Type 1-programmed dendritic cells drive antigen-specific latency reversal and immune elimination of persistent HIV-1

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Background: Despite the success of antiretroviral therapy (ART), latent HIV-1 continues to persist in a long-lived population of resting memory CD4+ T cells within those who are infected. Finding a safe and effective means to induce latency reversal (LR) during ART to specifically expose this latent HIV-1 cellular reservoir for immune elimination has been a major barrier to a functional cure.

Methods: In this study, we test the use of antigen-presenting type 1-polarized, monocyte-derived dendritic cells (MDC1) generated from chronic HIV-1-infected individuals on ART as a means to induce HIV-1 latency reversal in autologous CD4+ T cells harboring replication-competent provirus. We use the same MDC1 for ex vivo generation of autologous HIV-1 antigen-specific CD8+ cytotoxic T cells (CTL) and test their effector responses against the MDC1-exposed HIV-1-infected CD4+ T cell targets.

Findings: MDC1 presentation of either HIV-1 or cytomegalovirus (CMV) antigens to CD4+ T cells facilitated HIV-1 LR. This antigen-driven MDC1-mediated LR was sharply diminished with blockade of the CD40L/CD40 ‘helper’ signaling pathway. Importantly, these antigen-presenting MDC1 also activated the expansion of CTL capable of killing the exposed HIV-1-infected targets.

Interpretation: Inclusion of virus-associated MHC class II ‘helper’ antigens in MDC1-based HIV-1 immunotherapies could serve both as a targeted means to safely unmask antigen-specific CD4+ T cells harboring HIV-1, and to support CTL responses that can effectively target the MDC1-exposed HIV-1 cellular reservoir as a functional cure strategy.

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1. Introduction

Despite the success of antiretroviral therapy (ART), HIV-1 is managed as a chronic disease due to its persistence in a long-lived population of resting memory CD4+ T cells [1]. This latent reservoir of inducible, replication-competent HIV-1 in ART-suppressed individuals is considered a critical barrier to a cure [2], since the lack of viral protein expression in latently infected cells allows the reservoir to escape immune surveillance. The ‘kick and kill’ (or ‘shock and kill’) approach to controlling HIV-1 involves inducing HIV-1 latency reversal (LR) during ART to expose infected cells, while creating an arsenal of immune effector cells, such as cytotoxic T lymphocytes (CTL), capable of eliminating these targets [3]. Successful CTL targeting of the latent HIV-1 reservoir will require recognition of HIV-1-associated peptide epitopes presented on infected cells after LR. Besides latency itself, major hurdles for effective CTL elimination of HIV-1 infected cells include issues related to CD8+ T cell exhaustion [4], alterations in CTL epitopes, antigen processing, and antigen presentation associated with immune escape [5,6]; the establishment of epitope variants that act as partial agonists to induce dysfunctional noncytolytic cross-reactive memory CTL responses [7,8], and presentation of target antigen decoys by cells harboring defective virus [9]. Together, these points highlight the need to generate highly functional CTL either through induction of de novo CD8+ T cell responses, or subdominant memory responses targeting relevant conserved epitopes of the reservoir-associated virus.

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Finding a nontoxic, effective means to purge the latent HIV-1 reservoir remains a major obstacle to a functional cure. The 'kick and kill' approach to controlling HIV-1 involves inducing HIV-1 latency reversal (LR) during antiretroviral therapy (ART) to expose infected cells, while creating an arsenal of immune effector cells, such as cytotoxic T lymphocytes (CTL), capable of eliminating these targets. While pharmacological latency reversal agents (LRAs) have achieved limited success in ex vivo studies, they have yet to be found effective at reducing the latent reservoir in HIV-1-infected individuals. In addition, some LRAs have been shown to negatively impact antigen-specific CD8+ T cell effector responses in vitro. Besides latency itself, major hurdles for effective CTL elimination of HIV-1 infected cells include issues related to CD8+ T cell exhaustion, alterations in CTL epitopes, antigen processing, and antigen presentation associated with immune escape; the establishment of epitope variants that act as partial agonists to induce dysfunctional noncytolytic cross-reactive memory CTL responses, and presentation of target antigen decoys by cells harboring defective virus. Thus, an optimal cure strategy must address not only induction of proviral gene expression but also clearance of reactivated cells presenting HIV-1-associated peptide epitopes by either highly functional CTL, or through incorporation of other immune-based strategies, including vaccines and adjuvants, broadly neutralizing antibodies, or compounds modulating pro-apoptotic pathways.

**Add value of this study**

Conventional dendritic cells (DC) have been safely and widely used in both cancer and HIV-1 clinical trials for their capacity to induce antigen-specific T cell responses, but their HIV-1 LRA potential has been underexplored. Though not designed to specifically address their function as a therapeutic LRA, a recent study suggested a link between administration of a DC-based HIV-1 vaccine and increased residual viremia in ART-suppressed individuals prior to analytic treatment interruption. Here we report that antigen-presenting pro-inflammatory type 1-polarized monocyte-derived dendritic cells (MDC1) generated from chronic HIV-1-infected individuals on ART can induce HIV-1 LR in autologous CD4+ T cells. Importantly, this effect of driving the virus out of latency was facilitated through the strategic use both CMV- and HIV-associated antigen to specifically promote MHC-class II antigen restricted interactions between CD4+ T cells responsive to these pathogens and MDC1. Furthermore, we demonstrate the potential of this single MDC1-based therapeutic tool to promote both the antigen-specific exposure (the 'kick') and CTL killing (the 'kill') of CD4+ T cells harboring replication-competent provirus.

