T CELL EFFECTOR RESPONSES IN HIV-1 CURE STRATEGIES

by
Victoria Walker-Sperling

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ABSTRACT

HIV-1 is a virus that affects over 35 million individuals around the world, and yet despite present treatments, there remains a need for a cure. A current cure tactic widely researched is the "shock and kill" strategy, where cART-treated HIV-positive individuals would be given latency reactivating agents (LRAs) to induce HIV-1 production from latently-infected CD4+ T cells, allowing for the CD8+ T cell response to eliminate the latent reservoir. Here, I examined the capabilities of the CD8+ T cell response from HIV-positive individuals to eliminate macrophages, another cell type infected by HIV-1, and reactivated latently-infected primary CD4+ T cells, both reactivated with PMA and ionomycin and with LRAs. Both CD4+ and CD8+ T cells from elite suppressors, HIV-positive individuals with viral loads of less than 50 copies per milliliter of blood, were capable of suppressing virus production from HIV-infected monocyte-derived macrophages, with the CD8+ T cells actually killing the infected cells. As to the latently-infected CD4+ T cells, CD8+ T cells from chronic progressors were not consistently capable of eliminating the infected cells upon reactivation, although those from two of four viremic controllers were when PMA and ionomycin were used for stimulation. Treatment with LRAs such as bryostatin and romidepsin both alone and in combination significantly inhibited the CD8+ T cell response to HIV-1, with the mechanism for bryostatin inhibition being an increase in cell death, downregulation of CD3, and upregulation of exhaustion markers. Despite that the elite suppressor CD8+ T cell response was capable of inhibiting HIV-1 infection of macrophages and that the response of some viremic controllers and chronic progressors can do so for latently-infected CD4+ T cells, there remains a need for some sort of immunologic boost for the average

individual with HIV-1 infection. Worse, some LRAs and combinations of LRAs decrease the ability of the CD8+ T cell response to eliminate infected CD4+ T cells. Therefore, any given LRA or combination of LRAs should be examined for their effects upon the adaptive immune response prior to use in clinical investigations of HIV-1 cure strategies.

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I. INTRODUCTION

HIV-1 is a retrovirus that affects over 35 million people worldwide (1). Upon HIV-1 infection most individuals experience acute viremia accompanied by a decline in CD4+ T cells (HIV pathogenesis reviewed in 2). After the adaptive immune system and specifically the CD8+ T cell response is primed, the viremia declines to what is known as the viral set point, which is generally between 10,000-100,000 copies of HIV RNA per mL of blood, and the CD4+T cell count recovers (3). In the absence of therapy, an individual's immune systems will eventually lose what control it had, and the viremia will begin to increase as the CD4+ T cell count decreases. At a CD4+ T cell count of 200, an individual would be diagnosed with acquired immunodeficiency syndrome (AIDS) and, with the continuing decrease in CD4+ T cells and absence of therapy, would be susceptible to opportunistic infections and eventually die (4). The use of antiretroviral therapy in HIV-positive individuals is capable of suppressing viremia to levels below the limit of detection by clinical assays and increases life expectancy to similar levels as those who are HIV-negative (5). However, despite the existence of combined antiretroviral therapy (cART, formerly known as HAART), both a cure and a vaccine remain elusive.

Each HIV-1 virion contains two copies of the positive single-stranded RNA viral genome within the capsid (6). The genome of HIV-1 encodes nine genes: the structural proteins of *gag*, *pol*, and *env*; the accessory proteins *vif*, *vpu*, and *vpr*; and the early expressed proteins *tat*, *rev,* and *nef* (7). The life cycle of the virus begins when the viral envelope fuses with the cellular membrane of a CD4+ T cell or a macrophage (8). HIV-1 uses the primary receptor CD4 and either CCR5 or CXCR4, the preference for which of

the two coreceptors determines viral tropism (9-11). Once the viral capsid is within the cell, the viral genome undergoes reverse transcription and is shuttled as DNA into the nucleus. Inside the nucleus, the viral DNA is integrated into the host genome (12) and will remain there as a provirus until the appropriate transcription factors induce transcription and translation of the fully spliced transcript (13-15). Tat and Rev, two of the three early expressed proteins from the fully spliced transcript, allow for increased transcription of the HIV-1 genome as well as the alternative splice variants that encode for the rest of the genes (13-15). Once all of the viral proteins are available, they form new virions and are released from the target cell, usually inducing cell death (16).

