



Research paper

A targeted reactivation of latent HIV-1 using an activator vector in patient samples from acute infection

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ABSTRACT

Background: During combined anti-retroviral treatment, a latent HIV reservoir persists within resting memory CD4 T cells that initiates viral recrudescence upon treatment interruption. Strategies for HIV-1 cure have largely focused on latency reversing agents (LRAs) capable of reactivating and eliminating this viral reservoir. Previously investigated LRAs have largely failed to achieve a robust latency reversal sufficient for reduction of latent HIV pool or the potential of virus-free remission in the absence of treatment.

Methods: We utilize a polyvalent virus-like particle (VLP) formulation called Activator Vector (ACT-VEC) to 'shock' provirus into transcriptional activity. *Ex vivo* co-culture experiments were used to evaluate the efficacy of ACT-VEC in relation to other LRAs in individuals diagnosed and treated during the acute stage of infection. IFN- γ ELISpot, qRT-PCR and Illumina MiSeq were used to evaluate antigenicity, latency reversal, and diversity of induced virus respectively.

Findings: Using samples from HIV⁺ patients diagnosed and treated at acute/early infection, we demonstrate that ACT-VEC can reverse latency in HIV infected CD4 T cells to a greater extent than other major recall antigens as stimuli or even mitogens such as PMA/Iono. Furthermore, ACT-VEC activates more latent HIV-1 than clinically tested HDAC inhibitors or protein kinase C agonists.

Interpretation: Taken together, these results show that ACT-VEC can induce HIV reactivation from latently infected CD4 T cells collected from participants on first line combined antiretroviral therapy for at least two years after being diagnosed and treated at acute/early stage of infection. These findings could provide guidance to possible targeted cure strategies and treatments.

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1. Introduction

Despite the success of combination antiretroviral therapy (cART) at reducing HIV viral loads to undetectable levels, cART remains non-curative [1]. During HIV infection, rare integration events of replication-competent virus occurs, and for reasons not fully understood,

remain as transcriptionally silent provirus within regions of host DNA. The resulting viral reservoir is transiently non-replicating, temporarily hidden from immune surveillance and not susceptible to the protective actions of cART [2–5]. Following cART initiation, latent HIV undergoes a multiphasic decay, with the majority of provirus being present in long lived memory CD4 T cells [6,7]. Upon cART cessation, rapid viral recrudescence and resumption of disease occurs.

Many curative approaches during cART have been centered on “Shock and Kill”, where latency reversal agents (LRAs) reactivate transcription and translation of the otherwise latent provirus [8]. In

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Research in context

Evidence before this study

The HIV-1 latent reservoir becomes established early after primary infection and remains despite continuous cART administration. Numerous approaches have sought to induce transcriptional activity to eliminate the HIV-1 latent reservoir, including: HDAC inhibitors, TLR agonists, PKC agonists, BET bromodomain inhibitors, etc. These approaches have had variable degrees of success *in vitro* but are largely ineffective at either the clinical or pre-clinical stages of development. Historic and emerging studies suggest that HIV-1 latency reversal can be achieved via TCR engagement, and by using HIV-derived peptides to stimulate CD4 T cells. Furthermore, it is thought that a significant portion of the latent reservoir might be concentrated in the HIV-specific T cell population. The effect of a polyvalent stimulator capable of *specifically* targeting the predominant reservoir is largely unknown.

Added value of this study

Unlike other latency reversing agents to-date, ACT-VEC is a polyvalent formulation that can specifically target HIV-specific CD4 T cells for transcriptional reactivation. Our study identifies a novel immunotherapeutic strategy that is at least an order of magnitude more potent than other drugs currently under clinical development. Additionally, we show that the inducible HIV-1 proviral reservoir is enriched within HIV-specific CD4 T cells. Our study further elucidates several mechanistic steps that contribute to an optimized latency reversing response via antigen presentation.

Implications of all the available evidence

The ability of HIV-1 to establish latent provirus within the CD4 T cell reservoir is a major reason for virus recrudescence upon cessation of cART. Identifying novel mechanisms to deplete this reservoir are of great research priority, as current methods fail to effectively target latently infected cells for reactivation and eradication. Importantly, our work shows that the proviral reservoir established during acute infection is non-random and enriched for TCRs specific to HIV antigens. Furthermore, we are the first to describe a method to induce HIV latency reversal by targeting HIV-1 specific T cells using an immunotherapeutic approach.

theory an effective LRA therapy should induce de novo HIV-1 protein expression within previously latently infected cells, enabling their immune recognition and destruction, with any released virus neutralized by ongoing cART [9]. Histone Deacetylase Inhibitors (HDACi) are potential LRAs, based on their histone hyperacetylation, chromatin remodeling and pleotropic induction of gene expression. Potency of the HDACi shock can be significantly enhanced when combined with protein Kinase C (PKC) agonists [10]. However, high toxicity/low therapeutic index of most PKC agonists and HDACi, combined with their negative immuno-modulatory effects and lack of specificity has raised questions on their utility [11–13]. The modest effects of these LRAs to impact the reservoir size in clinical studies has renewed the urgent need to develop novel, safe, efficacious and targeted LRA approaches.

CD4 T cell populations are the major proviral reservoir, but only a fraction of these cells harbors inducible, replication competent virus (0.0001%). In fact, more recent studies have suggested that a mere 2.4% of proviruses are intact with the remainder harboring fatal

mutations and deletions [14]. Prior studies have suggested that latent HIV may be concentrated in CD4 T cells bearing T cell receptors (TCRs) specific for HIV antigens [15,16]. During acute/early infection, HIV drives clonal expansion of HIV-specific T cells, thereby providing an abundance of susceptible target cells to fuel exponential increase in HIV load [17]. By extension, in patients starting a cART regimen at acute/early infection, the latent HIV may be housed in the HIV-specific memory CD4 T cells. Interestingly, despite evidence suggesting the latent reservoir is established early after infection, data now supports that within infected individuals, the virus that was replicating immediately prior to suppressive cART may predominate within the HIV-1 DNA reservoir [18,19]. We therefore asked the question, could an immunotherapy based on a heterogeneous, polyvalent, HIV-1 vaccine consisting of HIV virus-like-particles (VLPs) (termed activating vector, ACT-VEC), serve as a highly specific and effective LRA “shock”? In this scenario, ACT-VEC would be presented to HIV-specific memory CD4 T cells, with signaling through the TCR activating transcription of the otherwise latent HIV-1 provirus, ultimately leading to expression of viral proteins and possible release of HIV. To address this question, we recruited HIV⁺ volunteers that were diagnosed and cART treated during acute/early infection (AC-cART). Purified CD4 T cells from these volunteers (AC-cART CD4 T cells) were either presented with a heterologous ACT-VEC VLP formulation or other non-HIV recall antigens by autologous monocyte-derived dendritic cells (MDDCs). We measured T cell stimulation and any HIV RNA production due to latency reversal. PMA/Iono, Bryostatin, various HDACi and non-HIV viral particles were used as LRA controls. These results show that ACT-VEC is a promising immunotherapeutic LRA for the induction of replication competent HIV-1 from latently infected CD4 T cells and that the inducible reservoir may indeed be enriched within HIV-specific CD4 T cells.

2. Materials and methods

2.1. Experimental design

The activator vector (ACT-VEC) LR agent was developed as an HIV-1 curative strategy. Based on previous studies suggesting that HIV-1 preferentially establishes a latent reservoir within antigen-specific CD4 T cells, we hypothesized that an HIV-1 antigenic formulation containing the epitopes that could be presented to cognate TCR bearing T cells, would be a promising transcriptional shock tactic. We therefore generated a highly diverse, polyvalent virus like particle (VLP) formulation by combining the HIV quasi-species from 5 chronic HIV-infected volunteer's plasma samples, taken immediately prior to cART initiation. The objectives of this study were succinct. In the first instance, we aimed to demonstrate a proof of concept that ACT-VEC could efficiently trigger viral LR in purified human CD4 T cells from patients receiving cART and show that the reservoir was indeed concentrated in HIV-specific memory CD4 T cells. Furthermore, we aimed to demonstrate that ACT-VEC induces replication competent virus i.e. the source of recrudescing virus upon cART cessation. Finally, we sought to compare the efficacy of ACT-VEC mediated LR to that induced by other LRAs such as HDACi and PKC agonists. To achieve the objectives of the study, we recruited cART-treated individuals that were diagnosed with HIV at acute/early stage of infection and who were able to durably suppress viremia. The recruited individuals consented to supply a large blood draw from which we could evaluate ACT-VEC LR on CD4 T cells *ex vivo*.

3. Ethics statement

HIV-infected individuals were recruited from the HIV adult clinical services at St Mary's Hospital (Imperial College NHS trust), through a protocol approved by the NHS Health Research Authority (protocol number: 14SM1988). Peripheral blood was obtained from

infected patient volunteers who had suppression of viremia to <50 copies HIV-1 RNA/mL for > 6 months on ART. All study subjects used in this manuscript were adults and gave prior written informed consent.

4. Virus like particle (VLP) latency reversal agents and their production

A highly diverse virus-like particle (VLP) formulation was constructed from the serum of HIV+ subtype B patient volunteers, identified and receiving cART at chronic phase of infection (isolated from patients infected for more than 1 year). Briefly, viral RNA was isolated from a banked plasma sample, prior to cART initiation, using a viral RNA isolation kit (Qiagen, USA). Viral RNA was reverse transcribed (Agilent Technologies, USA) to full length HIV-1 proviral cDNA as two fragments using two reverse primers to generate the 4561 bp 5' ($\Delta\psi$ region - Integrase) and 4880 bp 3' fragment (integrase -3'LTR) regions. The cDNA fragments were then utilized in a nested PCR protocol using 5' and 3' primer sets to create 5' and 3' overlapping fragments. Both the 5' and 3' overlapping primers for the two genomic halves contained mutations generating a AAH > RRK substitution in the Integrase (IN) coding region and destroys IN activity [20]. The 5' fragment ($\Delta\psi$ region - Integrase) was amplified with 5' primer that disrupts the ψ packaging region, preventing genomic RNA encapsidation [20]. The two subsequent (5', 4378 bp and 3', 4628 bp) half genome fragments were transfected into *Saccharomyces cerevisiae* in a 1:1 ratio (wt/wt) with 2 μ g SacII linearized plasmid, pREC Δ gag-U3/URA3. Resulting yeast colonies resulting from a triple recombination/gap repair into the pREC Δ gag-U3/URA3 to produce pREC_nfl_UH8.dS1.IN_{AAH>RRK} were selected on complete medium lacking leucine (C-Leu) plates supplemented with fluoroorotic acid (FOA). It is important to note that the pREC_nfl_UH8.dS1.IN_{AAH>RRK} also lacks the 5' LTR and produces defective gRNA that cannot be packaged (due lack of ψ) and cannot serve as a template for reverse transcription (due to lack of 5' LTR). The recombined plasmid vectors were isolated by yeast mini-prep, transformed into bacteria, purified using Maxiprep (Qiagen, CA) and then used to transfect 293T cells with Fugene 6 transfection reagent (3:1 vol/wt) (Promega, USA) to produce VLPs. Viral VLPs were first purified in cell-free supernatant by centrifugation through 100 KDa MWCO centrifuge tubes (Amicon, USA), before ultracentrifugation at 100,000g (Beckman, USA) with the resulting VLP pellet re-suspended in sterile PBS. To verify VLP particle production, the formulation was confirmed by dynamic light scattering, transmission electron microscopy, p24 production, radioactive reverse transcriptase assay for presence of Rev and Tat, Western blot and cell-cell fusion co-receptor tropism assay VERITROP for presence of Env and its functionality as described previously [20]. We also determined that our VLP formulations were non-infectious by performing a TZM-bl infectivity assay [61]. Following the aforementioned tests to determine safety and purity of our formulation, VLPs are referred to as ACT-VEC.

