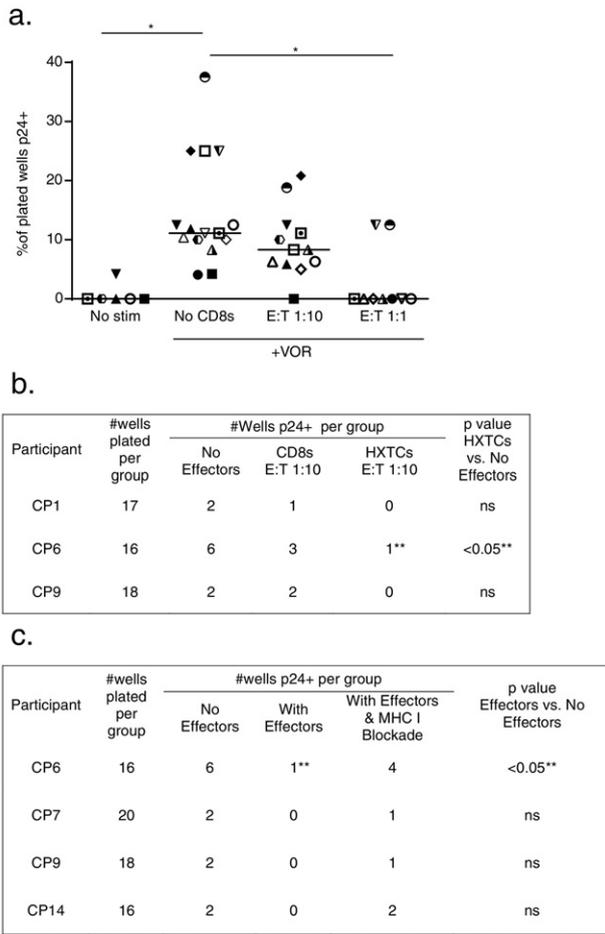


Fig. 2. VOR induces antigen detectable via TCR:MHC interactions. Resting CD4 T cells are exposed to 6 h of 335 nM of VOR, and then the LCA is performed (A) with or without unmodified, autologous CD8 cells at an E:T ratio of 1:10 or 1:1, (B) with unmodified, autologous CD8 cells or ex-vivo expanded HIV specific T cells (HXTC) at an E:T ratio of 1:10, or (C) with or without effectors in the presence or absence of MHC-I blockade (C). Results are shown as % of plated wells HIV Gag p24 positive ((#positive wells × 100) / total #plated wells). *Indicates p < 0.01 by Kruskal-Wallis with Dunn's posttest correction for multiple comparisons. **Indicates p < 0.05 by Fishers exact test compared to cultures without CD8 cells.

of HXTCs at the same E:T ratio of 1:10 led to a further reduction in virus recovery (Fig. 2B). In contrast, no reduction in virus recovery was seen with the addition of cells expanded ex-vivo against irrelevant, Flu-EBV-CMV antigens (irr (EBV)XTC) in the participant CP1, for whom an irr (EBV)XTC line made (same number of positive wells in the control with no CD8 T cells added and with irr (EBV)XTC added), indicating the observed reduction with HXTCs was HIV-specific.

The overall observed reduction in virus recovery by HXTCs at an E:T of 1:10, and CD8s at the E:T ratio of 1:1, is thus most likely reflective of induction of antigen detectable through a TCR:MHC interaction following VOR exposure. Demonstrating that this antiviral effect was the result of an HIV-specific response, MHC I blockade led to a substantial block of HXTC and CD8 mediated reduction in virus recovery (Fig. 2C). The observed reduction in virus recovery was not completely reversed, however, which could either be from incomplete blockade of MHC I given the high concentrations of cells used in the assay, the presence of MHC II mediated activity, or partial contribution of non-specific antiviral effects. However, the majority of the activity was reversed, confirming that the observed reduction in virus recovery was dependent on TCR: MHC interactions, at least in the 4 participants with sufficient cells to test at this condition.

3.3. CD8 T Cells From all Participants Show Detectable HIV-specific Responses

Frozen PBMCs from 13 participants were available to evaluate for the presence of HIV-specific responses, as measured by IFN γ Elispot. A conservative threshold for positive responses as >50 SFU/10⁶ PBMCs and >4× the Mock control was employed, based on prior literature (Goonetilleke et al., 2009; Goonetilleke et al., 2006; Liu et al., 2013). Using this criteria, PBMCs from all 13 participants demonstrated responses to at least one of the three tested peptide pools (Gag, Nef, or Pol) (Fig. 3). Negative responses were only seen to the Nef peptide pool (in two participants); all 13 participants demonstrated a positive detectable response to the Gag and Pol peptide pools, perhaps reflecting the more conserved nature of these proteins. A range of magnitudes of positive responses were observed across participants: 95 to 942 SFU/10⁶ PBMCs to the Gag peptide pool, 59.5 to 807 SFU/10⁶ PBMCs to the Nef peptide pool, and 62.8 to 1279 SFU/10⁶ PBMCs.