**Implications of all the available evidence**

To date, clinical trials of pharmacological LRAs have demonstrated minimal or no reduction in the latent reservoir in vivo. De- liberatedly programmed to release high amounts of the critical CTL-inducing cytokine IL-12p70 upon interaction with the CD4+ T helper cell factor CD40L, clinically applicable MDC1 are uniquely capable of both activating HIV-1 transcription in latently infected CD4+ T cells and inducing broad HIV-1-specific CTL responses. Strategic inclusion of virus-associated MHC class II 'helper' antigens in MDC1-based HIV-1 immunotherapies could serve both as a targeted means to safely unmask virus antigen-specific CD4+ T cells harboring HIV-1, and to support CTL responses that can effectively target the MDC1-exposed HIV-1 reservoir as a functional cure strategy.

Finding an effective means to expose and purge the latent reservoir in a nontoxic manner has been elusive and remains a major hurdle to this cure approach. While pharmacological latency reversal agents (LRAs) have achieved limited success in ex vivo studies, none have been shown to reduce the latent reservoir in HIV-1-infected individuals [10]. Although the protein kinase C (PKC) agonist bryostatin-1 was shown to achieve T cell activation comparable to that induced by PHA/IL-2 stimulation in vitro and ex vivo [10,11], its use in cancer clinical trials resulted in serious adverse events [12–15]. Furthermore, in a phase I HIV-1 clinical trial, tolerable but conservative drug dosing of bryostatin-1 prevented it from reaching detectable systemic concentrations associated with PKC activation and from reactivating latent HIV-1 reservoirs [16].

Some LRAs, specifically histone deacetylase inhibitors and PKC modulators, can negatively impacting antigen-specific CD8+ T cell effector responses in vitro [11,17,18]. However, it is unclear if this negative effect on HIV-1-specific immunity occurs with their use in vivo. More promising data, have emerged from studies utilizing TLR agonists as LRAs, including those targeting TLR7 [19–22]. This strategy to target such germline-encoded innate immune activation receptors effectively contributes to impacting the latent reservoir and delaying viral rebound. In nonhuman primate models, particularly when combined either with therapeutic vaccination using Ad26/MVA (recombinant adenovirus serotype 26 prime/modified vaccinia Ankara boost) expressing gag, pol and env [20], or with HIV-broadly neutralizing antibody (bNAb) therapy [21]. While promising, the potential for toxicity associated with TLR-induced broad activation of innate immune cells exists. These findings support the need to continue exploring eradication strategies to reverse HIV latency without inducing nonspecific global immune activation [23], while enhancing HIV antigen-specific adaptive immunity.

Although conventional dendritic cells (DC) have been safely used in HIV-1 clinical trials for their capacity to induce antigen-specific T cell responses [24–27], their HIV-1 LRA potential has been underexplored. Interestingly, a study from our group suggested a link between administration of a DC-based HIV-1 therapeutic and increased residual viremia in ART-suppressed individuals prior to analytic treatment interruption [28]. However, that study was not designed to specifically address the use of the DC therapeutic as an LRA. Thus a number of important questions remain, including the roles that DC polarization status and antigen presentation could have in the noted phenomenon, and the underlying mechanisms involved.

We hypothesize that under optimal conditions, a DC-based therapeutic strategy can be designed to safely facilitate both the 'kick' and 'kill' of the latent HIV-1 reservoir. Here, we show that clinically applicable, type 1-polarized, monocyte-derived DC (MDC1) are uniquely capable of both activating HIV-1 transcription in latently infected CD4+ T cells harboring replication-competent virus and inducing broad HIV-1-specific CTL responses that can effectively target the exposed infected cells. To promote strong HIV-1-specific CTL responses, these antigen-presenting MDC1 are deliberately programmed to subsequently release high amounts of the critical CTL-inducing cytokine IL-12p70 upon interaction with the CD4+ T helper cell factor CD40L [29]. We found that the strategic inclusion of heterologous cytomegalovirus (CMV)-associated antigen designed to encourage such CD4+ T cell ‘helper’ activity through MHC class II presentation also facilitated MDC1-mediated LR. The demonstrated antigen-dependent LRA activity of CMV and HIV-1 antigen-presenting MDC1 suggests that CD4+ T cells having antigen specificity to these viruses contribute to the latent reservoir, thus
offering a safe and directed means to immunologically expose and target this compartment as part of a functional cure strategy for HIV-1 infection.

2. Materials and methods

2.1. Study participants

HIV-1-infected ART-treated participants of the Pittsburgh clinical site of the Multicenter AIDS Cohort Study (MACS) were selected for this research. These participants were documented as having begun ART with a median virally controlled treatment duration of 12.3 years (range 1.7–20.8 years; Table S1). Whole blood products from HIV-1-negative blood donors were purchased from the Central Blood Bank of Pittsburgh. Written informed consent was obtained from participants prior to inclusion in the study. The University of Pittsburgh Institutional Review Board approved this study.

2.2. Isolation of monocytes and peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMC) obtained from buffy coat or whole blood were isolated by standard density gradient separation using Lymphocyte Separation Medium (Corning Cat# 25–072-CV). PBMC were further separated into monocytes and peripheral blood lymphocytes (PBL) using a positive selection human CD14 microbeads kit (Miltenyi Biotec Cat# 130–05-201; RRID: AB_2665482) according to manufacturer’s specifications, and the differentially isolated cells were cryopreserved until use.