4.1. Isolation of resting CD4 T lymphocytes and monocyte derived dendritic cells (MDDC)

Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation. CD4 T lymphocytes were enriched by negative depletion (CD4 T cell Isolation Kit, Miltenyi Biotec) using magnetic microbeads. The purity of these cells was analyzed by flow cytometry using a LSR II (BD Biosciences, USA) and FlowJo software (Treestar). To obtain immature DCs, PBMC were cultured for 2 h in a T-75 tissue culture flask (Sarstedt, Ca) and allowed to plastic adhere for 2 h in a 5% CO₂ incubator at 37 °C. The adherent monocytes were washed gently with complete RPMI to remove non-adherent cells. Isolated monocytes were then differentiated into monocyte derived dendritic cells (MDDC) by culturing in complete RPMI

(10%FCS + 2mM L-Glutamine) supplemented with GM-CSF and IL-4 (1000 and 500 U/mL respectively) in T-75 flasks for 6 days with 50% of the media replenished with fresh complete RPMI (IL-4 + GM-CSF) every 3 days.

5. Latency reversal assays

Purified CD4 T cells were co-cultured overnight with day 7, antigen-pulsed, autologous MDDC at a ratio of 1 DC per 4 T-cells. Antigen pulsing consisted of overnight stimulation of MDDC with either 1) media, 2) 5 μ g/mL (based on p24) heterologous ACT-VEC, 3) recall antigen cocktail CTF [250 ng/peptide/mL Flu M2 protein (bei Resources, USA), 2 μ g/mL Tetanus toxoid (Statens Serum Institut, DK) and 5 μ g/mL pp65 HCMV peptide pool (NIH AIDS Reagent Program, USA)]. On the day of MDDC-T cell co-culture either, 1/5000 phorbol 12-myristate 13-acetate (PMA) + Ionomycin (Iono) (ebioscience, Ca), 10 nM PKC agonist Bryostatin-1 (Sigma, USA) or 335 nM Histone Deacetylase inhibitor (HDACi) Vorinostat (Cayman Chemicals, USA), 40 nM Romidepsin (Cedar lane labs, CA) or 30 nM Panobinosat (Sell-ckchem), was added to the remaining un-pulsed MDDC-T cell co-cultures to serve as controls and competitor LRAs. All HDACi and PKC agonists were used at previously published concentrations known to upregulate viral RNA transcripts in primary T cells and cell lines. The fusion inhibitor, Enfuvirtide (T-20) was added after 24 h to all co-cultures at a concentration of 10 μ M to block any re-infection of T cells by the re-activated latent virus. In all cases, stimulated co-cultures were loaded onto IFN- γ ELISpot plates to assess T cell activation.

For studies involving measurements of LR occurring as a result of cell-cell contact or soluble secreted factors, ACT-VEC pulsed MDDC were pulsed overnight before, washing and incubating with/without autologous CD4 T cells in the apical or basolateral chamber of a 0.4 μ m Spin-X Columns (Sigma, Ca). For viral propagation studies, MOLT4 CCR5 (RRID: CVCL_S545) cells were added to the bottom chamber of the Spin-X column to allow the propagation of replication competent virus released from patient CD4 T cells. Supernatants were harvested every 3 days and HIV-1 infection was determined by p24 ELISA (NCI Frederick, USA) and assessed by qRT-PCR. To evaluate the impact of various signal transduction pathways on ACT-VEC mediated latency reversal, 10 nM Dasatinib (Sigma, Ca), 40nM Methotrexate (Sigma, Ca) and 1.7 μ M R7050 (Santa Cruz, Ca) were added to CD4 T cells 6h prior to coculture with ACT-VEC pulsed MDDC. For studies involving the titration of Dasatinib and stimulation with the T cell mitogens, PMA/Iono, PHA-L, and α CD3/ α CD28, purified CD4 T cells were culture in the presence of inhibitor overnight prior to flow cytometric analysis. For inhibition studies involving Pepstatin A (2 μ M, Sigma, USA) and Chloroquine (100 μ M, Sigma, USA), inhibitors were added to MDDC overnight prior to overnight pulsing with ACT-VEC.

5.1. IFN- γ antigen-specific ELISpot assays

Human IFN- γ enzyme-linked immunosorbent spot (ELISpot) assays (Mabtech, USA) were carried out on MDDC-CD4 T cell co-cultures as per the manufacturer's instructions. Briefly, anti-IFN- γ pre-coated plates were washed with sterile PBS and then blocked for 30 min using complete RPMI. Plates were again washed before addition of 1×10^6 cells/mL CD4 T cell. The MDDC stimulated CD4 T cells were then incubated for 16 h to assess the number of HIV-specific CD4 T cells. Unstimulated and PMA/Iono (eBioscience, Ca)-stimulated cells, diluted 1/500 (v/v) in media, served as controls. To detect spots, biotinylated anti-IFN- γ antibody was added at 1 μ g/mL for 2 h before washing and incubating with streptavidin-HRP for 1 h. Plates were washed as described above, and 100 μ l/well of TMB substrate was added.

5.2. Flow cytometry

For latency reversal in J-Lat 6.3 (AIDS Reagent Program; RRID: CVCL_8280) cell studies, cells were seeded overnight, in a 6 well tissue culture plate, at 1×10^6 cells/mL along with the various stimuli. Samples were washed three times using FACS buffer (2.5% FCS in PBS) and then fixed in 1.5% methanol-free paraformaldehyde (Polysciences, USA) in PBS. Samples were analyzed for GFP expression on a FACS LSR II instrument with FACS Diva software. Data analysis was performed with FlowJo (Treestar Inc., OR, USA).

For evaluation of MDDC primary cell maturation, monocytes from HIV negative donors were purified via plastic adherence and matured into MDDCs using IL-4 and GM-CSF as described previously. MDDCs were pulsed using either ACT-VEC VLP, CTF, HIV Consensus B Env/Gag/Pol peptide pools (2 μ g/peptide/mL; NIH AIDS Reagent Program, USA), or remained un-pulsed for the negative control. Cells were stained for the cellular maturation markers CD11c (Clone: 3.9, eFluoro450; eBioScience, Ca), CD209 (Clone: eB-h209, PE-Cy7; eBioScience, Ca; RRID: AB_10853336), CD40 (Clone: 5C3, BV711; BioLegend, USA; RRID: AB_2564212), CD80 (Clone: L307.4, BD Pharmingen, USA), and CD83 (Clone: HB15e, FITC; BioLegend, USA; RRID: AB_314513). Samples were fixed and analyzed as described previously.

For polyfunctional CD4 T cell stimulation studies, purified CD4 T cells were pulsed with the various stimuli for 6h and stained using anti-human CD3 (Clone: SP34-2, APC-H7; BioLegend, USA) and anti-human CD4 (Clone: L200, BV711; BioLegend, USA), anti-human IFN- γ (Clone: 4S.B3, FITC; BioLegend, USA), anti-human IL-2 (Clone: MQ1-17H12, PE-Dazzle 594; BioLegend, USA; RRID: AB_2564090), and anti-human TNF- α (Clone: MAb11, APC; BD Bioscience, USA; RRID: AB_398566). Samples were fixed and analyzed as described previously.

5.3. Detection and quantification of cell-free supernatant HIV-1 mRNA

After 18h of stimulation, HIV-1 mRNA was extracted using a Viral RNA extraction kit (Qiagen, CA) from 0.2 mL of cell-free culture supernatant, which corresponds to 200,000 purified LRA-activated CD4 T cells. cDNA synthesis was performed using AccuScript High fidelity RT-PCR system kit (AccuScript, USA). Real-time PCR was performed using SensiFAST SYBRTM No-ROX kit (Bioline, USA) on a Rotorgene 6000 Real-Time PCR machine (Corbett Research, USA) or QuantStudio5 (Applied Biosystems, Ca). Primers sets are listed in table S1. A 2-step cycling condition was carried out as follows: 1) 95 °C for 2 min, 2) 30 cycles of 95 °C for 5 s and then 63 °C for 30 s. Molecular standard curves were generated using 1/10 serial dilutions of in house prepared pREC_5'LTR-SBF-1 plasmid which has a known concentration and contains an intact 5'LTR and gag sequence from clade B NL4-3. The human TBP (TATA-box binding protein) primer set and control plasmid was used as an endogenous control (a kind gift from Dr Jimmy Dikeakos).

5.4. Verification of LRA-induced HIV-1 RNA by deep gene sequencing

5.4.1. Library preparation for next generation illumina sequencing

Amplicon products from the 5'LTR to Gag were generated from cDNA by external-nested PCR using a hot start polymerase (Platinum Taq, Invitrogen). External PCR primers included 5LTRPA and GAGREV. For the nested, PCR primers with Illumina overhang adapter sequences were used: MiS_PBSdt-F and MiS_GAGdt-R. Amplicons were further produced for the V2-V3 region of Env using the external PCR primers EnvB and ED14. The nested PCR utilized primers with Illumina adapter sequences E80_IlluminaTag and E125_IlluminaTag. PCR cycle conditions were 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1min (external) or 45 s nested, and a final extension of 72 °C for 10min. The PCR products were run

on a 1% agarose gel at 100V for 45 min to verify products of 350 bp (5' LTR-Gag) or 481 bp (Env). Products of the appropriate band size were excised from agarose gel using a QIAquick Gel Extraction Kit. Concentrations were determined using a NanoDrop 2000c Spectrophotometer (Thermo Fischer, USA).

5.4.2. Index PCR for the Illumina MiSeq system

The Nextera XT Index kit system (NexteraXT Index Kit v2 Set A, Illumina) was used to attach the Illumina dual-index sequence adapters to the amplified and purified PCR products using a limited cycle index PCR (KAPA HiFi HotStart, Kappa Enzymes). PCR cycle conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 30s, 55 °C for 30 s and 72 °C for 45 s, and a final extension of 72 °C for 10 min. The PCR products were run on a 1% agarose gel at 100 V for 45 min.

