Inconsistent Reversal of HIV-1 Latency Ex Vivo by Antigens of HIV-1, CMV, and Other Infectious Agents

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Abstract

Background

A reservoir of replication-competent but latent virus is the main obstacle to a cure for HIV-infection. Much of this reservoir resides in memory CD4 T cells. We hypothesized that these cells can be reactivated with antigens from HIV and other common pathogens to reverse latency.

Results

We obtained mononuclear cells from the peripheral blood of antiretroviral-treated patients with suppressed viremia. We tested pools of peptides and proteins derived from HIV and from other pathogens including CMV for their ability to reverse latency *ex vivo* by activation of memory responses. We assessed activation of the CD4 T cells by measuring the up-regulation of cell-surface CD69. We assessed HIV-expression using two assays: a real-time PCR assay for virion-associated viral RNA and a droplet digital PCR assay for cell-associated, multiply spliced viral mRNA. Reversal of latency occurred in a minority of cells from some participants, but no single antigen induced HIV-expression *ex vivo* consistently. When reversal of latency was induced by a specific peptide pool or protein, the extent was proportionally greater than that of T cell activation.

Conclusions

In this group of patients in whom antiretroviral therapy was started during chronic infection, the latent reservoir does not appear to consistently reside in CD4 T cells of a predominant antigen-specificity. Peptide-antigens reversed HIV-latency *ex vivo* with modest and variable activity. When latency was reversed by specific peptides or proteins, it was proportionally greater than the extent of T cell activation, suggesting partial enrichment of the latent reservoir in cells of specific antigen-reactivity.

Background

By preventing cells from becoming newly infected, antiretroviral therapy (ART) can suppress HIV-1 replication to nearly undetectable levels. However, latently infected cells persist, are remarkably stable, and once activated yield new infectious virus [1–3]. Consequently, interruption of ART is followed by a relapse of viral replication and viremia, which ultimately causes depletion of CD4 T cells and immunodeficiency if left untreated [4].

Latently infected cells form a "viral reservoir" that is established early after infection with replication-competent proviruses integrated into the genomes of CD4 cells, with a substantial proportion in resting memory CD4+ T cells [5–8]. During acute infection, HIV causes immune activation, preferentially infecting activated HIV-specific CD4 T cells [9]. This suggests the possibility that the viral reservoir is biased toward such HIV-specific memory cells.

A currently favored approach to eradicate latently infected T cells is to reactivate viral transcription using small molecules as latency reverting agents (LRAs) while maintaining ART [10]. The goal of this approach is to activate latent viruses that are integrated into host memory CD4 T cells so that the cells die from viral cytopathic effect or are eliminated by immune surveillance. These strategies have so far shown very limited success [11–14]. Agents under investigation as LRAs include various types of protein kinase C (PKC) activators (for example, bryostatin-1), HDAC inhibitors (for example, panobinostat and romidepsin), and BRD4 inhibitors (for example, JQ1), among others [15–19]. Most of these LRAs lack specificity for the cells that contain the viral reservoir. Consequently, some of them have a potential for toxicity; for example, agents targeting PKC-signaling might broadly affect cellular metabolism [20,21]. In contrast, activation of latently infected memory CD4 T cells by their cognate antigens is specific. However, the effectiveness of an antigen as a LRA will likely depend on the fraction of the latent reservoir that resides in cells that respond to it.

Since HIV preferentially infects activated CD4 T cells [5,9], and since the reservoir is partly established early during infection when most of those cells are responding to HIV, many reservoir cells might be HIV-specific. Alternatively, if HIV-infection is left untreated while CD4 T cells are responding to either chronic persistent infections such as those caused by CMV or EBV or recurrent infections such as influenza, then reservoir cells might be specific for those pathogens. More generally, if reservoir cells have a limited breadth of antigen specificity, then only a few antigens might be needed to reverse latency in the majority of cells while activating only a small fraction of the CD4 T cell population.

In this study, we hypothesized that the latent reservoir can be reactivated using antigens to activate specific memory CD4 T cells harboring latent provirus. We evaluated whether peptide antigens specific to HIV, as well as to common pathogens such as CMV, could act as LRAs to reactivate the HIV reservoir without causing general T cell activation. Support for this hypothesis came from previous studies suggesting that peptide antigens of HIV induced viral protein expression in CD4 T cells from participants with suppressed viremia [22–24]. Additionally, we tested the hypothesis that the presentation of antigens by dendritic cells optimizes the reversal of latency (reviewed in [25]). We found that in some cases antigenic peptides or proteins reversed latency, but they rarely did so to a substantial extent, and no particular antigen was active in all cases. When peptides or protein antigens reversed latency, they did so to an extent greater than would be predicted based on their induction of T cell-activation. This suggests that in occasional individuals the latent reservoir is disproportionally present in cells of specific antigen-reactivity, a finding potentially consistent with clonal expansion of specific memory CD4 T cells.

Results

Study participants and cell preparation

We obtained up to 300 ml of peripheral blood from 19 HIV positive participants (one on two occasions); three were ultimately excluded from the data presented, because their cells did not show evidence of latency reversal after stimulation with antibodies to CD3 and CD28 (the positive T cell activation control).

All participants had a CD4 T cell count of at least 500 cells/μl of peripheral blood and fully suppressed viremia (less than 20 copies/ml) for at least one year. For the eight participants for whom data are available, HIV-infection was diagnosed five to over twenty years before their blood was obtained for this study.

We isolated peripheral blood mononuclear cells (PBMC) by density centrifugation. To ensure optimal antigen presentation to CD4 T cells, we kept professional antigen presenting cells - B cells and monocytes - in the cell population by depleting CD8 T cells from the PBMC rather than isolating the CD4 T cells. Our rationale for removing CD8 T cells was to avoid potential killing of reactivated reservoir cells as well as the potential secretion of suppressive mediators. CD8 T cell depletion from participant PBMC was confirmed by flow cytometry, and residual CD8 T cells were usually < 0.1% (data not shown).