2.3. Generation of monocyte-derived DC

Immature DC were generated from monocytes isolated and cultured in Iscove’s Modified Dulbecco’s Media (IMDM; Gibco Cat# 12404–053) containing 10% fetal bovine serum Atlanta biologicals media alone, and incubating with CD107a-FITC (clone H4A3; BD Biosciences Cat# 6604665). Antigen-specific CTL responses were assessed by exposing CTL to HIV-1 Gag 9–mer peptides (1 μg/mL) or media alone, and incubating with CD107a-FITC (clone HA5.2B7; Beckman Coulter Cat# IM2729U), CCR7-FITC (clone 150503; BD Biosciences Cat# IM2218U), CD86-PE (clone 3D12; BD Biosciences Cat# 558164; RRID: AB_3961344) or LeafProtin® influenza A (H1N1), eEnzyme Cat# IA-1M01-023P, or PepTivator® Influenza A (H1N1) MP1, Miltenyi Biotec Cat# 130-097-285). Briefly, total CD4+ T cells were isolated from cryopreserved PBL derived from HIV-1-infected MACS participants by negative magnetic bead separation using an EasySep™ Human CD4+ T Cell Enrichment Kit (STEMCELL Technologies Cat# 19052). CD4+ T cells were cocultured with DC in complete IMDM at a ratio of either 1:7 (100,000 DC: 750,000 CD4+ T cells) or 1:10 (100,000:1 × 10^6) for seven days in 48-well plates. Total CD4+ T cells from HIV-1-infected MACS participants were treated with Dynabeads® Human T-Activator CD3/CD28 (Life Technologies Cat# 11131D) and implementation as a positive control in LR experiments. The cytokines rhIL-2 (Proleukin®, 100 U/mL; Prometheus Laboratories, Inc. Cat# N301), Stop Solution (Thermo Scientific Cat# N100), TMB Substrate Solution (Thermo Scientific Cat# N301), Stop Solution (Thermo Scientific Cat# N600).

2.6. Induction of HIV-1 LR in CD4+ T cells

MDC1 were tested for their ability to induce HIV-1 LR by coculture with autologous CD4+ T cells in the presence of sebm (Sigma-Aldrich Cat# #54481), CMV pp65 (CM/PP65 Recombinant Protein, Miltenyi Biotec Cat# 130-091-824, or PepTivator® CMV pp65, Miltenyi Biotec Cat# 130-093-435), HIV-1 Gag (HIV-1 IIB PR55 Gag Recombinant Protein, NIH AIDS Reagent Program Cat# 3276; HIV-1 Gag Recombinant Protein, Sigma-Aldrich Cat# H0160; or HIV-1 Consensus 15-mer Peptides (Sigma-Aldrich), or influenza M1 antigen (Influenza M1 Protein [A/California/04/2009] (H1N1), eEnzyme Cat# IA-M1-023P, or PepTivator® Influenza A (H1N1) MP1, Miltenyi Biotec Cat# 130-097-285). Briefly, total CD4+ T cells were isolated from cryopreserved PBL derived from HIV-1-infected MACS participants by negative magnetic bead separation using an EasySep™ Human CD4+ T Cell Enrichment Kit (STEMCELL Technologies Cat# 19052). CD4+ T cells were cocultured with DC in complete IMDM at a ratio of either 1:7 (100,000 DC: 750,000 CD4+ T cells) or 1:10 (100,000:1 × 10^6) for seven days in 48-well plates. Total CD4+ T cells from HIV-1-infected MACS participants were treated with Dynabeads® Human T-Activator CD3/CD28 (Life Technologies Cat# 11131D) and implemented as a positive control in LR experiments. The cytokines rhIL-2 (Proleukin®, 100 U/mL; Prometheus Laboratories, Inc. Cat# N301), Stop Solution (Thermo Scientific Cat# N100), TMB Substrate Solution (Thermo Scientific Cat# N301), Stop Solution (Thermo Scientific Cat# N600).
2.7. Relative quantification of HIV-1 gag RNA

Culture supernatants were ultra-centrifuged (Sorvall Stratos Biofuge) at 45,000 x g for 1 h at 4 °C to obtain viral pellets from which total RNA was isolated by the RNA-Bee™ method (TEL-TEST, Inc. Cat# CS-105B). Five microliters of RNA were used for detection of reverse transcription using TaqMan® Reverse Transcription Reagents (Life Technologies Cat# N8080234), according to the manufacturer’s protocol. A 20 μl TaqMan® PCR was performed by mixing 5 μl cDNA with TaqMan® Universal PCR Master Mix (Thermo Fisher Cat# 4364340), 500 nM each of forward (5′-CCATGTGTTTACGATCCAA-3′), reverse (5′-CCATGTTTACGATCCAA-3′) primers and probes (5′-CCATGTTTACGATCCAA-3′), and 250 nM FAM/-MGB-labeled probe (5′-CCATGTTTACGATCCAA-3′; Thermo Fisher, Cat# 4316033, Thermo Fisher, Cat# 4316033, Thermo Fisher). Real-time PCR was performed using the ViA 7 A&B Applied Biosystems instrument (Life Technologies) and the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Real-time PCR primers and probes were based on the HIV-1 pNL4.3 sequence encoding the gag region. Serially diluted pNL4.3 plasmid DNA ranging from 101 to 106 copies applied to each PCR assay served as the HIV-1 standard curve. A no template control was included in each assay to control for cross-contamination, and each sample was assayed in triplicate. QuantStudio™ Real-time PCR Software (Applied Biosystems, Foster City, CA) was used for PCR data analysis.