5.4.3. Sample library purification and quantification

All PCR products were purified using Magnetic beads (AMPure XP, Berkin Elmer) with a bead:DNA ratio of 1:1. The DNA was eluted in 50 μ l 10 mM Tris (pH 8.5). Libraries were then fluorometrically quantified (QuantIT PicoGreen, Invitrogen), normalized to 500ng and pooled together.

5.4.4. Illumina sequencing

The pooled samples were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; <http://www.lrgc.ca>) using the Illumina MiSeq (Illumina, Inc., San Diego, CA). The library was run on an Agilent (Agilent Technologies Inc., Palo Alto, CA) High Sensitivity DNA Bioanalyzer chip (Caliper Life Sciences, Mountain View, CA) to assess size distribution and qRT-PCR (Kapa Biosystems, Inc., Wilmington, MA) was used to assess the quantity. The library was diluted to 4 nM and sequenced (according to the Illumina protocol Preparing Libraries for Sequencing on the MiSeq, Rev. C) on an Illumina MiSeq using a 2 \times 300 paired end run with 10% PhiX.

5.4.5. Data analysis

Raw FASTQ paired reads (R1 and R2) files were quality checked with FastQC to visually evaluate the presence of Nextera paired-end adapters and to use a successive trimming strategy for detection and elimination of low quality base calls. Adapter removal and trimming were performed with Trimmomatic using the SLIDINGWINDOW option (cutting the remaining sequence once the average quality within a window of 4 nucleotides falls below a threshold of 20). The trimmed FASTQ outputs were then utilized for mapping against HIV reference genome HXB2 (GenBank accession number K03455) using bowtie2 with local alignment and using an iterative re-mapping strategy to adapt the reference genome to the sample. Finally, the mapping results were visualized with IGV for detection of 5'LTR and the δ S.1 mutation.

For *env* sequence analysis of proviral and induced virus, we analyzed a 277-nucleotide long fragment (HXB2, coordinates 6845-7121), which we subsequently collapsed to identify the unique sequences using the MiCall pipeline (<https://github.com/cfe-lab/MiCall>). The multiplicity values of the sequences were used to calculate the 1% cut off for the total number of input sequences for each sample. The resulting sequences were then defined as "unique sequences". The unique sequences were analyzed using the Los Alamos National Laboratory (LANL) Neighbor TreeMaker tool. The LANL database was used to acquire reference sequences. The output style was modified for clarity, to show symbols using Powerpoint.

6. Statistical analysis

Mann-Whitney non-parametric, or Wilcoxon matched pairs signed rank test was used to determine intra- and intersample statistical significance where indicated. We considered $P < 0.05$ to be

statistically significant. Correlations between sample conditions were evaluated using a two-tailed Spearman Rank Correlation where indicated. HIV-1-infected adult volunteers meeting the criteria for sustained viral load suppression (>6 months prior to sample donation) and free from any viral blips were enrolled. As samples from all patients were handled in the same way, there was no randomization or blinding.

7. Results

7.1. HIV[±] volunteer characteristics

The primary objective was to determine if heterologous ACT-VEC VLPs are an effective LRA for CD4 T cells derived from HIV⁺ volunteers ($N=9$, mean age of 38), diagnosed and receiving cART at acute/early stage of infection (AC-cART patients) (Table 1). The average time between the estimated date of HIV seroconversion (EDS) and diagnosis was 69 days (range = 6.5–207.5 days) while the time between HIV diagnosis and first line cART initiation was 32 days (range = 18–54 days). During cART treatment, volunteers rapidly suppressed viral loads to <50 copies/mL and maintained suppression throughout the study (Fig. 1). Average duration of cART treatment (with undetectable viremia) prior to PBMC sampling for this study was 884 days (range = 618–1206 days). At the time of PBMC sample collection, the CD4 nadir ranged from 456 to 1179 cells/ μ l (mean = 772 cells/ μ l). All volunteers were Hepatitis B and C negative at enrollment (Table 1). The mean frequency of HIV DNA⁺ cells, representing the full spectrum of the proviral reservoir (i.e. replication competent, replication defective and graveyard viruses) was determined to be 1/4133 CD4 T cells by qRT-PCR.

7.2. ACT-VEC can cause HIV-1 latency reversal in CD4 T cells

During acute HIV infection, the population of CD4 T cells with TCRs specific to viral antigens expands significantly. These activated CD4 T cells either: 1) provide help for anti-viral immune responses and eventually undergo caspase 3-mediated apoptosis, 2) revert to a resting memory phenotype and avoid apoptosis, 3) become productively HIV-infected and die via viral cytopathic effects (vCPE) or cytotoxic responses or 4) become latently infected during the transition into a memory phenotype [21–26]. Based on these cellular fates and because HIV preferentially infects activated CD4 T cells, we propose that initiation of cART during acute/early infection may trap latent HIV within the HIV-specific CD4 T cell population. Therefore, cellular reactivation via the TCR and associated co-stimulatory molecules should lead to the initiation of multiple intracellular signaling cascades and increased expression of a plethora of cellular transcription factors, which may in turn, lead to mRNA transcription from the HIV proviral DNA [27].

The heterologous ACT-VEC LRAs used in this study are VLPs derived from the quasi-species of patients infected with subtype B HIV-1 [28]. ACT-VEC is morphologically indistinguishable from wild type virus, containing all viral proteins, but is devoid of HIV RNA [28]. Monocyte derived dendritic cells (MDDC), derived from the PBMCs of AC-cART patients, were antigen-loaded with ACT-VEC (ACT-VEC +MDDC) overnight before washing the MDDC and then co-culturing with the autologous purified AC-cART CD4 T cells for three days (Fig. S1). When ACT-VEC+MDDC were co-cultured with AC-cART CD4 T cells, HIV genomic RNA was readily detectable in culture supernatants from each of the nine HIV⁺ donor samples (Fig. 2, A and B), representing an average 7.1-fold increase in HIV RNA compared to that from T cells incubated with unpulsed MDDC (Fig. 2G). To ensure *de novo* HIV production was from only latently infected cells and not due to HIV propagation, HIV entry inhibitor, Enfuvirtide, was added (20 μ M) to all samples to block new HIV infections. A 6.8 and 4.98-fold increase in HIV RNA was released from AC-cART CD4 T cells into

supernatant when they were treated with ACT-VEC+MDDC than when treated with polyclonal T cell activator PMA/Iono, or when MDDC were antigen loaded with a cocktail of CMV peptide, Tetanus Toxoid, and Flu M1 (CTF) (Fig. 2G). To exclude the possibility that ACT-VEC-associated increases in HIV RNA resulted from heightened levels of MDDC maturation, we measured the surface maturation markers CD40, CD80, and CD83 before and after stimulation via flow cytometry. Maturation induction in response to ACT-VEC particles was compared to both i) pre-processed peptide, in the form of Env/Gag/Pol pooled peptide and ii) a mixed peptide/protein formulation of CTF. Compared to CTF, ACT-VEC expressed similar levels of MDDC maturation across all three markers, indicating that any increased detection of HIV RNA in these studies were not, due to drastically differing levels of maturation of the MDDC (Supplemental Fig. 2, A and B). To determine the potential of contaminating CD4⁺ T cells within the plastic-adhered MDDC population, flow cytometry was routinely performed using HLA-DR, CD80, CD83, CD40 and CD11c. In all instances, the purity of the MDDC population 6-days after plastic adherence was between 95% and 99%. As additional non-HIV based latency reversal controls, and to help demonstrate the antigen-specificity of the observed latency reversal associated with ACT-VEC VLP, SIV-mac251 and FIV viral particles were made and both failed to induce detectable amounts of HIV-1 RNA (>30 copies/reaction) within culture supernatants (Supplemental Fig. 4G). This further suggests that only HIV and no other related lentiviruses presented by MDDC, can trigger HIV latency reversal in AC-cART CD4 T cells.

It is important to note HIV-1 RNA was measured in supernatant with a qRT-PCR assay targeting the gRNA 5'LTR region (R-U5) found only within inducible wild type HIV-1 but absent in genomeless ACT-VEC VLPs [28]. In support of this detection specificity, no induced HIV-1 or contaminating ACT-VEC was detected in any HIV⁻ donor control experiments using this qRT-PCR assay (Supplemental Fig. 3, A and E). As a further verification that the HIV-1 RNA detected by qRT-PCR was not derived from ACT-VEC, we RT-PCR amplified and then sequenced the HIV-1 5'LTR region derived from supernatant following latency reversal with the CD4 T cells. Illumina MiSeq confirmed the presence of HIV-1 5'LTR sequences and also the absence of our previously published mutations introduced into the DNA vector used to generate ACT-VEC VLP [28]. In the AC-cART volunteers, only a single HIV-1 sequence was identified per volunteer by sequencing which is consistent with the high levels of conservation of the 5'LTR and low levels of diversity of HIV provirus within acute HIV infections [29].

We suspect that the ability of ACT-VEC to induce latency reversal relates to the specific activation of memory CD4 T cells with TCRs specific to HIV as an antigen. However, HIV-specific memory CD4 T cells may only comprise a small proportion of all memory T cells within infected individuals [15] despite large numbers of these cells being generated during early infection. Our adult participants likely encountered flu, CMV and/or tetanus (as well as other antigens) multiple times during their life through vaccination, infection or environmental exposure. Based on these assumptions and the fact that standard vaccines such as Influenza, Hepatitis B, Pneumococcus and oral cholera vaccine have been described to cause transient elevations in HIV expression within individuals on suppressive antiretroviral treatment, we measured the number of AC-cART CD4 T cells that were responsive to ACT-VEC as well as to common recall antigens, such as CTF [30–35]. Additionally, cells were stimulated with the T cell mitogen, PMA/Iono, to determine the effect of a potent, non-specific activation on latency reversal. We utilized PMA/Iono as our assay positive control over other strong T cell mitogens such as α CD3/CD28, and PHA-L, based on our preliminary findings using flow cytometry (Supplemental Fig. 2, C to E) and IFN- γ ELISpot based protocols (Supplemental Fig. 2, F and G). In these initial experiments, the culminating result demonstrated that PMA/Iono was the most potent control stimulation, followed by α CD3/CD28 and PHA-L. This was

Table 1
Baseline characteristic of study volunteers.