Reverse transcription is highly error-prone process, introducing mutations into the various proteins of HIV-1 and allowing the virus to evade the adaptive immune response (17-18). The variable regions of *env* in particular are highly prone to mutation, making the development of an antibody vaccine that covers even a single subtype of HIV-1 very difficult (19). That said, multiple antibody-based vaccine candidates have been suggested in recent years and have shown great promise (20-21), but the CMV-based SIV vaccine tested in the rhesus macaques induced a CD8+ T cell response capable of eliminating SIV infection to the point that no DNA was detected in multiple tissues (22).

While no sterilizing cure owing to the immune response has been seen in humans, CD8+ T cell-mediated control of HIV-1 has been observed in HIV-positive long-term non-progressors (LTNPs) for decades (23-24). Although these individuals are HIV-positive, they do not progress to AIDS in the absence of cART. Elite suppressors (ES) are a related group of HIV-positive non-progressors who have a viral load of less than 50 copies of HIV-1 per milliliter of blood in the absence of antiretroviral therapy (25).

Overall, ES tend to have qualitatively superior CD8+ T cell responses to HIV-infected CD4+ T cells: the ability of the CD8+ T cells to kill via granzyme B or perforin as well as the cells' ability to produce antiviral cytokines such as IFN-gamma and TNF-alpha are extensively documented to be superior in ES as compared to the average chronic progressors (26-31). The immunologic control exhibited by the ES in their immune responses may be considered a model for an effective immune response to HIV-1 needed for a functional cure (32).

The need for an effective adaptive immune response is not solely limited to the vaccine effort. HIV-1 preferentially infects activated CD4+ T cells, but owing to T cell biology, an infected CD4+ T cell may return to a resting state prior to virus-mediated cell death but following integration of the provirus (reviewed in 33). Normally, once CD4+ T cells see their cognate antigen, they become activated and perform their effector functions. Most of these cells die after the infection associated with the antigen is cleared, but not all of the activated cells die and some instead return to a resting state thus preserving immunologic memory. Many of the transcription factors associated with T cell activation (such as NFkB and NFAT) are in fact necessary for the transcription of HIV-1 provirus (34-37), and when an infected T cell returns to a resting state, these transcription factors are sequestered away from the nucleus, rendering HIV-1 more or less transcriptionally silent. Without production of antigen, these infected yet transcriptionally silent cells unable to be detected by the cytotoxic T lymphocyte response (38). Antiretroviral therapy likewise has no effect on these CD4+ T cells as the antiviral drugs target only the replication steps in the viral life cycle. These infected

CD4+ T cells make up what is termed the latent reservoir and are effectively a major reason that cART alone cannot cure individuals (39).

Current efforts to eliminate the latent reservoir are focused on a strategy termed "shock and kill" (reviewed in 40 and 41). In this strategy, a patient would theoretically be treated with latency reactivating agents (LRAs), drugs that specifically induce HIV-1 transcription but not global T cell activation, to reactivate the provirus present in resting CD4+ T cells (40-41). Once HIV-1 transcription initiates, antigen is then produced, allowing for the CD8+ T cell response to identify and eliminate the infected cells. Strong candidates for LRAs include HDAC inhibitors and PKC agonists (42-45). In vitro data with latently infected CD4+ T cells from patients have shown that while some LRAs such as romidepsin, panobinostat, and bryostatin-1 are capable of inducing production of HIV mRNA, combinations of LRAs from separate drug classes are likely to be necessary (46). That said, there have been conflicting reports regarding the effects of the HDAC inhibitors upon the adaptive immune system (47-48). The archived proviruses of individuals fully suppressed by cART will also likely have escape mutations to which the CD8+ T cell response of the infected individual may not be able to effectively respond (49), further supporting the likelihood that some sort of booster or therapeutic vaccine will likely be necessary for the elimination of the latent reservoir.