Identification of antigen specific CD4 T cell responses by INF-γ ELISpot assay

Production of IFN-γ by CD4 T cells is a hallmark of the Th1-type CD4 T cell phenotype and is typically associated with an effective host defense against intracellular pathogens (reviewed in [26]). Therefore, we first used an IFNγ ELISpot assay to evaluate the ability of peptide pools to activate and induce an immune response in the CD4 T cells within the CD8-depleted cell populations.

We screened IFN-γ production in response to peptide pools containing 15mer overlapping peptides from: 1) Cytomegalovirus (CMV) matrix protein 65 (pp65); 2) *Candida albicans* mannoprotein MP65; 3) a mixture of 14 previously identified optimal MHC class-II restricted epitopes from *human cytomegalovirus* (HHV-5), *Epstein-Barr virus* (HHV-4), *influenza A*, and *Clostridium tetani* toxoid (pool named CEFT); 4) HIV-1 Gag; 5) HIV-1 Pol; 6) HIV-1 Env; and 7) HIV-1 Nef. For each condition, we stimulated 100,000 CD8-depleted PBMC with 1 μg/ml per individual peptide. We used anti-CD3/CD28-coated beads as well as phorbol myristate acetate in combination with ionomycin (PMA/Iono) as a positive control for maximal T cell stimulation. Both positive controls typically saturated the signals on the ELISpot plate and did not allow quantification (Fig. 1A). We used DMSO as a negative control, because the peptide pools and individual peptides were reconstituted in DMSO. Wells treated with DMSO typically showed < 10 SFC/million cells. IFNγ responses of varying magnitudes were measured for these peptide pools in CD8-depleted PBMCs from a subset of participants (Fig. 1A, which shows example ELISpot results for each of the peptide pools; in addition to the example shown, two additional participants tested showed no response to peptides from *C.albicans*). For three participants, we tested the 123 Gag 15mer peptides singly at a concentration of 12.5 μg/ml per peptide with 100,000 CD8-depleted PBMC per well to identify the specific peptides within the pool that induced IFN-γ production in the CD4 T cells (Fig. 1B and data not shown). The peptides that yielded > 50 SFC/million CD8-depleted PBMC were subsequently pooled to generate a participant-customized ELISpot selected Gag peptide pool used in some of the latency reactivation experiments. We verified for one participant that the observed IFN-γ production was not a result of contamination with CD8 T cells by comparing two ELISpot assays, one with CD8 depleted PBMC and one with isolated CD8 T cells. The ELISpot assay with the isolated CD8 T cells showed IFN-γ production in response to different peptides compared to the assay with CD8 depleted PBMC, suggesting

that the epitopes recognized by class I MHC and class II MHC were different (data not shown). Overall, these data indicated that with the possible exception of the peptides derived from *Candida* MP65, the peptide pools were able to activate a small fraction of CD4 T cells, presumably in an antigen-specific manner.

Virion-release from latently infected cells after antigenic stimulation measured by real-time RT-qPCR

For nine participants, we stimulated five million CD8-depleted PBMC with each peptide pool in quadruplicate for seven days in the presence of the integrase-inhibitor raltegravir [1 μM]. Raltegravir prevented viral spread and ensured that the measured cell-free, virion-associated (cf)-RNA was released directly from reactivated reservoir cells and not a consequence of propagation of virus in the culture. Depending on the yield of isolated cells from each participant, we stimulated the cells with overlapping peptide pools from Gag, CMV, Candida, CEFT, Pol, Env, and Nef at a concentration of 1 μg/ml per peptide. DMSO was used as negative control and platebound antibodies against CD3 and CD28 were used as a maximum-stimulation control.

We first assessed the activity of the peptide pools with respect to immune activation by measuring the early activation marker CD69. After two days of culture, an aliquot of ~ 50,000 cells from each condition was stained for cell-surface CD69 and CD4 and analyzed by flow cytometry. The DMSO controls showed baseline CD69-positive CD4 T cells ranging from 1–12%, whereas the platebound anti-CD3 and anti-CD28-stimulated cells showed the maximum T cell activation for each participant, which ranged from 45–87% CD69-positive CD4 T cells (Fig. 2A). In general, none of the peptide pools activated a substantial fraction of the CD4 T cells.

After seven days of antigen-stimulation in the presence of raltegravir, we measured released virion-associated RNA to assess reversal of latency. The culture supernatants were cleared of debris and cellular contaminants by low-speed centrifugation, and the virions were isolated by ultracentrifugation through 20% sucrose. Cell-free (cf)RNA was isolated and cf-HIV Gag RNA was measured using real-time RT-qPCR (Fig. 2B). The results were normalized to the cf-RNA yields after maximum stimulation with antibodies to CD3 and CD28 for each participant. One participant was evaluated twice, at times approximately one year apart (Fig. 2B, filled circles and open circles). At the first evaluation, this participant's cells showed relatively high fractional levels of latency-reversal in response to several peptide pools including the Gag and CEFT pools, despite a limited up-regulation of CD69 (Fig. 2A, filled circles). At this time, this participant's cells yielded cf-RNA in response to the *C. albicans* peptide pool, although this pool did not show activity in the IFN-γ ELISpot assay using cells from other participants (Fig. 1A and data not shown). Also at this time, this participant's cells had the highest baseline expression of cfRNA in the DMSO control. At the later time (open circles in Fig. 2B), this participant's cells showed a different pattern of latency reversal: under 5% of the positive control values in all cases except for the Nef peptide pool, which was over 10% of the positive control value. Cells from the other participants shown in Fig. 2 yielded modest latency reversal after exposure to the peptide pools (less than 15% of the positive

control values). The HIV Nef peptide pool appeared the most consistent among multiple participants, although the fraction of latency reversal relative to the positive control was less than 15%.