Volunteer	Age	Time between EDS and first and positive test	Days before ART initiation after first positive test	Days on ART prior to PBMC donation	Days on ART until viral suppression	ART regimen since diagnosis	Baseline VL at PBMC donation	Baseline CD4 levels at PBMC donation	HIV-1 DNA frequency in CD4 T cells	Hep B/C Status
P1	33	61.5	34	748	125	Atripla	<20	710	1/400	Negative
P2	45	33.5	45	1206	159	Atripla	<20	684	1/9025	Negative
P3	47	77	22	809	231	Atripla	<20	1179	N.D.	Negative
P4	34	6.5	34	1206	188	Atripla	<20	456	1/1324	Negative
P5	36	26	18	795	116	Atripla	<20	805	1/320	Negative
P6	31	207.5	20	685	139	Eviplera	<20	684	1/715	Negative
P7	41	102	54	618	65	Tenofovir, Emtricitabine, Daunavir	<20	561	N.D.	Negative
P8	25	46.5	40	855	268	Atripla	<20	1038	1/688	Negative
P9	52	61	25	1033	407	Eviplera	<20	1220	1/16458	Negative
Average	38	69	32	884	155	N/A	<20	815	1/4275	Negative

N.D. = Not Determined. DNA frequency could not be determined due to limited sample availability.

Average HIV-1 DNA frequency was determined using the average of all samples for which a value could be determined ($n=7$). Samples labelled N.D. were excluded from this calculation.

Nine HIV-1 infected volunteers were enrolled in this study. Volunteers were identified through the SPARTAC (Short Pulse Anti-Retroviral Therapy at HIV Seroconversion) study and recruited through the HIV adult clinical services at St Mary's Hospital (Imperial College NHS trust). All study participants provided written informed consent. All nine volunteers were recruited based on early HIV diagnosis and treatment with unabated cART-mediated suppression of plasma viremia (<50 copies/ml HIV-1 RNA). The participants, age, date of HIV diagnosis, date of cART initiation, cART regimen, viral loads, CD4⁺ T cell numbers, time of enrolment and HepB/C status were all recorded. The estimated date/time of seroconversion (EDS) was calculated as the midpoint between the most recent negative and the first positive test for patients.

corroborated by findings from other research groups showing PMA/Iono has accelerated latency reversing kinetics when compared to the other stimulations, while reaching a similar level of peak activation [36,37]. As shown, ACT-VEC+MDDC was clearly antigenic when presented to the CD4 T cells with generation of approximately 4-fold more CD4 T cell spot forming units (SFU)/10⁶ cells relative to the untreated controls (average of 891 versus 222 SFU/10⁶, $**p<0.0296$) (Fig. 2, C and D). Stimulation of T cells with CTF+MDDC ($**p=0.0012$) and PMA/Iono ($***p=0.0001$) resulted in 7.31 and 36.4-fold increases in IFN- γ SFU relative to the untreated controls (Fig. 2F) and were stronger stimuli than ACT-VEC+MDDC (1.8 and 9.1-fold increase in SFUs, respectively).

We next measured the ratio of HIV-1 RNA production versus CD4 T cell activation (Fig. 2E and Supplemental Fig. 3C). If latent HIV is housed primarily in HIV-specific CD4 T cells, the virus released into supernatant per activated CD4 T cell should be greatest under the MDDC+ACT-VEC conditioning, which was indeed the case (Fig. 2G). The numbers of ACT-VEC activated CD4 T cells (based on SFU/10⁶) correlated with the induced HIV copy number ($**p=0.0045$) (Supplemental Fig. 4, A and B). Significantly less HIV-1 RNA in culture supernatant was observed per activated CD4 T cell when treated with either unpulsed MDDC, CTF+MDDC, and even PMA/Iono controls (Fig. 2G). As a non-specific CD4 T cell activator, the mitogen PMA/Iono stimulates substantially more cells than either ACT-VEC or CTF and yet, less HIV is released from reactivated latent pool in these PMA/Iono activated T cells. This finding coupled with the inability of CTF to trigger significant HIV latency reversal supports our hypothesis that a sizable reservoir of latent HIV is housed primarily in HIV-specific CD4 T cells of volunteers treated with cART at acute/early infection. Importantly, each of these participants will exhibit differences in viral load (associated with increased transmission risk), infecting viral subtype B isolate, and timing of infection, diagnosis, and treatment initiation. All these factors will result in considerable variability with respect to the amount of latent provirus in each participant. Since we are working with an outbred population, we fully anticipated to see a heterogenous latent HIV-1 pool amongst the volunteers as well as differences in their immune response to the various stimulations; alternatively, a homogenous response would be unlikely within this population. No HIV-specific CD4 T cell activation or induced HIV genome was detectable in CD4 T cells of HIV-naïve donors co-cultured with autologous MDDC pulsed with ACT-VEC (Supplemental Fig. 3, A and E).

7.3. ACT-VEC mediated latency reversal induces replication competent virus

Within HIV infected individuals receiving cART, proviral DNA integrated into host DNA may be inert or can be induced to produce replication competent as well as defective and dead HIV. While reactivation and elimination of all inducible, replication competent provirus will be necessary for a sterilizing cure, any reductions in proviral reservoir might significantly reduce the length of time individuals may be required to take cART for the chances of viral rebound to be diminished. To evaluate whether HIV antigens delivered by ACT-VEC induces replication competent HIV from activated CD4 T cells, we monitored HIV propagation following ACT-VEC stimulation (in the absence of T-20) in an assay where MDDC+ACT-VEC/CD4 T cells were separated by a 0.4 μ m membrane from the permissive MOLT4 CCR5 cells (Fig. 3A). In this assay, HIV produced from activated T cells in the apical chamber can flow through, infect and propagate in the susceptible MOLT4 CCR5 cells in the basolateral chamber. By removing the apical chamber, we can accurately quantify the induced and replication competent virus in the basolateral chamber without a continuous influx of virus from the apical chamber confounding the results. Furthermore, the format of this viral outgrowth experiment enabled us to propagate virus indeterminately, using a low number of stimulated cells (1×10^5 T cells) and without the need for sub-culturing. Culture supernatants were collected every 3 days and assessed for viral RNA using qRT-PCR (Fig. 3B). If replication competent virus was induced by the stimuli and subsequently infected the MOLT4 CCR5 cells within the basolateral chamber, we would expect a marked increase in the amount of viral RNA in culture supernatants between early and later time points. In this outgrowth format, we detected a pronounced increase in HIV-1 RNA via qRT-PCR by day 6, while day 3 was undetectable. This represents 2-3 log amplification of virus. After day 6, there was a marked reduction in detectable viral RNA (Fig. 3B). The detection of virus on day 6 compared to day 3 represented a slight delay when compared to the previous latency reversal ELISpot format (Fig. 2), which can be explained by the greater volume of culture supernatant involved in the assay and the fact that the induced virus is infecting the MOLT4 CCR5 cells. To further evaluate this finding, we conducted latency reversal experiments on samples from two volunteers under sub-culturing conditions (1/3 split) to determine if we could detect viral propagation (Fig. 3C). As shown, we detected significantly increasing levels of HIV-1 p24 and viral RNA in

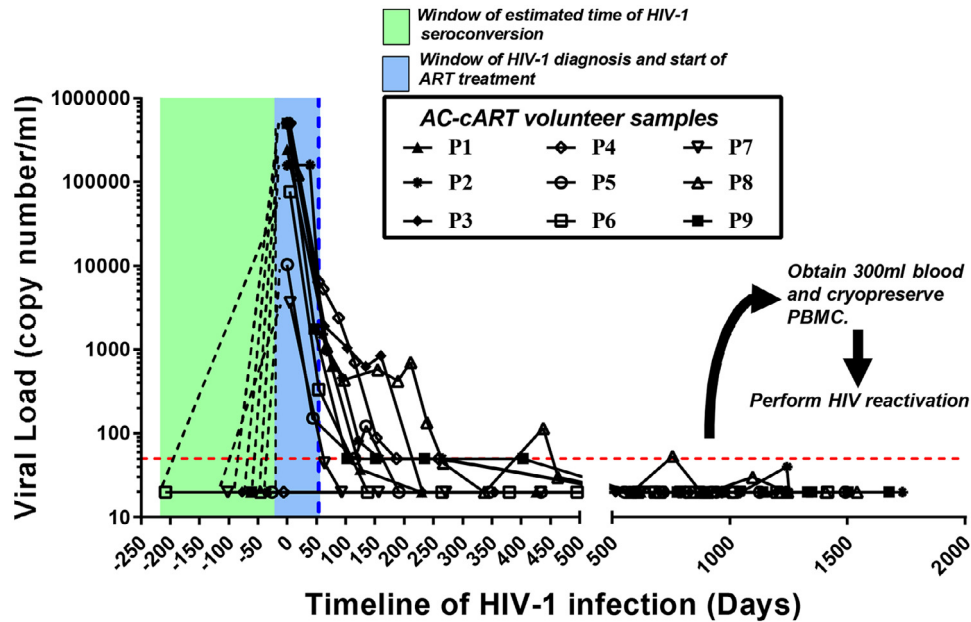


Fig. 1. All nine HIV volunteers enrolled in this study durably suppressed viremia upon cART initiation. A total of nine HIV infected individuals that were diagnosed and treated during acute/early stage of HIV infection were enrolled into this study. Upon diagnosis and initiation of cART, the treated HIV+ volunteers durably suppressed viral replication to <50 HIV copies/mL (---). All volunteers were fully consented to provide a large blood draw (300–500 mL) for PBMC isolation and use in the latency reversal studies. The estimated date/time of seroconversion (EDS) was calculated as the midpoint between the most recent negative and the first positive test for the volunteers and is shown by the green shaded window (■). The time window in which HIV diagnosis and cART treatment occurred is shown by the blue shaded area (■).

culture supernatants over time (** $p=0.0078$), which culminated at days 9 and 12 (Fig. 3, C and D). The increased production/propagation of HIV over time in this sub-culturing experiment suggests ACT-VEC induces infectious virus from the latent pool.

Deep gene sequencing of the propagated virus (C2-V3 region of Env) following ACT-VEC stimulation revealed 2 distinct virus clusters (Fig. 4, A and B). For each of two AC-cART patients, replicate ACT-VEC stimulations ($\times 7$) of a limited number of CD4 T cells resulted in the propagation of a population of related HIV clones as well as some HIV-1 clones unique to only specific replicates. Of note is the identification of cellular proviruses (Red) with genetic homology to the induced viruses (Blue) in both volunteer samples (Fig. 4B). Within each latency reversal assay (LR1–7) and excluding for the possibility that latently infected CD4 T cell clones might be represented more than once in each well [38], if each unique virus was induced from one latently infected CD4 T cell, we can extrapolate the data and estimate the minimal ACT-VEC inducible reservoir to be ~ 14 cells/million. This is substantially greater than the 1 cell/million cited in earlier publications and less than the 60 cells/million that has been estimated in other studies [39–41].