The following studies therefore examine the CD8+ T cell response of HIV-positive individuals. In the first study, the efficacy of elite suppressor CD4+ and CD8+ T cell responses were measured in relation to infected macrophages, the major cell type besides CD4+ T cells infected by HIV-1. These cells are thought to be another reservoir in patients on cART (50). Both CD4+ And CD8+ T cells were found to be capable of

suppressing virus release by infected macrophages, with the CD8+ T cells actively causing cell death (51). In the second study, the kinetics of HIV-1 reactivation were determined in regards to production of both intracellular HIV-1 mRNA transcript and virions, and HIV-specific CD8+ T cells from HIV-positive chronic progressors were found to be inefficient at eliminating newly reactivated latently infected CD4+ T cells (52). In the third study, the effect of both PKC agonist and HDAC inhibitor latency reactivating agents on the CD8+ T cell response was found in many cases to be inhibitory, specifically due to an increase in cell death and CD8+ T cell exhaustion (53). Together, these three studies underline the need for both a therapeutic vaccine to be used in cure research as well as careful examination of the effects of latency reactivating agents prior to use in the clinic.

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II. Comparative analysis of the capacity of elite suppressor CD4+ and CD8+ T cells to inhibit HIV-1 replication in monocyte-derived macrophages

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INTRODUCTION

Elite suppressors (ESs) are rare patients who control human immunodeficiency virus type 1 (HIV-1) replication without antiretroviral therapy (1). Many studies have shown that CD8+ T cells from ESs are more effective at inhibiting viral replication in CD4+ T cells than CD8+ T cells from chronic progressors (CPs) (2,–11). Furthermore, HIV-1-specific CD4+ T cells from ESs have high-avidity T cell receptors and are more likely to maintain responses that are either proliferative, polyfunctional, or cytotoxic than effector CD4+ T cells from CPs (12,–19).

While HIV-1 also infects macrophages, these target cells are rarely examined in the context of immunologic control. Macrophages are thought to be more difficult to infect with HIV-1 than activated CD4+ T cells, in part due to differences in the level of expression of retroviral restriction factors, such as tetherin, SAMHD1, and APOBEC3 (20,–22). SAMHD1 specifically contributes to the lower concentration of deoxynucleoside triphosphates already found in macrophages, greatly inhibiting reverse transcription (23, 24). Even though CD4+ T cells are the major reservoir of HIV-1 infection, the infection of macrophages remains a concern, especially since these cells

can directly infect CD4+ T cells with HIV-1 in an efficient manner (25, 26). Thus, examining the cellular immune response to HIV-1-infected macrophages will contribute to the rational design of an HIV-1 vaccine.

While some CD8+ and CD4+ T cell clones and cell lines have previously been shown to suppress HIV-1 or simian immunodeficiency virus (SIV) replication in infected macrophages (27,–30), less is known about the inhibitory capacity of unstimulated primary T cells. Interestingly, in the macaque model of elite suppression, freshly isolated SIV-specific primary CD8+ T cells were able to inhibit viral replication in CD4+ target cells but not in macrophages (31).

In order to determine whether primary human ES T cells were capable of suppressing viral replication in macrophages, we compared the replication kinetics of a laboratory HIV-1 isolate in monocyte-derived macrophages (MDMs) in the presence and absence of freshly isolated primary CD4+ and CD8+ T cells. Our results provide guidance for the development of an effective therapeutic vaccine against HIV-1 infection that can elicit immune responses similar to those observed in ESs.