Viral cell-associated mRNA induction after antigenic stimulation measured by droplet digital (dd)PCR

To confirm and extend the results obtained measuring cf-RNA, we evaluated latency reversal in a second group of participants by measuring cell-associated (ca) HIV mRNA using a ddPCR assay that detects multiply spliced Tat/Rev transcripts [27]. We stimulated nine million CD8-depleted PBMC from six participants with each peptide pool, again in the presence of 1 μM raltegravir to prevent viral spread. Depending on the yields of participant cells, CD8-depleted PBMC were stimulated with peptide pools from CMV, CEFT, Gag, or a participant-customized ELISpot-selected Gag peptide pool at a concentration of 1 μg/ml and plated in three-fold limiting dilutions and six replicates. As before, we used DMSO as the negative control and platebound antibodies to CD3 and CD28 as a maximum-stimulation control. Cellular activation was measured by the up-regulation of CD69 after 48 hours of incubation. Consistent with the results above (Fig. 2A), we observed that the peptide pools did not activate a substantial fraction of the CD8-depleted PBMC (Fig. 3A).

After five days of culture in the presence of 1 μM raltegravir, we collected the cells and isolated caRNA to measure the amounts of multiply spliced Tat/Rev mRNA using the ddPCR assay. Overall, the ddPCR mRNA data showed no consistent induction of the expression of multiply spliced Tat/Rev mRNA by the different peptide pools. With some exceptions the peptide pools reversed latency only modestly when compared to the positive control (Fig. 3B). In cells from one participant (indicated by open diamond), the Gag peptide pool induced Tat/Rev mRNA to 75% of the positive control value, but for this participant the DMSO control value was also unusually high (24%). Notably, in cells from another participant (indicated by "x"), the ELISpot-selected Gag-peptide pool induced Tat/Rev mRNA to 50% of the positive control value, while the DMSO control value in this case was low (under 3%). This participant also had partial latency reversal in response to peptide pools of CMV (18% of the positive control) and the CEFT mixture (29% of the positive control).

Antigen Presentation By Autologous Monocyte-derived Dendritic Cells (dc)

We considered that suboptimal antigen presentation might render the above experiments less sensitive to the potential activity of antigens as LRAs. Therefore, we isolated, differentiated, and matured autologous monocyte-derived DC from two additional participants and used these mature DC as antigen-presenting cells. We also used complete proteins in addition to peptide pools as antigens to better simulate natural antigen processing and presentation (Fig. 4 and Table 1). In these co-culture experiments, like those above, we included the integrase inhibitor raltegravir to block the spread of infection during the seven day incubation (Fig. 4B), but for one participant we also omitted the raltegravir and incubated the cells for 18 days to determine how allowing viral propagation would affect the results (Table 1).

Table 1

RNA measurements of latency reversal after presentation of the indicated antigens by dendritic cells for one of the participants shown in Fig. 4; cells were cultured with or without raltegravir. Values in parentheses are the percent of the α-CD3/α-CD28 control.

Assay	DMSO	α-CD3 + α-CD28	CMV pp65	HIV-1 p55 Gag	SIV p55 Gag	CMV pp65 peptide pool[1]	CEFT[2] peptide pool	HIV-1 Gag peptide pool
cf[3]-RNA raltegravir[4]	n.d.[5] (0)	24,000 (100)	n.d. (0)	130 (0.5)	n.d. (0)	107 (0.4)	n.d. (0)	8300 (35)
cf-RNA no raltegravir[6]	n.d. (0)	1.2×10^{10} (100)	n.d. (0)	1×10^{10} (87)	370 (3×10^{-6})	440 (4×10^{-6})	n.d. (0)	33,000 (2.7×10^{-4})
ca[7]-RNA raltegravir[4]	n.d. (0)	151 (100)	3 (2)	n.d. (0)	n.d. (0)	14 (9)	30 (20)	96 (64)
ca-RNA no raltegravir[6]	n.d. (0)	1.2×10^{9} (100)	n.d. (0)	7.5×10^{6} (0.63)	n.d. (0)	n.d. (0)	n.d. (0)	50 (4×10^{-6})

[1]all peptide pools were used at 1 μg/ml each peptide

[2]CEFT: pool of class II restricted peptides from CMV, EBV, influenza virus, and tetanus toxoid.

[3]cf: cell-free, virion-associated RNA in copies/ml

[4]raltegravir was used at 1 μM to block viral propagation and the cells were stimulated for 7 days

[5]n.d.: not detected

[6]cells were stimulated for 18 days in the absence of raltegravir

[7]ca: cell-associated, Tat/Rev mRNA in copies/μg RNA

Consistent with our experiments using CD8-depleted PBMC, the use of autologous DCs to present antigen did not cause substantial T cell activation measured by CD69 up-regulation (Fig. 4A), nor did it yield consistent latency reversal by any peptide or protein antigen (Fig. 4B). For both participants, we used cf-RNA for the initial readout (Fig. 4B). The data of one participant indicated that in the presence of raltegravir, the Gag peptide pool reversed latency to almost 40% of the positive control value, but the other peptide pools and complete proteins were inactive.

For the participant in whom the Gag peptide pool appeared active (square symbol in Fig. 4B), we also omitted raltegravir from parallel cultures: in this condition the intact p55 Gag protein yielded substantial cf-RNA (1×10^{10} copies/ml) reflecting marked viral outgrowth, but neither SIV Gag nor any other antigen did (Table 1). Notably, although the Gag peptide pool did not yield amounts of cf-RNA comparable to maximum-stimulation or the p55 Gag protein, it differed from the other conditions, yielding 3×10^4 copies/ml of cf-RNA compared to the low or undetectable copies for DMSO and the other antigens (Table 1). For this participant (square symbol in Fig. 4B), we also used ddPCR of ca Tat/Rev mRNA as the readout (Table 1). The data indicated that in the presence of raltegravir, the CMV, CEFT, and Gag peptide pools each reversed latency substantially. The activity of the Gag peptide pool (64% of maximum-stimulation) measured by the ca-RNA (Tat/Rev mRNA) readout in the presence of raltegravir was consistent with that measured using the cf-RNA (virion-RNA) readout (35% of maximum-stimulation). In contrast, when raltegravir was omitted, only HIV p55 protein yielded detectable induction of Tat/Rev mRNA: stimulation with p55 yielded 7.5×10^6 copies/ml compared to 1.2×10^9 copies/ml after maximum stimulation. Overall, these results suggested that antigen presentation by DCs did not markedly increase the degree or consistency of latency reversal by these peptides and proteins. The results also suggested that prolonged culture can change the conclusions of these latency reversal experiments relative to short-term, "single-cycle" readout, either amplifying or reducing the signals.