7.4. ACT-VEC is a more promising LRA compared to HDACi's and PKC agonist monotherapies

ACT-VEC as a promising LRA was compared to the clinically relevant LRAs including Panobinostat, Vorinostat, Romidepsin and Bryostatatin (Fig. 5). We initially tested the various HDACi and PKC agonists on the J-Lat 6.3 cell line to verify concentrations of drug that enable detectable latency reversal. As shown, the PKC agonist and HDACi alone or in combination, induced HIV-1 latency reversal in the J-Lat cell line (Supplemental Fig. 5, F and G), consistent with previous studies. However, while we were able to detect latency reversal in the J-Lat cell line, we were unable to detect significant levels of HIV-1 RNA release from AC-cART CD4 T cells treated with HDACi's or Bryostatatin monotherapies. While HDACi and PKC agonists were poor inducers of HIV in primary cells, ACT-VEC+MDDCs ($*p=0.0159$) again showed

significant latency reversal properties (Fig. 5, A and B). Interestingly, ACT-VEC did not induce latency reversal in the J-Lat cell line, which suggests that, by itself, it is unable to induce latency reversal, probably because there is no MDDC-mediated presentation. Of note, Bryostatatin in combination with Panobinostat ($*p=0.031$) or Romidepsin ($**p=0.0079$) was able to induce significant levels of detectable HIV-1 latency reversal, but this was less than observed with ACT-VEC. These findings demonstrate that ACT-VEC treatment of latently infected CD4 T cells is a stronger LRA than the tested HDACi and PKC agonists in primary human cells.

In these studies, ACT-VEC+MDDC was also found to be slightly more antigenic than the unstimulated control and individual HDACi's (Fig. 5, C and D) as measured by IFN- γ ELISpot analyses. However, when comparing Bryostatatin alone ($*p<0.05$) and the combination of Bryostatatin with Romidepsin or Panobinostat, ACT-VEC was found to be less immune-stimulatory for AC-cART CD4 T cells. Again, when ACT-VEC latency reversal is evaluated based on the number of activated CD4 T cells, we demonstrate that ACT-VEC induces substantially more HIV-1 release on a per T cell basis than these HDACi or PKC agonists, suggesting ACT-VEC might be specifically targeting latency reversal in HIV-specific cells.

7.5. ACT-VEC mediated HIV latency reversal is partially controlled by MDDC mediated contact with CD4 T cells and signaling through the Src family member Lck

Understanding exactly how ACT-VEC functions as a latency reversal agent is now a priority, as is determining the fate of the cell reservoir upon ACT-VEC mediated latency reversal. Antigen processing, cell-cell contact, TCR-MHC II and co-stimulatory interactions, as well as soluble secreted factors are all likely to be important mediators involved in latency reversal. With that said, we noted that volunteer P7, whose CD4 T cells were consistently the weakest responders to ACT-VEC + MDDC (Fig. 6A), was the only donor to be on a cART regimen involving a protease inhibitor (PI). The fact that PIs have previously been shown to alter antigen processing and presentation,

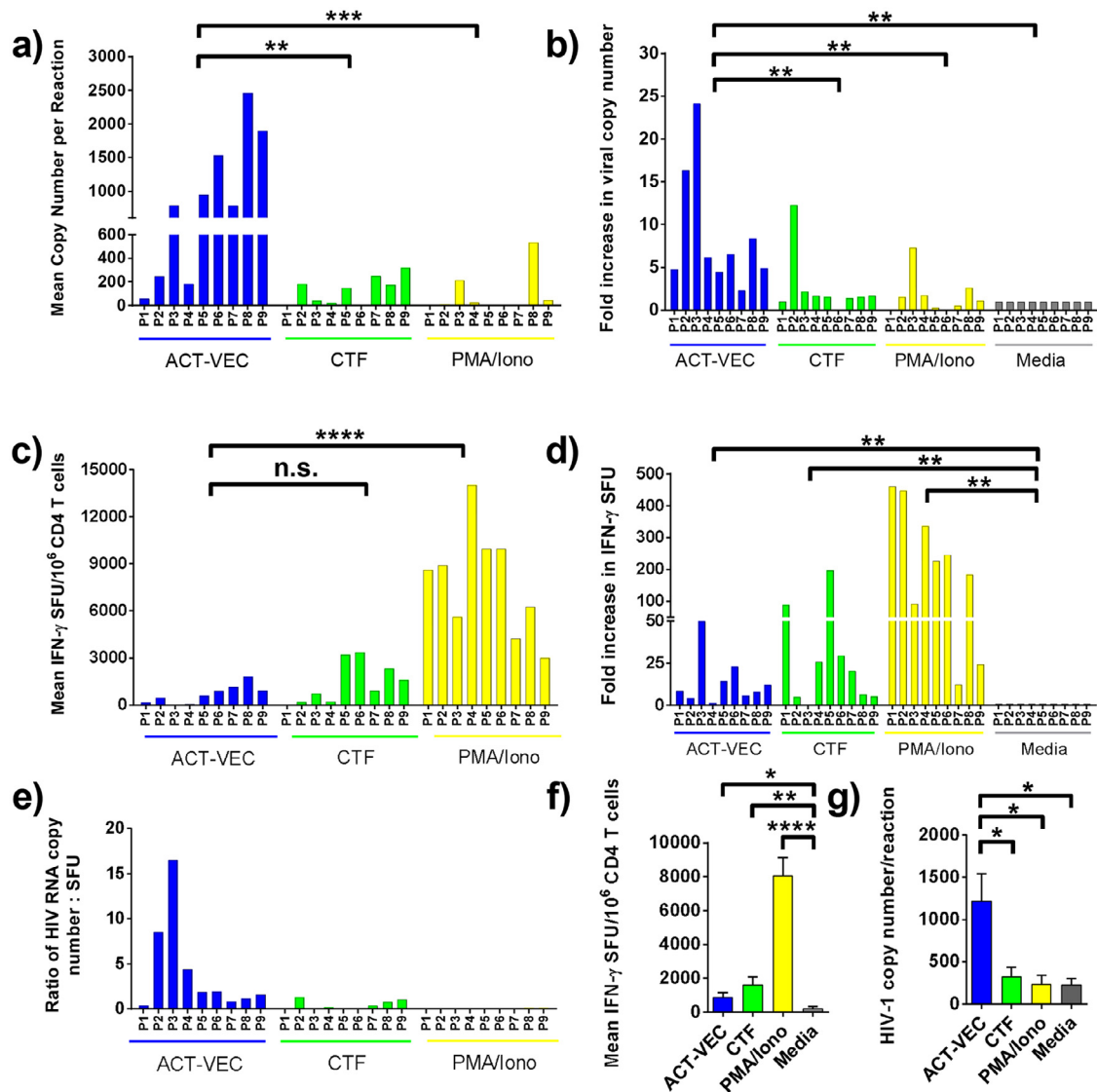


Fig. 2. ACT-VEC induces potent latency reversal within patient-derived CD4 T cells. cART treated HIV+ volunteers ($n=9$) with durable viral suppression (<50 HIV copies/mL) were enrolled in this study. ACT-VEC (5 μ g / mL) or a cocktail of Flu M1, Tetanus toxoid, CMV peptide pool (CTF) were used as HIV-specific or non-specific stimuli to pulse volunteer MDDCs. The pulsed MDDC were then co-incubated with autologous CD4 T cells. PMA/Iono and media served as assay positive and negative controls, respectively. (a) After 3 days of co-culture, mean viral copy number was determined via qRT-PCR from culture supernatants. (b) Copy numbers derived from culture supernatants were represented as fold increase over the media control. (c) Cells stimulated with ACT-VEC, CTF and PMA/Iono were cultured overnight in an IFN- γ ELISpot to determine the CD4 T cell antigenicity. (d) Volunteer-specific IFN- γ spot forming units (SFUs) were counted /10⁶ CD4 T cells, and fold increase calculated over the media control (\pm SEM). Statistical significance was calculated using a Wilcoxon matched pairs signed rank test. (e) The relationship between CD4 T cell activation and the quantity of induced HIV-1 virus is shown. Grouped samples were used to determine the mean effect (\pm SEM) of the stimulations on antigenicity and induced copy number (f and g) with statistical significance ascertained by Mann-Whitney unpaired t -test (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$; **** $P < 0.00005$).

leading to changed frequencies and patterns of MHC displayed peptides and, 2) have reduced HIV reactivation potential, further suggests that antigen presentation and contact between MDDC and CD4 T cells may be important [42–44]. To confirm the role of MHC presentation on T cell activation and subsequent latency reversal, we performed our latency reversal assays in the presence of either the PI, Pepstatin A, or Chloroquine (CQ). Pepstatin A alters antigen processing and abrogates MHC presentation of HIV-specific peptides [45,46]. Chloroquine is a lysosomotropic agent that prevents acidification of the endosome [47], and further prevents the fusion of the endosome and lysosome. This ultimately leads to insufficient peptide processing and poor presentation by MHC complexes. By limiting MHC II presentation of HIV derived peptides with either Pepstatin A or CQ, CD4 T cell activation was reduced by 72.9% and 96.2%, respectively, as compared to ACT-VEC stimulation in the absence of an inhibitor (Fig. 6B). This marked reduction in T cell activation also had implications on latency

reversal, wherein ACT-VEC+PI and ACT-VEC+CQ conditions both reduced HIV copy number by approximately 55% (Fig. 6C). To build upon the previous result, we sought to determine the importance of MDDC - CD4 T cell contact at influencing HIV-1 latency reversal. Therefore, we co-cultured ACT-VEC+MDDC/CD4 T cells or separated the cells using a 0.4 μ m membrane. As expected, we show that MDDC-CD4 T cell contact is important for the high levels of latency reversal and viral budding seen in these studies (Fig. 6, D and E). It is, however, difficult to sort out the master “regulator” of latency reversal by ACT-VEC, considering the multipronged signaling cascade from the TCR, co-stimulatory molecules and cytokine/chemokine receptors following this antigen presentation. This is further complicated by the diversity in proviral integration site, meaning multiple signals are probably required to initiate latency reversal. Despite this, we evaluated the impact on ACT-VEC mediated CD4 T cell activation and latency reversal by using an inhibitor of T cell activation i.e. Dasatinib.