2.8. Generation and characterization of HIV-1-infected CD4+ T cell targets

Total CD4+ T cells cocultured with antigen-presenting MDC1 and were tested weekly for the presence of HIV-1 p24 antigen by intracellular flow cytometry staining with KC57-FITC antibody (clone FH190–1–1, Beckman Coulter Cat# 6604665). Target cells were pre-screened for p24 expression, and cryopreserved for later use as targets when they reached at least 10% positivity.

2.9. Induction and expansion of autologous CTL

Total CD8+ T cells were isolated from cryopreserved PB L by negative magnetic bead separation using an EasySep™ Human CD8+ T Cell Enrichment Kit (STEMCELL Technologies Cat# 19053). To induce CTL responses as previously described [7], CD8+ T cells were cocultured with autologous differentially matured DC loaded with either HLA-A2-restricted Gag p24 Gag151–159 9-mer peptide epitopes when using HIV-1-negative blood donors, or Gag p17/p24 overlapping 15-mer peptides (1 μg/mL, Sigma-Aldrich) when using HIV-1-infected patients. The cocultures (75,000 DC: 750,000 CD8+ T cells) were treated with or without the addition of either 25,000 gamma-irradiated (5000 rad) CD40L-transfected J588 cells [30] or MEGACD40L® Protein (0.25 μg/mL; Enzo Life Sciences Cat# ALX-522-110-C010) where stated. On day 5, rhIL-2 (250 U/mL) and rhIL-7 (10 ng/mL) were added to the cultures and every three days thereafter. On day 12, T cell cultures were restimulated with either gamma-irradiated HLA-A2+ T2 cells (for induction of primary CTL responses in HLA-A2+ HIV-1-negative donors) or differentially matured autologous DC loaded with autologous 9-mer peptides (1 μg/mL) corresponding to the viral antigens and DC type used in the initial stimulation. Antigen-specific readout assays were performed between days 20–24 to assess CTL activity.

2.10. IFN-γ ELISPOT assays

Autologous CTL (3 x 106/well) were tested for reactivity to individually and pooled Gag 9-mer peptide antigens (1–10 μg/mL) by ELISPOT assay using anti-human IFN-γ and biotin monoclonal antibodies (clones 1-D1K and 7–B6–1; Mabtech Cat# 3420–6–1000) as previously described [7,8]. Recorded values were net responses compared to control wells consisting of CTL exposed to assay medium alone.

2.11. HIV-1 antigen-expressing cell killing assays

CTL effector function was assessed as described previously, with modifications [8]. Briefly, MDC1-stimulated total CD8+ T cells were cocultured with autologous MDC1-induced CD4+ target cells at various effector:target (E:T) ratios for 18 h at 37 °C. Harvested cocultures were stained for surface expression of CD8 (PerCP-Cy5.5, clone SK1; BD Biosciences Cat# 341051; RRID: AB_400298) and intracellular expression of HIV-1 p24 (KC57–FITC, clone FH190–1–1; Beckman Coulter Cat# 6604665). Effector CD8+ cells were excluded from analysis gating, and the percent reduction in infected CD4+ T cells was determined at each E:T ratio. For the colorimetric cytolytic assays described in the supplemental material, autologous CD4+ T cells were stained with either CFSE (eBioscience Cat# 65–0850) or CellTrace™ Violet (Thermo Fisher, Cat# C34557) dyes following the manufacturer’s protocols. Target cells (CFSE) were then loaded with individual peptides at 100 ng/mL in PBS for 60 min at room temperature (RT); excess unbound peptide was removed by washing. The CFSE and CellTrace Violet dye-labeled cells were mixed in equal numbers and coincubated for 18 h with autologous CTL at various E:T ratios. The antigen-specific killing of HIV-1 peptide-loaded CD4+ T cells (green) was calculated based on relative changes in percentages of the differentially stained target cells remaining, using by flow cytometry analysis.

2.12. Viral outgrowth assays

Total CD8+ T cells were cocultured with autologous p24-expressing CD4+ target cells at various E:T ratios as described for the CTL kill assay. Cultures were maintained for eight days, after which culture supernatants were harvested and tested by p24 ELISA (Frederick National Laboratory for Cancer Research, Frederick, MD) for CTL-induced viral suppression [7].

2.13. Quantification of replication-competent HIV-1

Culture supernatants harvested from LR and viral outgrowth assays were spinoculated onto TZM-bl cell (NIH AIDS Reagent Program Cat# 8129–422; RRID: CVCL_B478) monolayers (30,000 cells/well) at 300 hours at 300 g and cocultured for 48 h. Beta-Glo® reagent (Promega Cat# E4740) was added to PBS-washed TZM-bl cell monolayers and incubated for 1 h at room temperature. Control supernatants from cultured CD4+ T cells of an uninfected donor were treated in parallel. Chemiluminescence from the TZM-bl cells was detected by luminometer as previously described [31]. Sample wells were considered positive for the presence of replication-competent virus if the chemiluminescent signal exceeded the mean + 2 S.D. of a control sample.