Peptides And Peptide Pools

For antigen stimulation of CD8-depleted PBMC we used pools of 15mer peptides overlapping by 11 amino acids. These peptide pools were specific for: HCMV pp65 (138 peptides), PepMix Candida (MP65) (JPT – 92 peptides), PepMix CEFT-MHC-II-pool (JPT – 14 peptides selected from defined HLA class II-restricted T-cell epitopes of *Clostridium tetani*, Epstein-Barr virus (HHV-4), Human cytomegalovirus (HHV-5), and Influenza A), HIV consensus B Gag (123 peptides), HIV consensus B Env (211 peptides), HIV consensus B Pol (249 peptides), HIV consensus B Nef (49 peptides). The 123 peptides of HIV consensus B Gag peptide-pool were also tested individually using an IFN-γ ELIspot assay (see below).

For experiments using matured monocyte-derived DC, we used proteins of CMV pp65 (Miltenyi Biotec), HIV-1 Gag p55 (Abcam) and SIV Gag p55 (Proteinsciences) as a control.

The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HCMV pp65 Peptide Pool (cat# 11549) [33–35], HIV-1 Consensus B Gag Peptide Pool (cat# 12425), HIV-1 Consensus B Gag Peptide Set (cat# 8117), HIV-1 Consensus B Pol Peptide Pool (cat# 12438), HIV-1 Consensus Subtype B Env Peptide Pool (cat# 12540), HIV-1 Consensus Subtype B Nef Peptide Pool (cat# 12545), and HIV-1 Consensus Subtype B Nef Peptide Set (cat# 5189).

Generation Of Mature Monocyte Derived Dendritic Cells (dc)

CD14 monocytes were isolated from PBMC using magnetically labelled anti-CD14 antibodies (Stemcell Technologies). Monocytes were cultured for five days in Iscove's Modified Dulbecco's Media (Gibco) complemented with 10% fetal bovine serum, penicillin/streptomycin (Gibco), granulocyte-monocyte colony-stimulating factor (GM-CSF, 1000 IU/ml, R&D Systems), and interleukin4 (IL-4, 1000 IU/ml, R&D Systems). On day five, maturation factors [interferon (IFN)-α (1000 IU/ml, R&D Systems), IFN-γ (1000 U/ml, R&D), IL1β (10 ng/ml, R&D Systems), tumor necrosis factor (TNF)-α (25 ng/ml, R&D Systems), and polyinosinic:polycytidylic acid (20 ng/ml, Sigma-Aldrich)] were added to the cultures for 48 hours, as previously described [36]. DC-maturation was confirmed by staining the cells with antibodies against CD11c, CD80, CD83, CD86, CD143, CD169, CD197 (all antibodies were obtained from Biolegend) and analysis by flow cytometry. Mature DC were loaded with the antigen for two hours, washed in IMDM media and subsequently co-cultured with autologous CD4 T cells in complete IMDM in the presence of the integrase inhibitor raltegravir [1 μM] at a ratio of 1:10 (100,000:1 million) for seven days (or in the absence of raltegrvir for 18 days) in 12-well plates. Culture supernatants and cells were collected on day seven (presence of raltegravir) or day 18 (absence of raltegravir) for quantitation of HIV cf-RNA and ca-RNA.

Interferon-gamma ELISpot assay

Freshly isolated CD8 depleted PBMC were seeded in 96-well polyvinylidene difluoride-backed plates (MAIP S45, Millipore) that had been coated with an anti-IFN-γ MAb 1-D1k (0.5 μg/ml, Mabtech) overnight at 4 °C.

HIV-specific CD4 T-cell responses were quantified, as described previously [37]. Overlapping peptides were added to 1×10^5 CD8 depleted PBMC per well at a final concentration of 12.5 μg/ml. The plates were then incubated for a 14–16 hours at 37°C and 5% CO_2. IFN-γ positive were detected using a biotinylated secondary anti-IFN-γ MAb 7-B6-1 (0.5 μg/ml, Mabtech), a streptavidin-alkaline phosphatase conjugate (Mabtech) and TMB substrate (Mabtech).

IFN-γ-producing cells were counted and expressed as spot-forming cells (SFC) per 10^6 PBMC. Negative controls were always < 10 SFC per 10^6 cells. As positive controls, we incubated PBMC with phytohemagglutinin or anti-CD3/CD28 coated beads. Wells were considered positive if they had at least 20 SFC/10^6 PBMC.

Real-time Qrt-pcr

Five million CD8 depleted PBMC were stimulated with the respective peptide pools at a concentration of 1 μg/ml and plated on a 6-well cell culture plate. After seven days of culture in the presence of the integrase inhibitor raltegravir [1 μM] supernatant was collected from each well and centrifuged for five minutes at 300 x g to remove cells and debris. Virions were harvested by centrifugation of cell-free culture supernatants through a 20% sucrose cushion at 23,500 x g for one hour at 4 °C. The pelleted virus particles were resuspended in 200 μl PBS and cell-free (cf)-RNA was isolated using the Viral RNA

extraction Kit (Roche) following the manufacturer's instructions. Cell free viral RNA was eluted in 20 µl and real-time RT-qPCR was performed using Superscript III Platinum One-step qRT-PCR Kit (Invitrogen) and 10 µl cf-RNA, in duplicate. The PCR reactions used Cy5-labeled *gag* probes together with the corresponding forward and reverse primers. Cycling was performed in an 7900HT Sequence Detection System (Applied Biosystems) with the following parameters: 50 °C for 15 minutes for the RT-step and 95 °C for 2 minutes, followed by 50 cycles of 95 °C for 15 seconds and 60 °C for 30 seconds, and a final step of 50 °C for 10 minutes. A standard curve of pNL4-3 plasmid DNA in duplicate, 10-fold serial dilutions was included on each plate, as well as water negative controls. The limit of detection of this assay was 100 copies/ml *gag* DNA.