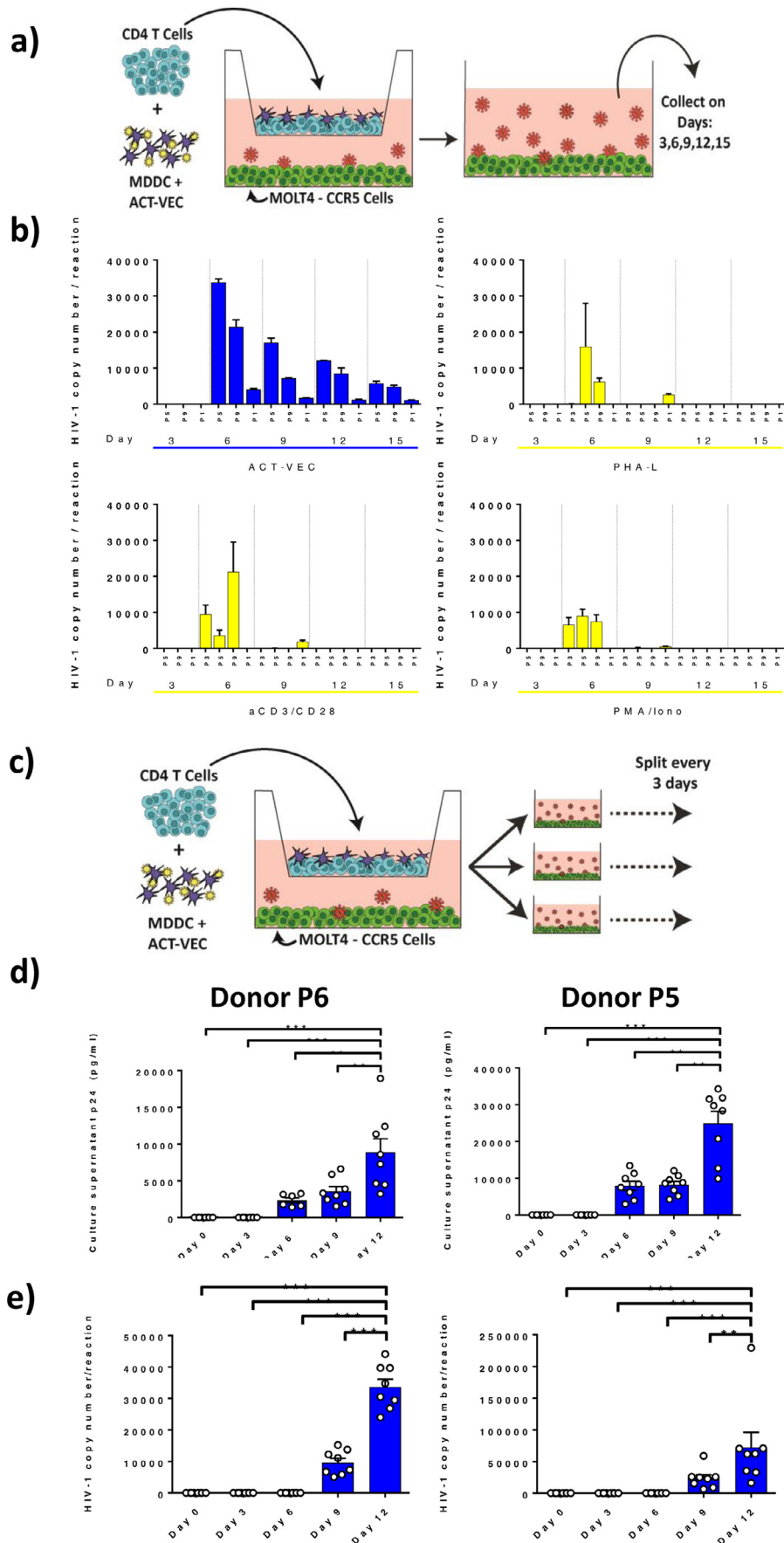


Fig. 3. MDDC presentation of ACT-VEC to T cells induces replication competent virus through contact dependent mechanisms. ACT-VEC pulsed MDDC were used to stimulate autologous CD4 T cells in Spin-X columns. (a) MOLT-4/CCR5 cells were added to the basolateral chamber of the column to propagate induced virus capable of replicating. After 3 days of

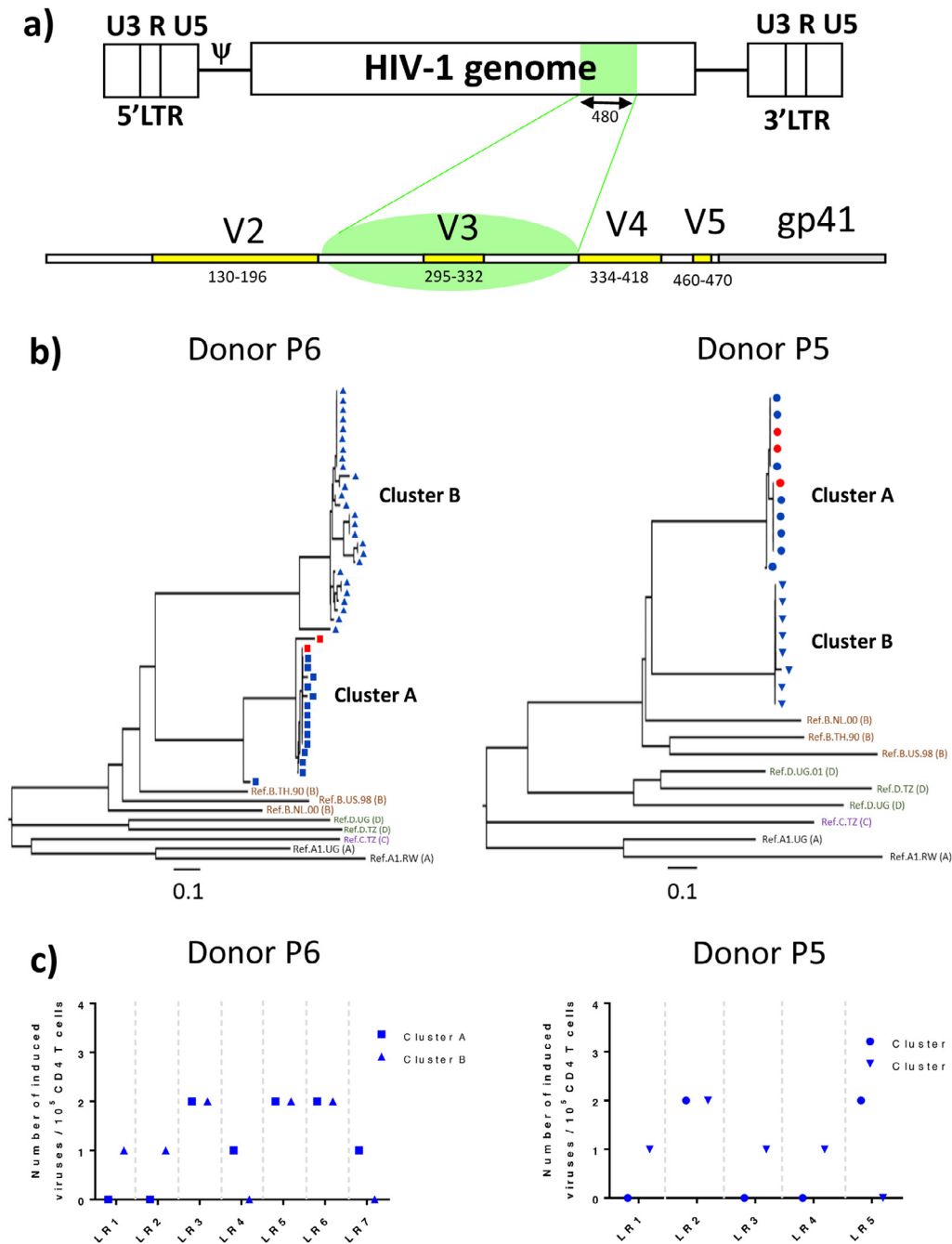


Fig. 4. ACT-VEC-induced HIV-1 exhibits limited genetic diversity. ACT-VEC pulsed MDDC were used to stimulate autologous CD4 T cells in Spin-X columns. MOLT-4/CCR5 cells were added to the basolateral chamber of the column to propagate induced virus capable of replicating. After 3 days of stimulation and co-culture, the apical insert containing the MDDC-CD4 T cells was removed and the MOLT-4 CCR5 cells were plated into a 24 well plate and cultured for a further 12 days. (a) Illumina MiSeq deep gene sequencing of the C2-V3 region of Env from ACT-VEC induced HIV-1 was carried out on day 12 culture supernatants. (b) Monophyletic viral clusters corresponding to the induced HIV-1 (Blue), were compared to HIV DNA from isolated provirus (Red) to demonstrate genetic homology. Reference sequences for subtypes A,B,C and D were added for comparison. (c) Latency reversal assays (LR1-7) were conducted multiple times with the induced virus sequenced to determine the number of times and variability that a particular viral sequence could be identified within culture supernatants.

The T cell mitogens used as controls in these latency reversal studies act through differing mechanisms. As shown in Fig. 6F, and as reported by others [48], Dasatinib works through inhibition of Lck,

which is a protein tyrosine kinase that acts proximal to the TCR. Hence, it is unsurprising that Dasatinib was ineffective at inhibiting T cell activations when PMA/Iono was the stimulus, as PMA/Iono is a

stimulation and co-culture, the inserts containing MDDC-CD4 T cells were removed and MOLT-4 CCR5 cells were plated into a 24 well plate and cultured for a further 12 days. (b) MOLT-4 CCR5 cells from outgrowth experiments were cultured for 15 days and viral copy number was analyzed in the culture supernatants every three days ($n=3,4$). (c) A similar viral outgrowth assay was set up whereby after 3 days of MDDC-CD4 T cell co-culture, the MDDC-CD4 T cell insert was removed and the MOLT4/CCR5 cells were split evenly into three plates. Samples were subsequently sub-cultured at 3-day intervals. (d) The two donor ($n=2$) samples that were sub-cultured were maintained over time to observe if viral propagation would occur. Viral p24 was measured by ELISA every 3 days. Viral p24 results are shown for days 9 and 12 (+/- SEM) (e) Viral copy numbers in culture supernatants were also determined by qRT-PCR every 3 days. Results for qRT-PCR are shown for days 9 and 12 (+/-SEM). Statistical significance was ascertained by Mann-Whitney unpaired t -test ($*P < 0.05$; $**P < 0.005$; $***P < 0.0005$; $****P < 0.00005$).