2.14. Statistical analyses

Statistical analyses for ELISA and ELISPOT data (Fig. 1b and c) were calculated using Wilcoxon matched-pairs signed-ranks test and a linear mixed model with 95% confidence intervals, respectively. Differences between MDC1-mediated LR were determined by multilevel mixed-effects tobit regression analyses (Figs. 2, 4a) and Wilcoxon matched-pairs signed-ranks test (Figs. 3, 4c–d).

3. Results

3.1. MDC1 effectively induce primary HIV-1-specific CTL responses with CD40L ‘help’

We initially compared the use of two clinically applicable, differentially activated DC preparations using blood products from HLA-A2+ HIV-1-naïve blood bank donors to test their capacity to induce primary HIV-1-specific CTL responses. MDC1 were characterized and defined by
their mature phenotypic status, expressing high levels of CD86 and CD83, and their high capacity to produce IL-12p70 upon subsequent stimulation with the CD4+ T cell 'helper' signal CD40L, while PGE2-DC were IL-12p70-deficient and less responsive to CD40L signaling (Fig. 1a and b, and Fig. S1) [29,32]. These DC types were loaded with HLA-A2-restricted HIV-1 peptide antigen and used as in vitro stimulators of autologous CD8+ T cells, in the presence of gamma-irradiated (5000 rad) CD40L-expressing J558 cells (J558-CD40L), which served as a CD40L+ TH cell surrogate. In doing so, we found that the MDC1 had a higher CTL priming capacity compared to PGE2-DC (Fig. 1c). Importantly, the effective in vitro induction of long-term CTL responses by MDC1 required the presence of CD40L 'help' during the initiation of the priming cocultures (Fig. 1d).

3.2. Antigen presentation by autologous MDC1 drives HIV-1 LR in CD4+ T cells

Recent evidence linked a DC therapeutic with an increase in residual HIV-1 viremia while the study participants were on ART, suggesting that the DC therapeutic acted in some way as an LRA [28]. However, the mechanisms involved in this phenomenon are not yet clear, including the role that antigen presentation may have played. Because MDC1 were shown to be strong inducers of primary CTL responses (Fig. 1), we next tested their capacity to reactivate or 'kick' latent HIV-1 from latency to expose the infected cells for subsequent CTL targeting. MDC1 were generated from ART virally suppressed HIV-1-infected MACS participants and cocultured with autologous peripheral blood CD4+ T cells in the presence or absence of SEB antigen. SEB was used because as a superantigen, it can effectively facilitate immune cross-talk between the antigen-presenting cells and a large percentage (~30%) of SEB responsive T cells [29]. CD4+ T cells treated with anti-CD3/CD28 mAb-coated beads were used as a positive LRA control [33]. qRT-PCR analysis of HIV-1 RNA presence in day 7 coculture supernatants revealed that MDC1 indeed acted as a strong LRA in an SEB antigen-dependent manner (Fig. 2, MDC1 alone vs MDC1 + SEB, P < 0.05).

3.3. Role of CD40/CD40L interaction in MDC1-mediated 'kick' of latent HIV-1

DC crosstalk with CD40L+ T cells plays a critical role in the induction and survival of long-term CTL responses [34–37]. Because we previously showed that MDC1 are particularly sensitive to CD40L signaling [29], and that this CD4+ T cell-derived 'helper' factor is required for effective MDC1-mediated in vitro priming of de novo CTL responses...
we wanted to determine if CD40/CD40L cross-talk between the MDC1 and CD4+ T cells was playing a role in the MDC1-mediated HIV-1 LR. Indeed, we found that blocking CD40/CD40L interaction strongly decreased the effectiveness of MDC1-mediated LR. The impact of this CD40L signaling inhibition on MDC1-mediated LR was clearly evident when analyzing the activated CD4+ T cells by flow cytometry, where the addition of an anti-CD40L blocking antibody resulted in a marked inhibition of CD4 downregulation (87.7% ± 3.1%, \( P < 0.05 \); Fig. 3a and b), a phenomenon associated with HIV-1 protein translation [38]. As expected, this inhibition of CD4 downregulation by addition of the CD40L blocking antibody was associated with abrogation of intracellular p24 expression (90.8% ± 7.0%) (Fig. 3a, c; \( P < 0.05 \)) induced in autologous CD4+ T cells, and with the reduction in HIV-1 RNA content in the day 7 coculture supernatants measured by qRT-PCR (Fig. 3d, 94.1% ± 6.1% inhibition). Importantly, the addition of an isotype control antibody to the MDC1:T cell cocultures had no significant impact on the induced changes in CD4 expression or HIV-1 expression resulting from LR (Fig. 3a-d). Taken together, these data support the required involvement of cognate antigen-driven bidirectional signaling events between MDC1 and antigen-responsive CD4+ T cells in HIV-1 LR.

We have shown that MDC1-mediated transcription of HIV-1 DNA (Fig. 2) and subsequent translation of p24 (Fig. 3a, c) are both dependent on the presence of SEB superantigen and on CD40/CD40L signaling. However, to simulate a clinically relevant method of HIV-1 LR, we posited that the inclusion of common viral MHC class II antigens as part of our MDC1-based therapeutic could promote interaction with CD40L-expressing CD4+ TH cells, to both provide immune ‘help’ for MDC1-mediated induction of HIV-1-specific CTL responses and facilitate MDC1-mediated exposure of viral antigen-specific CD4+ T cells harboring latent HIV-1.