3.4. VOR Induces Cell Surface Expression of Env, Detectable by DART Molecules

We also utilized HIV DART molecules (Sung et al., 2015b) to assess the LCA in the context of an alternative clearance approach. HIV DART molecules are antibody-based bispecific proteins that redirect polyclonal T cells to HIV-1 Env-expressing cells. We tested DART molecules with an HIV-specific domain that is based on the variable region of the broadly binding antibodies 7B2 (directed towards a gp41 epitope) or A32 (directed towards a CD4 induced gp120 epitope) (Acharya et al., 2014; Pincus et al., 2003). Thus, CD3 + effector cells are recruited to bind and kill HIV Env-expressing cells regardless of underlying TCR specificity. We have previously shown that HIV DART molecules are capable of redirecting T cells against autologous reservoir virus as well as against rare latent infection in resting CD4 + T cells (Sung et al., 2015b). Here, with the additional step of depletion of CD8 T cells prior to addition of allogeneic target cells, we show that HIV DART molecule-mediated redirection of effector T cells directly recognizes and clears latently infected cells expressing HIV Env in the 24-hour period following VOR exposure. Even at the E:T ratio of 1:10, the addition of HIV DART molecules to CD8 + T cells led to a significant reduction in recovery of p24 + wells (Fig. 4, p < 0.05 by Kruskal-Wallis with Dunn's post-test correction). In comparison, CD8 + T cells alone at an E:T ratio of 1:10 were unable to significantly reduce recovery of p24 + wells in this subset of 6

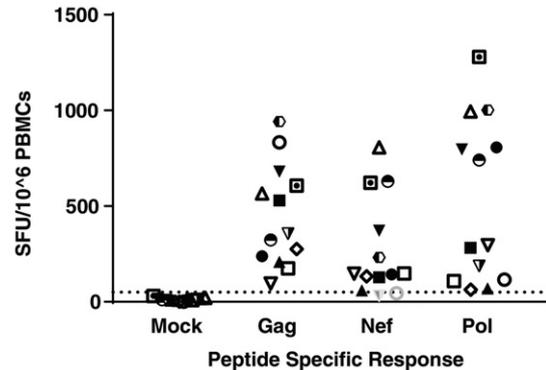


Fig. 3. HIV-specific responses of participants. PBMCs from 13 of the participants who had sufficient cells cryopreserved and stored for analysis from the same leukapheresis product used in the latency clearance assay were thawed and resting overnight. Cells were counted and plated in triplicate at 400 k cells/well on an IFN γ coated plate and stimulated with 2 μ g/ml of the indicated Clade B consensus peptide pool (Gag, Pol, or Nef or media alone for the Mock control). After 16 h of culture, Elispot plates were washed and incubated with secondary anti-IFN γ antibody for 24 h, after which plates were developed and spots counted. Results are shown as SFU per million PBMCs. A threshold of >4× Mock and >50 SFU/10⁶ were considered positive (dotted line). Detectable but negative responses by this threshold are shaded in gray.

of antigen production that might be relevant for viral clearance strategies. We used multiple tools and controls to interrogate the ability of VOR to induce detectable antigen expression, with consistent results across these approaches and conditions.

In summary, we have demonstrated that VOR is capable of inducing HIV MHC:peptide as well as Env antigen expression on the cell surface of latently infected, resting CD4 cells from ART suppressed individuals at levels sufficient for clearance by a variety of effector mechanisms. The same methods can be adapted to further understand the kinetics of latency clearance, modeling exposures to an array of LRAs (or combination approaches) and effector mechanisms. Such efforts will be essential for the rational design of optimal combination latency reversal and clearance strategies. Our ongoing clinical trials will attempt to validate the LRA and clearance activities measured in the ex vivo LCA. Latency clearance activity observed in this study suggests that HDAC inhibitors such as VOR may prove useful when used in appropriate combination with immune clearance strategies. Further, these findings underscore the potential value of evaluating latency reversing agents within the context of viral clearance ex vivo, prior to ultimate in vivo testing.

Conflict of Interest Statement

The authors report no conflicts of interest.

Funding

This work was supported by National Institutes of Health grant numbers U19-AI096113 and UM1AI126619 to DMM and the Collaboratory of AIDS Researchers for Eradication, R01 HL132791 to DMM and CMB, KL2 TR001109 to JMS, P30 CA016086 Cancer Center Core Support Grant to the UNC Lineberger Comprehensive Cancer Center for the UNC Flow Cytometry Core Facility, and the UNC CFAR P30 AI504100.

Author Contributions

JS wrote the manuscript and designed, supervised and performed experiments. DM, NA, JN, and CB edited the manuscript and provided input into and supervision for experiment design. KS and JK performed experiments and edited the manuscript. JDK and CG coordinated clinical samples and edited the manuscript.

Acknowledgments

We thank CM Rooney and the PACT through Baylor School of Medicine for their generous provision of HXTCs. We thank M Hudgens who provided statistical input. Importantly, we thank the HIV-positive participants involved in this project. JMS, NMA, and DMM designed and conducted experiments. CB provided advice on use of and experimental design involving HXTCs and JLN provided DART molecules and advice on their experimental use. JK and KS provided technical support and JDK and CLG provided clinical study management. JS authored the manuscript and all coauthors edited the manuscript.

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