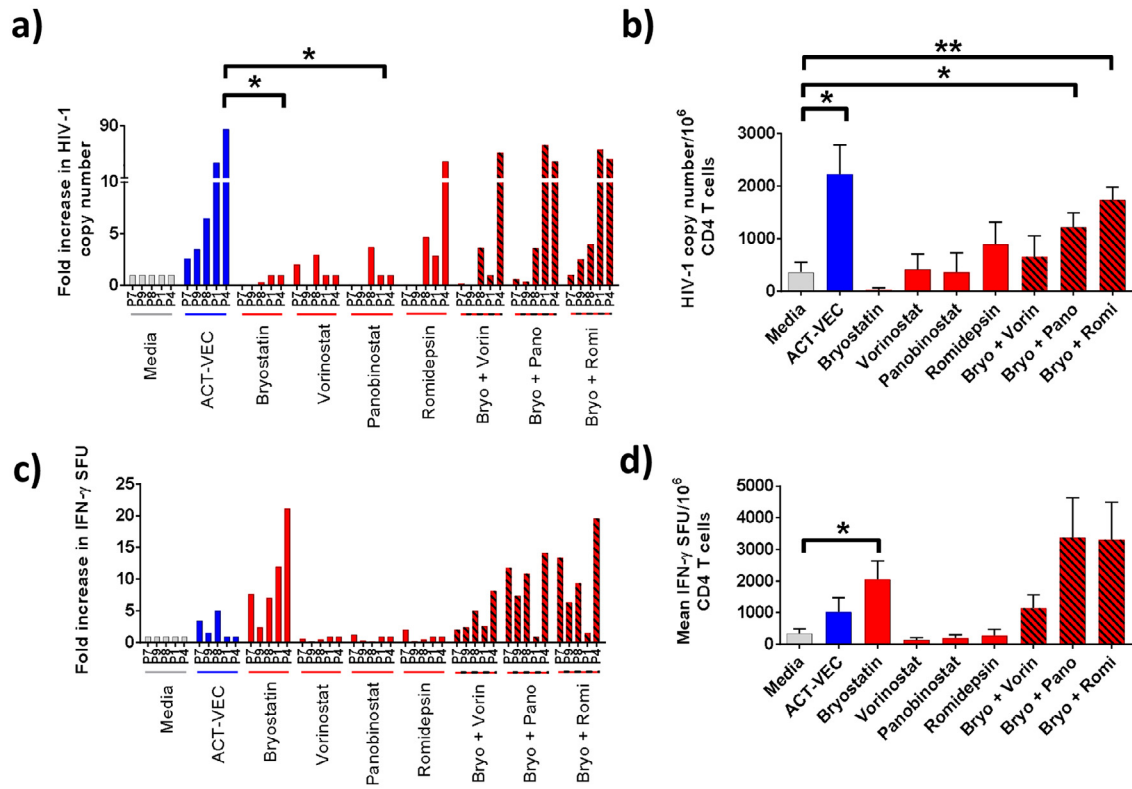


Fig. 5. ACT-VEC is a more robust LRA than HDACi and PKC agonist regimens. ACT-VEC+MDDC were used to stimulate autologous CD4 T cells from HIV infected donor cells (n=5). ACT-VEC stimulations were compared to Bryostatatin, Vorinostat, Romidepsin and Panobinostat monotherapies as well as Bryostatatin/Vorinostat, Bryostatatin/Romidepsin and Bryostatatin/Panobinostat combination therapies. (a and b) The induced viral copy number was determined after 3 days of cell culture in HIV donor samples by qRT-PCR. (c and d) To determine CD4 T cell activation, IFN- γ ELISpot was performed (100,000 cells/well) on HIV+ cells after overnight incubation. Results are shown as fold increase over media or group means of IFN- γ SFUs /10⁶ CD4 T cells (\pm SEM). Statistical analysis was assessed by Mann–Whitney unpaired t-test (* p < 0.05 and ** p < 0.005).

known PKC agonist and triggers T cell activation downstream of the TCR signaling cascade. This contrasts with the other mitogens tested that stimulate through the TCR, and where activation was severely impeded by Dasatinib. Interestingly, when Dasatinib was used as an inhibitor in our latency reversal reactions, a marked reduction in induced virus was detected in culture supernatants, approaching ~40% (Fig. 6G). As this is a noticeable albeit incomplete reduction, it suggests that there are other signals that are important for DC-T cell signaling in the context of an MHC:II response. Importantly, the dose (10 nM) and duration (8 hr) of Dasatinib treatment was well-tolerated in DC-T cell co-cultures, with minimal death observed as a result of treatment. This indicates the importance of signaling through the TCR, which is likely the mechanisms employed by MDDC pulsed with ACT-VEC.

While contact and signaling through the TCR appear important, inhibition of JAK-STAT and TNFR mediated signal transduction did not significantly reduce ACT-VEC mediated latency reversal in our hands, suggesting that cell secreted factors alone are not responsible for the observed latency reversal (Data not shown). Additional support for this is shown by ACT-VECs comparatively lower capacity to induce IFN- γ , TNF- α and IL-2 production from activated CD4 T cells compared to the polyclonal stimulator PMA/Iono (Data not shown), the latter being a poor latency reversal agent in these studies.

8. Discussion

Development of an HIV-1 cure is a significant challenge. Currently, only the Berlin patient has been cured of HIV-1, although it has recently been reported that a London patient and Düsseldorf patient have also been described to be in HIV-1 remission [49–52]. However, the myeloablation/stem cell transplantation therapy used to achieve

these remarkable feats are impractical for global roll out or use in the cART era [49]. Regardless, the addition of the London and Düsseldorf patient does add further credibility to the HIV cure effort and also adds supportive evidence that the cure seen in the Berlin patient was not an anomaly, despite the lack of HIV eradication seen in other clinical examples such as the Mississippi Child and the Boston patients. Alternative approaches to HIV cure have been based on *in vitro* stimulation of latently HIV infected cell lines and primary T cells using a diverse array of chemotherapeutic and immunomodulatory agents [53,54]. However, the initial promising *in vitro* effects of HDACi and PKC agonists on latency reversal have failed to translate into strategies capable of decreasing the reservoir size, suggesting a need for more effective alternatives to eliminate activated cells.

For full HIV latency reversal, an LRA must overcome key epigenetic barriers. The latent HIV genome is typically integrated into heterochromatin or genomic regions of limited transcriptional activity. TCR mediated stimulation induces a complex signaling cascade leading to increased levels of transcriptional factors NF- κ B, NFAT, AP-1, p-TEFb, and Ets-1 as well as other host factors, all of which interact with the promoter/enhancer elements in the LTR to activate, enhance and/or elongate HIV-1 mRNA transcription [55,56]. In the studies presented herein, we stimulated the T cells through HIV-specific TCRs using a targeted approach employing MDDC and a susceptible HIV VLP formulation. We chose AC-cART samples for this study as the minimal genetic diversity and lower reservoir size is likely to play a significant role within immunotherapeutic cure strategies. This differs significantly from most cure studies that evaluated latency reversal in samples derived from individuals diagnosed and treated at chronic stage of infection, where reservoirs and diversity are going to be larger. Our HIV-specific, CD4 T cell-targeted approach

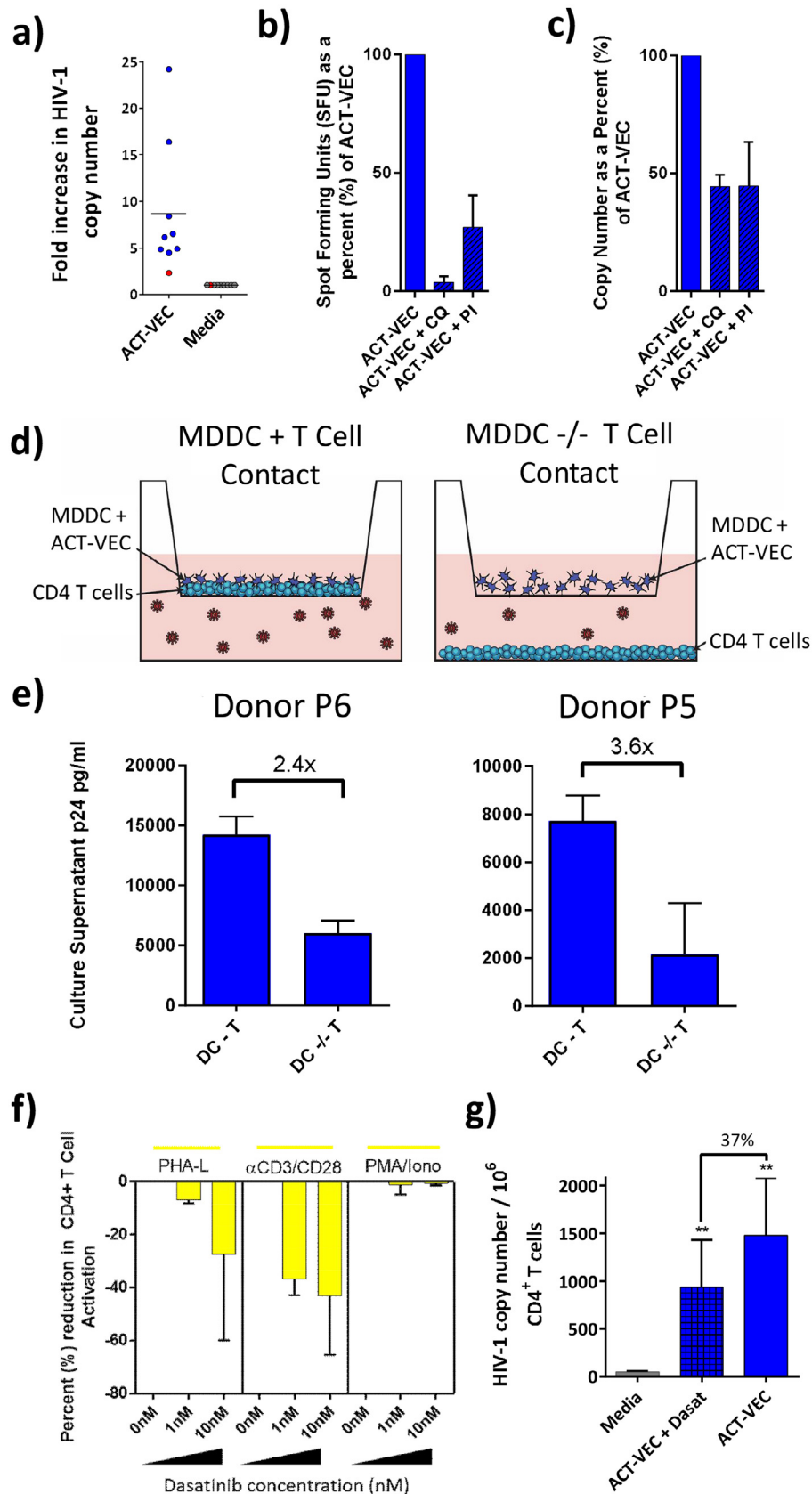


Fig. 6. MDDC presentation of ACT-VEC to T cells induces replication competent virus through contact dependent mechanisms. (a) PBMC from volunteers treated with cART containing a protease inhibitor induced the lowest fold change in viral reactivation by qRT-PCR. (b) MDDCs were pulsed with ACT-VEC in the presence or absence of either the protease inhibitor, Pepstatin A, or the lysosomotropic agent, Chloroquine (CQ), and subsequently co-cultured with autologous CD4 T cells. IFN- γ ELISpot SFUs were used to evaluate the percent (%) change in activation between conditions. (c) After 3 days in culture, supernatants were collected and viral copy number determined via qRT-PCR. (d) To evaluate if MDDC-CD4 T cell contact was necessary for HIV latency reversal, the MDDC-CD4 T cells were either cocultured in the apical insert of the Spin-X columns or separated by seeding them in the basolateral and apical chambers of the columns, respectively. (e) After MDDC were pulsed with ACT-VEC and the MDDC used to stimulate autologous CD4 T cells, induced virus