The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Raltegravir from Merck & Company, Inc (Cat # 11680).

Droplet Digital Pcr

Study participant samples

We received up to 300 ml blood whole from 19 study participants with the following inclusion criteria: HIV positive, suppressed viremia (< 50 cps/ml blood) for at least one year, and a CD4 T cell count of at least 500 cells/µl blood.

Isolation of PBMC, CD8 T cell depletion, and stimulation with peptide pools

Peripheral blood mononuclear cells (PBMCs) were isolated from 300 ml whole blood donations using Lymphoprep (Stemcell Technologies) density gradient centrifugation. CD8 T cells were depleted by positive selection using the EasySep™ Human CD8 Positive Selection Kit (Stemcell Technologies) and EasySep magnet (Stemcell Technologies). Depletion of CD8 T cells was confirmed by staining an aliquot of 50,000 CD8 depleted PBMC with anti-CD3-FITC (clone: OKT3), anti-CD4-PE (clone: OKT4) and anti-CD8-APC (clone: HIT8a) fluorescent antibodies and analysis with an Accuri C6 flow cytometer and software (BD Biosciences).

Antigen reactivity of CD8 T cell depleted PBMC to the peptide pools was determined where indicated by IFNγ ELISpot assay (see below), and cells were subsequently stimulated with the various antigen peptide pools (see below) at 1 µg/ml each peptide. After 48 hours of antigen stimulation, an aliquot of ~ 50,000 CD8 depleted PBMC of each condition was stained for anti-CD4-APC (clone: OKT4) and anti-CD69-PE (clone: FN50) and analyzed using the Accuri C6 flow cytometer and software (BD Biosciences). All antibodies for flow cytometry were obtained from BioLegend.

Declarations

Ethics approval and consent to participate: The study was approved by the UC San Diego and the San Diego VA Healthcare System institutional review boards. All participants gave written informed consent for participating.

Consent for publication: Not applicable.

Availability of data and materials: source data available on request.

Competing interests: the authors declare no competing interests.

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Authors' contributions: TV, AOA, BM, and MNK did the experiments; TV, MFO, DDR, and JG wrote the manuscript.

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References

1. Chun TW, Stuyver L, Mizell SB, Ehler LA, Mican JA, Baseler M, et al. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Natl Acad Sci USA. 1997;94:13193–7.
2. Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, et al. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science. 1997;278:1295–300.
3. Wong JK, Hezareh M, Günthard HF, Havlir DV, Ignacio CC, Spina CA, et al. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science. 1997;278:1291–5.
4. Davey RT, Bhat N, Yoder C, Chun TW, Metcalf JA, Dewar R, et al. HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. Proc Natl Acad Sci USA. 1999;96:15109–14.
5. Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. Proc Natl Acad Sci USA. 1998;95:8869–73.
6. Chun T-W, Finzi D, Margolick J, Chadwick K, Schwartz D, Siliciano RF. In vivo fate of HIV-1-infected T cells: Quantitative analysis of the transition to stable latency. Nat Med. 1995;1:1284–90.
7. Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, et al. Latent infection of CD4 + T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective

combination therapy. Nat Med. 1999;5:512–7.

8. Perelson AS, Essunger P, Cao Y, Vesanen M, Hurley A, Saksela K, et al. Decay characteristics of HIV-1-infected compartments during combination therapy. Nature. 1997;387:188–91.
9. Douek DC, Brenchley JM, Betts MR, Ambrozak DR, Hill BJ, Okamoto Y, et al. HIV preferentially infects HIV-specific CD4 + T cells. Nature Nature Publishing Group. 2002;417:95–8.
10. Hamer DH. Can HIV be Cured? Mechanisms of HIV persistence and strategies to combat it. Curr HIV Res. 2004;2:99–111.
11. Archin NM, Liberty AL, Kashuba AD, Choudhary SK, Kuruc JD, Crooks AM, et al. Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. Nature. 2012;487:482–5.
12. Marsden MD, Loy BA, Wu X, Ramirez CM, Schrier AJ, Murray D, et al. In vivo activation of latent HIV with a synthetic bryostatin analog effects both latent cell “kick” and “kill” in strategy for virus eradication. PLoS Pathog. 2017;13:e1006575.
13. Rasmussen TA, Tolstrup M, Brinkmann CR, Olesen R, Erikstrup C, Solomon A, et al. Panobinostat, a histone deacetylase inhibitor, for latent-virus reactivation in HIV-infected patients on suppressive antiretroviral therapy: a phase 1/2, single group, clinical trial. The Lancet HIV. 2014;1:e13–21.
14. Søgaard OS, Graversen ME, Leth S, Olesen R, Brinkmann CR, Nissen SK, et al. The Depsipeptide Romidepsin Reverses HIV-1 Latency In Vivo. PLOS Pathogens Public Library of Science. 2015;11:e1005142.
15. Boehm D, Calvanese V, Dar RD, Xing S, Schroeder S, Martins L, et al. BET bromodomain-targeting compounds reactivate HIV from latency via a Tat-independent mechanism. Cell Cycle Taylor Francis. 2013;12:452–62.
16. Bullen CK, Laird GM, Durand CM, Siliciano JD, Siliciano RF. New ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo. Nature Medicine Nature Publishing Group. 2014;20:425–9.
17. Laird GM, Bullen CK, Rosenbloom DIS, Martin AR, Hill AL, Durand CM, et al. Ex vivo analysis identifies effective HIV-1 latency–reversing drug combinations. J Clin Invest American Society for Clinical Investigation. 2015;125:1901–12.
18. Marsden MD, Wu X, Navab SM, Loy BA, Schrier AJ, DeChristopher BA, et al. Characterization of designed, synthetically accessible bryostatin analog HIV latency reversing agents. Virology. 2018;520:83–93.
19. Sloane JL, Benner NL, Keenan KN, Zang X, Soliman MSA, Wu X, et al. Prodrugs of PKC modulators show enhanced HIV latency reversal and an expanded therapeutic window. PNAS National Academy of Sciences. 2020;117:10688–98.
20. Clutton G, Xu Y, Baldoni PL, Mollan KR, Kirchherr J, Newhard W, et al. The differential short- and long-term effects of HIV-1 latency-reversing agents on T cell function. Scientific Reports Nature Publishing Group. 2016;6:30749.
21. Jiang G, Dandekar S. Targeting NF-κB Signaling with Protein Kinase C Agonists As an Emerging Strategy for Combating HIV Latency. AIDS Research and Human Retroviruses. Mary Ann Liebert, Inc.,

publishers; 2014;31:4–12.