In choosing which viral antigens to incorporate in our model of MDC1-mediated LR, we considered previous findings that a significant pool of latently infected CD4+ T cells are HIV-1-specific [39,40]. As such, an HIV-1-based vaccine or LRA construct could potentially reactivate this population while also facilitating the ‘kill’ through CTL priming. We also considered the fact that approximately 95% of HIV-1-infected individuals are coinfected with CMV [41], in whom CMV-specific CD4+ T cell memory infiltration occurs [42], with some having greater than 25% of their T cells specific to CMV [43]. Therefore, we
**Fig. 4.** CMV and HIV antigen presentation drives MDC1-mediated HIV-1 latency reversal. 

a) MDC1 were loaded with either CMV pp65, HIV-1 Gag, or influenza M1 antigen and tested for their ability to induce LR in autologous CD4^+^ T cells. Culture supernatants were assayed by qRT-PCR for detection of HIV-1 RNA at day 7. 

b) Representative flow cytometry plots of p24 expression of day 20 cocultures, gated on total CD4^+^ T cells. 

c) Graphical representation of MDC1/antigen-induced CD4 downregulation in cocultures described in (b), as measured by flow cytometry. 

d) Expression of p24 expression in cell populations detailed in (b). Each colour in (c) and (d) represents an individual study participant. 

P values comparing viral RNA levels were determined by multilevel mixed-effects tobit regression analyses. Error bars indicate mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.

**Fig. 5.** MDC1-induced broadly reactive HIV-1-specific CTL effectively kill HIV-1 infected CD4^+^ T cells exposed by MDC1. 

a) MDC1 generated from HIV-1-infected, ART-suppressed individuals were loaded with HIV-1 Gag peptides and used to induce broadly reactive antigen-specific autologous CTL as determined by flow cytometry staining for CD107a and IFN-γ. 

b) Polyclonal IFN-γ responses to individual Gag 9-mer epitopes by MDC1-induced CTL described in (a). 

c) HIV-1 latency reversal was induced by MDC1 and SEB or antigen in CD4^+^ T cells. Target cells (T) were coincubated with autologous MDC1-induced effector CTL (E) at various E:T ratios for 18 h. CTL-induced target killing was measured by loss of HIV-1 Gag p24-expressing target cells using flow cytometry. 

d) Summary of 5 independent flow cytometry cytotoxicity experiments. Square symbols represent MDC1/SEB-induced target cells; circles indicate MDC1/viral antigen-induced targets. Error bars indicate mean ± S.D.
hypothesized that inclusion of heterologous CMV antigen would effectively promote MDC1 interaction with CD4+ T cells to facilitate ‘help’ for HIV-1 specific CTL induction and to induce CMV antigen-specific MDC1-mediated LR.

We tested MDC1 alone or loaded with CMV pp65, HIV-1 Gag, or influenza A virus M1 protein (representing a common, non-persistent virus antigen) for their ability to induce latency reversal in autologous CD4+ T cells (Fig. 4). We found that MDC1-mediated LR was antigen-dependent, with CMV and HIV-1 antigen having notable LR activity, while the influenza virus antigen did not (Fig. 4a–d). MDC1/CMV- and MDC1/HIV-1–mediated increases in extracellular virion-associated HIV-1 RNA were significantly greater than those induced by MDC1 alone (Fig. 4A; P < 0.01 and P < 0.001, respectively). Importantly, MDC1 presenting either CMV or HIV-1 antigen exposed latent HIV-1 cellular reservoir targets, identified by a marked downregulation in CD4 expression (Fig. 4c) that corresponded to increases in intracellular p24 (Fig. 4b, d).

3.5. MDC1-induced CTL effectively kill MDC1-exposed CD4+ T cell targets harboring replication-competent HIV-1

MDC1 loaded with autologous HIV-1 Gag peptides were used to induce antigen-specific CTL, as determined by flow cytometry analysis for antigen-induced expression of CD107a and interferon (IFN)-γ (Fig. 5a) and by IFN-γ ELISPOT (Fig. 5b). Antigen-induced downregulation of CD8 expression, a characteristic previously shown to be associated with enhanced cytolytic capacity [7,44], was evident along with high expression of CD107a and IFN-γ in the CTL generated ex vivo using the HIV-1 antigen-presenting MDC1 (Fig. 5a). Also, the CTL responses induced by MDC1 were broadly reactive to a range of individual Gag 9-mer epitopes by IFN-γ ELISPOT (Fig. 5b). The antigen-specific killing capacity of these CTL was initially tested by coculturing them overnight with differentially labeled Gag 9-mer peptide antigen-loaded or antigen negative (control) autologous CD4+ T cell targets, which clearly showed the selective elimination of the antigen-loaded target cells as
determined by flow cytometry analysis (Fig. S2a, b). More importantly, the LR activity of CMV and HIV-1 antigen-presenting MDC1 resulted in the effective exposure of HIV-1-infected antigen-expressing target cells that were also recognized and efficiently controlled by the MDC1/Ag-induced HIV-1-specific CTL in short-term cytotoxicity assays (75.4% ± 14.3% killing), indicated by a CTL dose-dependent decrease of p24-expressing CD4+ T cells (Fig. 5c, d; S2c, d). This pattern was consistent regardless of the type of antigen used to induce MDC1-mediated LR. Furthermore, the CTL suppressed viral outgrowth from the infected cells in long-term cocultures by 74.4% ± 20.2%, as measured by p24 ELISA (Fig. 6a, Right).