METHODS

Patients. All blood was obtained from patients and healthy donors (HDs) after they provided written and informed consent and was handled as recommended by the Institutional Review Board of the Johns Hopkins University. The ESs (n = 12) had viral loads of less than 50 copies per ml, and the virus in highly active antiretroviral therapy (HAART)-treated CPs (n = 11) had been fully suppressed with antiretroviral therapy for at least 1 year. Seronegative controls comprised 20 healthy HIV-1-negative HDs.

Cell isolation and tissue culture. Peripheral blood mononuclear cells (PBMCs) isolated from whole blood via Ficoll-Paque Plus gradient centrifugation (GE Healthcare Life Sciences) underwent positive selection for $CD14^+$ monocytes using a magnetically activated cell sorting system (CD14 microbeads; Miltenyi Biotec). Monocytes were plated at 10^5 cells per well in a flat-bottomed 96-well plate with macrophage differentiation medium (RPMI 1600, 20% type human AB serum [U.S. origin; GemCell], 1% HEPES, 50 ng/ml human recombinant macrophage colony-stimulating factor [R&D Systems]) and incubated for 7 days at 37°C (32). PBMCs collected 7 days prior to infection were stimulated with phytohemagglutinin in activating medium (100 units interleukin-2 [IL-2]/ml) for 3 days before $CD4^+$ T cell targets were isolated by negative selection.

$CD4^+$ and macrophage suppression assay. PBMCs isolated from whole blood via Ficoll gradient centrifugation on the day of infection underwent positive selection for $CD8^+$ T cells (median purity, 94.3%; CD8 microbeads; Miltenyi Biotec) and negative selection for $CD4^+$ T cells (median purity, 95.8%; CD4 T cell isolation kit; Miltenyi Biotec) to isolate the effector cells used in the suppression assay. MDM and $CD4^+$ T cell

targets were spinoculated with HIV_{BaL}(500 ng p24 of virus stock per 10^6 cells was used for the standard assay; half that dose was used for MDMs in the experiment for the comparison with CD4 targets [32]) for 2 h at 1,200 × *g* and 37°C (33). Autologous $CD4^+$ and/or $CD8^+$ effectors were added to 10^5 target MDMs or target $CD4^+$ T cells at ratios of 1:1, 1:2, and 1:4. $CD4^+$ T cell and MDM targets were cultured in RPMI 1640–10% fetal bovine serum (FBS). Nonsuperinfected target cells incubated with effector cells were used to control for background HIV production. The same number of infected target cells used in the experimental conditions was included to determine the maximum amount of virus production for calculating percent inhibition, which was calculated as 100% · [1 – (experimental value/maximum virus production)]. All effectors remained in culture with infected target cells for the entire time span of each experiment. $CD4^+$ T cells and MDMs were cultured postinfection with 10 units/ml of IL-2, which was added every other day solely for the experiment comparing $CD8^+$ T cell-mediated effector inhibition between target cell types. All other suppression assays were conducted with RPMI 1640–10% FBS without IL-2. Culture supernatant was collected for analysis immediately after the addition of effectors (day 0) and on days 3, 5, and 7 postinfection.

Macrophage infection without spinoculation. Monocyte-derived macrophages were incubated with HIV_{BaL} (500 ng p24 per 10^6 cells) for 4 h at 37°C. Three-quarters of the virus inoculum was removed and replaced with RPMI 1640–10% FBS, and the cells were incubated overnight. The remaining virus was then removed and replaced with RPMI 1640–10% FBS. Culture supernatant was collected immediately after addition of effectors (day 0) and on days 3, 5, and 7 postinfection.