22. Demoustier A, Gubler B, Lambotte O, de Goër M-G, Wallon C, Goujard C, et al. In patients on prolonged HAART, a significant pool of HIV infected CD4 T cells are HIV-specific. AIDS. 2002;16:1749–54.
23. Shete A, Thakar M, Singh DP, Gangakhedkar R, Gaikwad A, Pawar J, et al. Short Communication: HIV Antigen-Specific Reactivation of HIV Infection from Cellular Reservoirs: Implications in the Settings of Therapeutic Vaccinations. AIDS Research and Human Retroviruses. Mary Ann Liebert, Inc., publishers; 2011;28:835–43.
24. Kristoff J, Palma ML, Garcia-Bates TM, Shen C, Sluis-Cremer N, Gupta P, et al. Type 1-programmed dendritic cells drive antigen-specific latency reversal and immune elimination of persistent HIV-1. EBioMedicine. 2019;43:295–306.
25. Kristoff J, Rinaldo CR, Mailliard RB. Role of Dendritic Cells in Exposing Latent HIV-1 for the Kill. Viruses. Multidisciplinary Digital Publishing Institute; 2020;12:37.
26. Kak G, Raza M, Tiwari BK. Interferon-gamma (IFN-γ): Exploring its implications in infectious diseases. Biomolecular Concepts De Gruyter. 2018;9:64–79.
27. Richman DD, Huang K, Lada SM, Sun X, Jain S, Massanella M, et al. Replication competence of virions induced from CD4 + lymphocytes latently infected with HIV. Retrovirology. 2019;16:4.
28. Ho Y-C, Shan L, Hosmane NN, Wang J, Laskey SB, Rosenbloom DIS, et al. Replication-Competent Noninduced Proviruses in the Latent Reservoir Increase Barrier to HIV-1 Cure. Cell. 2013;155:540–51.
29. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245–52.
30. Evans VA, Kumar N, Filali A, Procopio FA, Yegorov O, Goulet J-P, et al. Myeloid Dendritic Cells Induce HIV-1 Latency in Non-proliferating CD4 + T Cells. PLOS Pathogens Public Library of Science. 2013;9:e1003799.
31. Kumar NA, Sluis RM van der, Mota T, Pascoe R, Evans VA, Lewin SR, et al. Myeloid Dendritic Cells Induce HIV Latency in Proliferating CD4 + T Cells. The Journal of Immunology American Association of Immunologists. 2018;201:1468–77.
32. Rappocciolo G, Jais M, Piazza P, Reinhart TA, Berendam SJ, Garcia-Exposito L, et al. Alterations in cholesterol metabolism restrict HIV-1 trans infection in nonprogressors. mBio. 2014;5:e01031-01013.
33. Kern F, Faulhaber N, Frömmel C, Khatamzas E, Prösch S, Schönemann C, et al. Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides. Eur J Immunol. 2000;30:1676–82.
34. Kern F, Bunde T, Faulhaber N, Kiecker F, Khatamzas E, Rudawski I, et al. Cytomegalovirus (CMV) Phosphoprotein 65 Makes a Large Contribution to Shaping the T Cell Repertoire in CMV-Exposed Individuals. J INFECT DIS. 2002;185:1709–16.
35. Maecker HT, Dunn HS, Suni MA, Khatamzas E, Pitcher CJ, Bunde T, et al. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. J Immunol Methods. 2001;255:27–40.
36. Zaccard CR, Watkins SC, Kalinski P, Fecek RJ, Yates AL, Salter RD, et al. CD40L Induces Functional Tunneling Nanotube Networks Exclusively in Dendritic Cells Programmed by Mediators of Type 1

Immunity. The Journal of Immunology American Association of Immunologists. 2015;194:1047–56.

37. Altfeld MA, Trocha A, Eldridge RL, Rosenberg ES, Phillips MN, Addo MM, et al. Identification of Dominant Optimal HLA-B60- and HLA-B61-Restricted Cytotoxic T-Lymphocyte (CTL) Epitopes: Rapid Characterization of CTL Responses by Enzyme-Linked Immunospot Assay. J Virol. 2000;74:8541–9.
38. Strain MC, Lada SM, Luong T, Rought SE, Gianella S, Terry VH, et al. Highly precise measurement of HIV DNA by droplet digital PCR. PLoS ONE. 2013;8:e55943.