Recent studies have posited that CTL preferentially target cells containing defective HIV-1 proviruses, which in effect act as decoy targets to prevent elimination of the true latent reservoir [9,45,46]. Therefore, we sought to determine whether MDC1-mediated LR activity unmasked cells harboring replication-competent virus that could be subsequently recognized and killed by the CTL. To do so, culture supernatants collected from viral outgrowth assays at various effector-to-target ratios (Fig. 6a) were subsequently cultured on TZM-bl reporter cells [31] for quantification of infectious HIV-1 (Fig. 6b). Importantly, we found that MDC1 LR activity exposed those targets harboring replication-competent virus, whose dose-dependent elimination (Fig. 6b, Left) resulted in 84.5% ± 6.7% suppression of replication-competent HIV-1 (Fig. 6b, Right). Thus, MDC1-primed HIV-1-specific autologous CTL were capable of eliminating HIV-1-infected cells harboring replication-competent virus following their subsequent unveiling through the LR activity of MHC-class II antigen-presenting MDC1.

4. Discussion

We have shown that antigen-presenting MDC1 are capable of inducing both HIV-1 latency reversal in infected CD4+ T cells isolated from ART-treated HIV-1+ MACS participants, and HIV-1 antigen-specific CTL responses that can effectively kill the MDC1-exposed HIV-1-infected targets. This MDC1-mediated ‘kick’ was found to be antigen-dependent, and bidirectional signaling events between the MDC1 and CD4+ T cells involving the CD40/CD40L signaling pathway contributed to this process. Other studies have explored the LRA potential of immature DC using in vitro models of HIV-1 latency with infected immortalized cell lines [47,48], through in vitro establishment of HIV-1 latency in primary CD4+ T cells of uninfected donors [49], or by addressing their nonspecific impact on in vitro pre-expanded polyclonal-activated T cells [50]. However, we demonstrate, in a natural setting of chronic HIV-1 infection, the use of an effective, clinically relevant autologous mature DC type specifically programmed to both mediate LR in freshly isolated CD4+ T cells derived from individuals undergoing successful ART, and to induce effector cells capable of recognizing and eliminating the infected cells. Our current data imply that a component of the HIV-1 reservoir is contained within the pool of both CMV- and HIV-1-specific CD4+ T cells. Importantly, our study was limited to a small number of viral antigen sources, and to one target protein antigen for each of the respective viruses tested. Therefore, the levels of LR induced most likely underrepresent the magnitude of HIV-1 reactivation possible if the number and selection of antigens had been optimized. This is especially true when considering antigen-specific CMV immunity, where CD4+ T cell responses to pp65 and IE-1 protein antigens comprise less than 12% of total CD4+ T cell responses to CMV in coinfected individuals [43]. As CMV is one of the largest and most complex viruses, with a genome encoding over 200 open reading frames [51], our study has room for optimization through incorporation of other CMV antigens that could enhance the effectiveness of our MDC1-based LR strategy. Nevertheless, our study supports the concept that HIV-1 LR can be achieved to expose cells infected with replication-competent virus for CTL elimination, in both a safe and directed antigen-specific fashion.