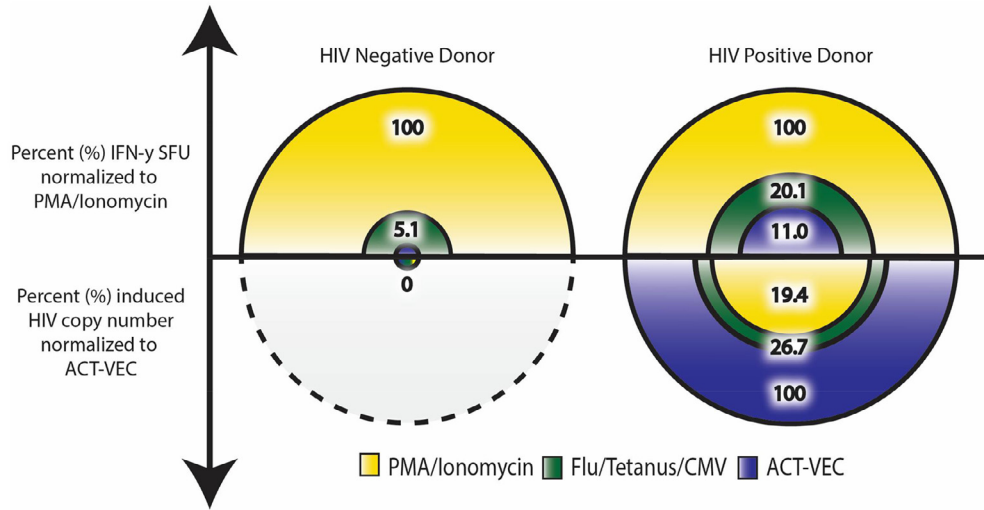


Fig. 7. Schematic representation of latency reversal in human acute/early CD4 T cells. Representation of the frequencies (%) of CD4 T cell activation and the quantities of induced HIV-1 virus detected in culture supernatants using PMA/Iono (yellow), Flu M1/Tetanus Toxoid/CMV peptide pool (green) and ACT-VEC (blue). Frequencies of induced virus (below the x-axis) and activated CD4 T cells (above the x-axis) are normalized to the condition eliciting the greatest response (i.e. ACT-VEC for induced virus and PMA/Iono for activated CD4 T cells). The schematic demonstrates that ACT-VEC is a stronger latency reversal agent than non-specific stimuli whilst being substantially less antigenic.

was based on several studies suggesting that these HIV-specific T cells may be the primary “fuel” for HIV production, especially during acute/early infection [15,16]. HIV antigens, however, would only be an effective transcriptional shock if CD4 T cells expressing HIV-specific TCRs served as a significant reservoir for latent HIV, which is indeed the phenomenon that we observed. Our VLP formulation presents self-assembling viral proteins in their native conformation and, therefore, would exhibit greater DC-mediated uptake, and stability relative to peptide-based therapies. Additionally, maximal stimulation requires the presentation of peptides that encompass the high diversity present within the host quasi-species, a feature we have incorporated into our VLP formulation. Simply put, ACT-VEC+MDDC resulted in a pronounced increase in the production and release of HIV from CD4 T cells of volunteers receiving cART during early infection (AC-cART). This latency reversal and release of HIV-1 by our immunotherapy was significantly greater than that observed when the same AC-cART CD4 T cells were treated with common recall antigens, HDACi and even potent mitogens (Fig. 7). Importantly, use of the latently infected J-Lat 6.3 cell line confirmed that all therapeutics (HDACi, PKC agonist) were used at effective doses consistent with the literature [37]. Moreover, these studies confirmed that ACT-VEC-based latency reversal is dependent on DC-T cell contact, as delivery of ACT-VEC to J-Lat cells alone was unable to induce transcriptional reactivation.

Using deep gene sequencing of the induced, propagating virus, we were able to estimate the numbers of latently infected cells that were inducible within patient samples. Our results suggest there are ~14 CD4 T cells per million with infectious but latent HIV provirus that can be stimulated to produce HIV in our acute samples. This falls within current estimates in the literature of between 1-60 CD4 T cells /million harboring latent but potentially infectious HIV provirus during stable cART [39–41]. Interestingly, a study by Baxter *et al* demonstrated that in a FISH/Flow assay, the median frequency of PMA/Iono inducible cells harboring latent HIV was 3.56 CD4 T cells/10⁶, which is substantially less than our calculated 14 cells/million and agrees with the low level reactivation that we see in these studies [57]. Of

note, the results presented herein were limited by patient availability, as identifying/treating donors treated during acute infection is not as easily obtained as individuals treated during the chronic stage of infection. In the future, studies using more powerful technologies, such as the before mentioned FISH/Flow, and newer assays that can discriminate between intact and defective forms of virus, such as the Intact Proviral DNA Assay (IPDA), would likely shed important insights into the effectiveness of our shock approach while more accurately characterizing the reservoir size [58].

Unlike many latency reversing agents, many of which non-specifically activate CD4 T cells, ACT-VEC-mediated latency reversal is targeted towards activating CD4 T cells expressing HIV-specific TCRs. Indeed, HIV-specific CD4 T cells have previously been described as a potential reservoir for HIV-1 [15]. However, it is possible that, replication competent, infectious HIV resides in CD4 T cells that express TCR against non-HIV antigens. For instance, vaccines such as Influenza, Hepatitis B, Pneumococcus and oral cholera vaccine have previously been shown to cause transient elevations in HIV expression within individuals diagnosed at chronic stage of infection [30–35]. Certainly, the incidence of opportunistic infections is likely to increase as an individual remains in the untreated phase of HIV infection, generating pools of activated CD4 T cells that can become infected by the virus. While the volunteer samples used in this study were diagnosed and cART treated at acute stage of HIV infection, they would be expected to have a limited reservoir seeding in non-HIV-specific CD4 T cells, owing to the short timeframe between infection and treatment. This is likely to be different in samples from individuals diagnosed and treated at chronic stage of HIV infection. In the later case, future studies involving combinations of FLOW/FISH and qVOA, along with the testing of a wider panel of non-HIV immunogens might be able to sufficiently delineate the TCR specificity of cells that contain inducible, replication competent virus from graveyard virus.

While the fate of the cellular reservoir upon viral reactivation is unknown, activation of HIV-specific memory CD4 T cells by ACT-VEC could provide a strong T helper response for CTL or antibody-based immune control of this reactivated latent HIV. Therefore, it is important to note that we have previously shown ACT-VEC induced cell-

in culture supernatants was quantified by p24 ELISA. (f) To evaluate the effect on latency reversal by stimulating through the TCR, we used an inhibitor of the TCR signaling pathway, Dasatinib. To determine the best concentration of Dasatinib to use in latency reversal studies, we titrated Dasatinib on CD4 T cells at varying concentrations (0 nM, 1 nM, 10 nM) prior to activation with either PHA-L, αCD3/CD28, or PMA/Iono. Percent activation was determined via flow cytometry. (g) ACT-VEC-pulsed MDDCs were co-cultured with CD4 T cells in the presence or absence of Dasatinib, and supernatants were analyzed for viral RNA concentrations (copy number +/- SEM) after 24 h (*P < 0.05; **P < 0.005).

mediated cytotoxic Granzyme B responses in these AC-cART treated volunteer PBMC [28]. In the context of immunotherapeutic cure, particularly in patients treated during acute/early infection, an antiviral cytotoxic response induced by ACT-VEC may be very effective in eliminating the relatively homogenous HIV-1 presented by infected cells via MHC I. Thus, ACT-VEC, delivered via adoptive transfer of *in vitro* generated, antigen-loaded DCs, or *in situ* targeting of DCs could prime for a possible “kill” along with the observed “kick” that we see. Over the years, a number of studies have used DC-based approaches to present HIV-1 antigens in the non-human primates and in clinical trials to enhance an immune activated “kill” of the cell reservoir and/or residual virus [59–63]. Notable reductions in viral RNA and DNA levels were observed in the cells from various anatomical compartments of the vaccinated animals, a finding that has yet to be substantiated in human trials. However, these studies were focused on a “kill” by CD8+ T cytotoxic lymphocytes rather than exploring how DC mediated presentation of these SIV antigens may have activated virus-specific CD4 T cells and thereby triggering latency reversal.

While these studies were conducted using samples from patients, cART-treated at acute stage of subtype B infection, it should be noted that the vast majority of individuals are diagnosed much later in infection. During the chronic stage of infection, untreated individuals would be exposed to a plethora of opportunistic infections which might change the TCR demographics of cells harboring latent provirus. Equally, it should be considered that subtype B infection accounts for a considerably smaller proportion of HIV infection, with subtype C constituting the majority of the global epidemic. Therefore, in future studies, it would be essential to expand on these preliminary studies to evaluate ACT-VEC in the context of i) infection by HIV of other subtypes (e.g. A,C,D) and ii) among individual receiving cART during chronic stage of infection. Both of these studies are ongoing with chronically infected patient receiving cART in Uganda, Canada, and the USA with larger study groups, to stratify for the length of infection prior to cART, to account for size of the latent pool as well as variability in the replication-competent, defective, and dead reservoir, and finally, to determine potential for CD4 T cell exhaustion in chronic patient samples.

In conclusion, we demonstrate that ACT-VEC efficiently reactivates transcriptionally silent HIV-1 from CD4 T cells of patients treated at acute/early infection. Furthermore, we can show viral translation occurs and that the released virus is replication competent. Our study also demonstrates that this immunotherapy is targeted to HIV-specific CD4 T cells, which may house a large proportion of the latent HIV reservoir. Taken together, this approach establishes ACT-VEC as a highly promising immunotherapeutic LRA. Future planned *in vitro*, *in vivo* non-human primate and human clinical studies will suitably address whether ACT-VEC can indeed cause reservoir reduction/elimination and whether ACT-VEC can induce protective humoral and cytotoxic T cell responses *in vivo*.

Declaration of Competing Interest

JFSM and EJA hold an issued patent and a pending patent related to the data presented herein.

Author contributions

JFSM, JP, KK, RG, DC and EJA designed experiments. JFSM, JP, KK, CNW, RP, and YG designed and built VLP and ACT-VEC constructs. RP, JP, KK, and CWN performed qRT-PCR experiments and performed analysis. KK and JP performed deep gene sequencing. AFYP, MA, and JFSM analyzed deep gene sequencing data and created trees. JP and JFSM performed immunogenicity and co-culture assays. JP performed flow cytometry. DFLK, SF, RJS, and PFM recruited, consented, and processed blood samples from sero-positive donors. JM extracted data from clinical charts. JFSM, JP, and CWM consented and processed

blood from sero-negative donors. JFSM, JP, KK, and EJA wrote the manuscript. All authors reviewed the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ebiom.2020.102867](https://doi.org/10.1016/j.ebiom.2020.102867).

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