Figures

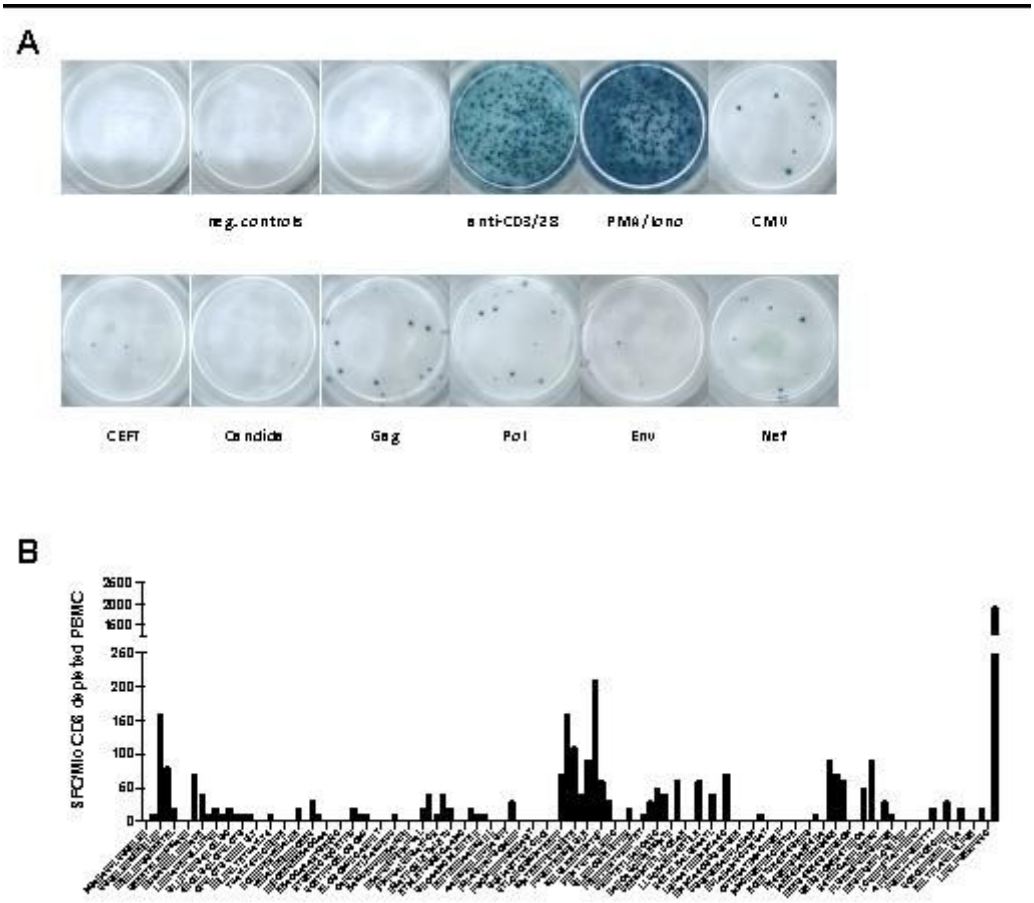

Figure 1

Representative IFN-γ ELISpot. A: CD4 T cell response to pooled 15mer peptides with 11 amino acid overlap, specific for CMV pp65 (138 peptides), CEFT (14 peptides selected from defined HLA class II-restricted T-cell epitopes of Clostridium tetani, Epstein-Barr virus (HHV-4), Human cytomegalovirus (HHV-5), and Influenza A), HIV consensus B Gag (123 peptides), HIV consensus B Env (211 peptides), HIV consensus B Pol (249 peptides), HIV consensus B Nef (49 peptides). Example wells are shown for each peptide pool; the examples are from different participants. B: CD4 T cell responses of one representative participant towards 123 individual overlapping 15mer HIV consensus B Gag peptides. The x-axis shows the sequence of every other single peptide.

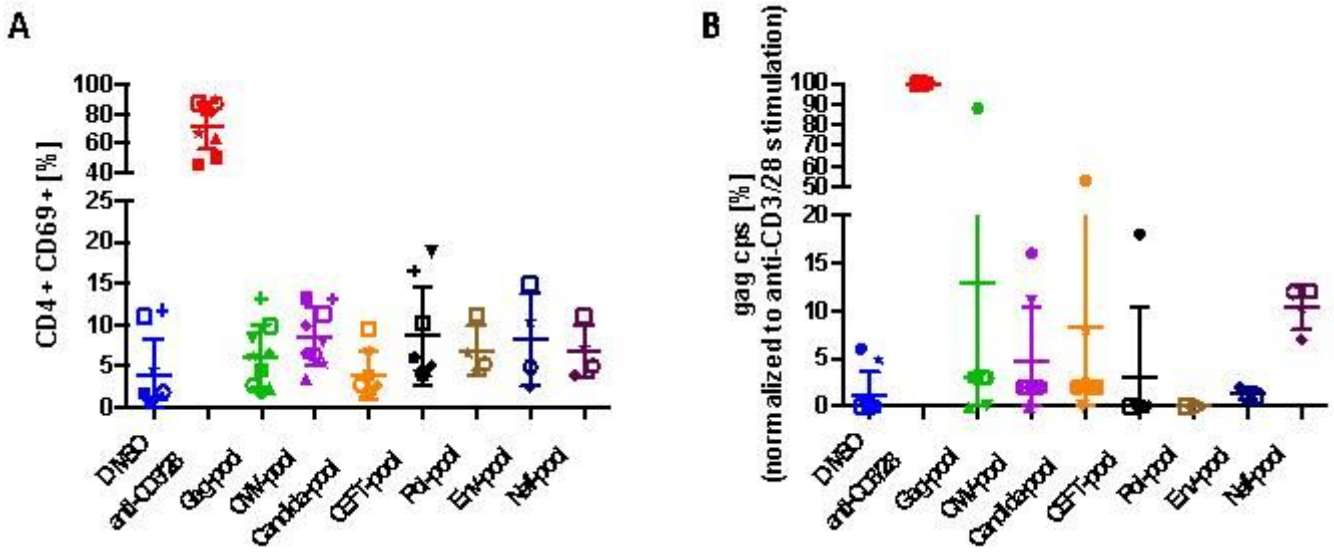

Figure 2

Immune activation and latency reversal after stimulation of CD8-depleted PBMC with antigenic peptide pools measured by real-time RT-qPCR of released virions. A: Flow cytometric detection of CD69 expression levels on CD4 T cells after 48 hours of stimulation with the indicated peptide pools. All peptide pools were used at a concentration of 1 µg/ml per peptide. B: Expression levels of cell-free HIV Gag RNA after seven days of stimulation with the indicated peptide pools measured by real-time RT-qPCR detecting HIV Gag (n = 9). Cells were incubated in the presence of raltegravir to prevent viral propagation. The values graphed are the means of quadruplicate samples (5x106 CD8-depleted cells for each sample) for each participant and each condition. Each participant's cells are shown using a distinct symbol; the open and closed circles are cells from the same participant obtained on two occasions approximately one year apart. Values were normalized to the positive control (antibodies to CD3 and CD69) for each participant. DMSO is the negative control.