Previous studies in HIV-1/CMV coinfection indicate that HIV-1-specific CD4+ T cells are preferentially infected and depleted by HIV-1 [40,52], and that a portion of latently infected cells that remain during ART are indeed HIV-1-specific [39]. Interestingly, in contrast to our findings, it has been reported that CMV-specific CD4+ T cells are less susceptible to HIV-1 infection in vivo [53]. In spite of this, a large body of data exists to support the notion that CMV antigen-specific CD4+ T cells, in particular, likely contribute to a sizeable portion of the latent HIV-1 cellular reservoir. For example, CMV infection is frequent in HIV-1-infected individuals, with a seroprevalence of approximately 95% [41]. Furthermore, CMV occupies an inflated proportion, on average 10%, of memory T cell responses in healthy individuals, and CMV-specific CD4+ T cells persist at high levels in HIV-1 and CMV coinfected individuals [42,51,54,55], with greater than 1 out of 4 of the total number of CD4+ T cells in peripheral blood being CMV-specific in some individuals [43]. Subclinical CMV replication often occurs in the mucosal and peripheral tissues of HIV-1-infected individuals, contributing to T cell dysfunction, impaired immune recovery, and chronic immune activation during ART [56–59]. Consequently, recruitment of target cells to sites of inflammation that are also major sites of HIV-1 persistence, such as gut and other lymphoid tissues, creates a favorable environment for reservoir seeding [60–63]. Recent evidence supporting this scenario was provided by a study of ART-suppressed individuals in whom CMV replication in the gut was associated with inflammation, mucosal barrier damage, and microbial translocation [60]. Manipulation of HIV-1 co-receptor expression by CMV could also serve as a mechanism for establishment of the latent reservoir in susceptible target cells. For example, CMV upregulates CCR5 expression on newborn umbilical cord blood central memory CD4+ T cells that could facilitate in utero transmission of HIV-1 [64]. In addition, in vitro studies demonstrate the ability of CMV to manipulate AP-1 and NF-κB signaling for induction of HIV-1 gene expression in infected bystander cells through direct transactivation of the HIV-1 LTR [65–68]. Each of these mechanisms could potentially lead to enhanced HIV-1 infection of target cells in CMV-coinfected individuals. Once established, the latent HIV-1 cellular reservoir could also be subject to CMV-mediated proliferation or clonal expansion. In support of this theory, cross-sectional studies have shown a correlation between CMV replication in blood and semen and higher levels of HIV-1 DNA in both ART-naïve and ART-suppressed individuals [69,70]. In a related longitudinal study, CMV replication in peripheral blood mononuclear cells (PBMC) of men initiating early ART was associated with delayed decay of HIV-1 DNA reservoirs [63]. Furthermore, proviral and integration site analyses in ART-suppressed individuals have implicated clonal expansion of latently infected CD4+ T cells as a major mechanism of HIV-1 persistence [71–75], and recent findings estimate that these expanded clones comprise 50–60% of the latent HIV-1 reservoir [76–78]. Of note, in a study of 15 HIV-1-infected patients who underwent myeloablative chemotherapy for CMV- and Epstein-Barr virus (EBV)-associated malignancies, increases in HIV-1 DNA were preferentially found in CMV- and EBV-specific CD4+ T cells after immune reconstitution [79]. It is possible that self-renewal of stem cell memory T cells (TSCM) contributes to homeostatic proliferation of the latent HIV reservoir [80]. This subset of memory T cells plays a significant role in the maintenance of long-term immunological memory and contains the most copies of integrated provirus per cell in HIV-1-infected individuals [80]. Finally, CMV-infected individuals possess functional CMV-specific TSCM cells that could promote expansion of the HIV-1 reservoir in CMV/HIV-1-coinfected persons through homeostatic proliferation, even during ART [81]. Recent studies in vivo studies utilizing TLR agonists to promote HIV-1 LR have shown promise [19–22], and are currently being studied in human trials (NIH Clinical Trials NCT02854801 (GS-9620; NCT03060447; NCT03837756). However, the specifics of how these innate immune receptor activators are leading to reactivation of the latent reservoir have yet to be fully elucidated. Evidence from both human and non-human primate studies points toward the IFN-α-producing plasmacytoid DC (pDC) as being an important cellular component in this process [19–22]. It is worth noting that the combination of factors...
used in the maturation and generation process of the specialized antigen-presenting type-1 programmed MDC1 used in our study, which includes IFN-α, IFN-γ, and a TLR3 agonist, was designed to mimic maturation events expected to occur as a result of DC crossstalk with responding IFN-α-producing pDC and IFN-γ-producing NK cells during the early stages of a successful antiviral immune response [32,82]. The factors produced by these early immune responders programs the maturing DC for being hyper-responsive to subsequent signaling factors they receive during antigen cognate interactions with CD4+ T cells. While the underlying mechanisms of the MDC1-mediated LR observed in our study have not been fully identified, our findings do indicate that bidirectional antigen-driven cross-talk between MDC1 and CD4+ T cells involving the CD40/CD40L signaling pathway contributed to the noted LR activity. Based on gene chip analysis of CD40L-activated MDC1 that revealed upregulations in galectin-9, TNF-α, and IL-15 mRNA expression (data not shown), all of which have been implicated as inducers of LR [50,83,84], the potential contribution of these factors warrants further investigation.

There are a number of caveats and limitations to our study that we acknowledge. Although our results point to CMV- and HIV-1-specific CD4+ T cells as harboring latent provirus, it is likely that CD4+ T cells specific to other viruses that manifest as chronic infections, including EBV and herpes simplex virus, and others contribute to the latent reservoir as well. Moreover, while we did not observe influenza antigen-mediated LR ex vivo, others have documented increases in cell-associated HIV-1 RNA expression in HIV-1-infected individuals receiving influenza vaccination during suppressive ART [85,86]. However, deep sequencing studies pre- and post-vaccination suggested nonselective induction of proviral expression from a broad pool of HIV-1-infected bystander cells [86]. While the LR we show in our study was antigen-driven, we did not truly identify the antigen specificity of the infected CD4+ T cells, and therefore we do not rule out the possibility that reactivation of the virus could reflect nonspecific bystander effects of a potent MDC1-mediated antigen-specific response, rather than a direct impact on an antigen-specific reservoir. Furthermore, targeting the latent reservoir poses numerous challenges with regard to antigen delivery by a DC vaccine to certain sites that are anatomical sanctuaries of HIV-1, such as lymph node B cell follicles [87]. Because of this, the LRA potential of antigen-presenting B cells and antigen-presenting DC-derived exosomes should be explored for their ability to drive HIV-1 LR and to target these anatomical sites. Alternatively, developing strategies for direct antigen delivery to the antigen presenting cells residing in these areas in vivo should be considered. Nonetheless, our results provide strong rationale for the incorporation of MDC1 and ‘helper’ antigen derived from heterologous viral sources (such as CMV) into the design of a dual therapeutic approach to address both the ‘kick’ and the ‘kill’ of latent HIV-1.

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Declaration of interests

The authors have nothing to disclose.

Author contributions

J Kristoff performed experiments, analyses, and co-wrote the manuscript. ML Palma and TM Garcia-Bates provided technical assistance and contributed to experimental data. C Shen performed statistical analysis. N Sluis-Cremer provided intellectual input and edited the manuscript. P Gupta provided intellectual input and data interpretation. CR Rinaldo provided intellectual input, supervision, and edited the manuscript. and RB Mailliard designed the study, provided supervision, technical assistance, and co-wrote the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiomedi.2019.03.077.

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