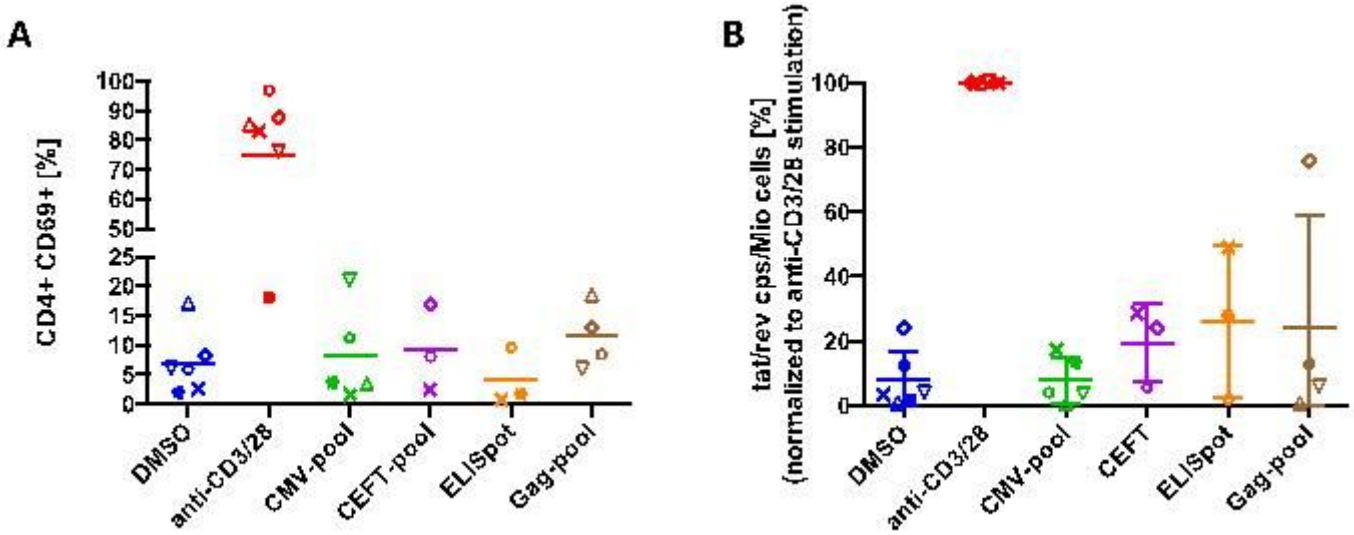

Figure 3

Immune activation and latency reversal after stimulation of CD8-depleted PBMC with antigen peptide pools measured by droplet digital (dd)PCR of cellular Tat/Rev mRNA. A: Flow cytometric detection of CD69 expression levels on CD4 T cells after 48 hours stimulation with the indicated peptide pools. All peptide pools were used at a concentration of 1 µg/ml per peptide. B: Expression levels of cell-associated multiply spliced HIV Tat/Rev after five days stimulation with the indicated peptide pools (n = 6). Nine-million cells from each participant were stimulated as indicated, then plated in three-fold limiting dilutions and six replicates. "ELISpot" refers to a custom, participant-specific Gag-peptide pool selected by INF-γ ELISpot assay of individual peptides. Cells were incubated in the presence of raltegravir to prevent viral propagation. Each participant's cells are shown using a distinct symbol. Some participant's cells were not tested with every peptide pool. Values were normalized to the positive control (antibodies to CD3 and CD69) for each participant. DMSO is the negative control.

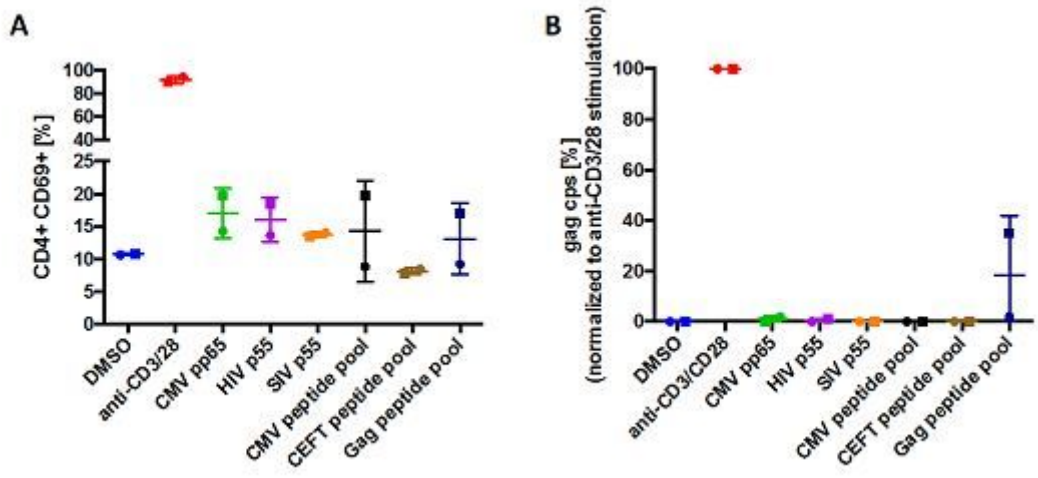

Figure 4

Latency reversal by co-culture with antigen-loaded dendritic cells (DC). A: Flow cytometric detection of CD69 expression levels on CD4 T cells after 48 hours stimulation with the indicated peptide pools or whole proteins and DCs. All peptide pools were used at a concentration of 1 µg/ml per peptide. B: Expression levels of cell-free HIV Gag RNA measured by real-time RT-qPCR after seven days of co-stimulation with DC presenting the indicated proteins and peptide pools at a concentration of 1 µg/ml per peptide in the presence of 1 µM raltegravir (RAL). Each participant's cells are shown using a distinct symbol (n = 2).