Transwell macrophage suppression assay. Monocytes isolated 7 days prior to infection were plated at 5×10^5 cells per well on flat-bottomed 24-well plates in macrophage differentiation medium for 7 days at 37°C. After spinoculation with HIV_{BaL} (2 h at 1,200 × *g* and 37°C; 500 ng/10^6 cells), $CD4^+$ and $CD8^+$ effectors were isolated from fresh PBMCs and added to the monocyte-derived macrophages at a 1:1 ratio either directly or in a transwell (Corning transwell permeable supports). Supernatant was collected immediately after addition of effectors (day 0) and on days 3 to 7 postinfection.

Viral output and effector cell infection. Viral production in the culture supernatants was determined via p24 enzyme-linked immunosorbent assay (ELISA; PerkinElmer) per the manufacturer's instructions. For fluorescence-activated cell sorting, T cell effectors were stained with the extracellular markers CD3-Pacific Blue and CD8-allophycocyanin-H7 (BD Biosciences). Cells were permeabilized with Cytofix/Cytoperm (BD Biosciences) and then stained with Coulter clone K57-RD1 (HIV-1 core antigen, FL-2 channel). Fluorescence data were collected on a BD FACSCanto II flow cytometer and analyzed with FlowJo software.

Cytotoxicity assay. Macrophages were differentiated and infected with HIV_{BaL} as described above for the suppression assay. PBMCs were acquired on the day of macrophage infection and stimulated with Gag peptides (10 μg/ml) and 10 units/ml of IL-2 for 1 week. After 7 days of culture after HIV_{BaL} infection, the macrophage culture medium was changed. Stimulated and unstimulated primary CD4 and CD8 effector cells were added at a 1:1 effector cell/target cell ratio. Supernatant was harvested from the cultures at 24 h after addition of the effectors and analyzed for lactate dehydrogenase (LDH) release using a Cytotox-96 nonradioactive cytotoxicity assay (Promega) per the

manufacturer's instructions (34). Infected macrophages without any effectors were used as a spontaneous target cell death control, and effectors cultured alone were used as a spontaneous effector cell death control. Maximum LDH release was determined by treating macrophages with 0.5% Triton-X for maximum cell death. Percent cytotoxicity was calculated as follows: 100% · (experimental LDH release – spontaneous target cell LDH release – spontaneous effector cell LDH release)/(maximum LDH release – spontaneous target cell LDH release).

Statistical analysis. Statistical analyses were performed using GraphPad Prism (version 6) and Microsoft Excel software. Unpaired, two-tailed Student's *t* tests were used for comparing ES and HAART effector responses in the macrophage suppression and cytotoxicity assays. A paired, two-tailed Student's *t* test was used to examine directly applied and transwell effector responses. Linear analyses used Pearson correlations.

RESULTS

In order to examine the T cell response to macrophage infection in HIV-positive individuals, we first examined the susceptibility of monocyte-derived macrophages (MDMs) from 12 ESs, 11 CPs on suppressive HAART regimens, and 19 HDs to HIV-1 infection. Using spinoculation and a replication-competent virus (HIV_{BaL}), we found that there was no difference in the amount of virus produced by MDMs from ESs, CPs, and HDs by day 7 postinfection (Fig. 1A and B). To determine whether there were subtle differences in the susceptibility to infection that were masked by spinoculation, we performed the infectivity assay without spinoculation in macrophages from randomly selected subsets of ESs and HDs ($n = 4$ for each; Fig. 1C). We found that, under these conditions, HIV_{BaL} was similarly capable of infecting MDMs from both ESs and HDs. Taken together, these data suggest that there is no significant difference in the susceptibility of MDMs from ESs, CPs, and HDs to HIV-1 infection.

Strong $CD8^+$ T cell responses to HIV-1-infected $CD4^+$ T cells have been documented in many ESs; thus, we examined the ability of unstimulated primary ES $CD8^+$ T cells to suppress viral replication in MDMs using a variation of a previously described inhibition assay (4, 35). The responses elicited by the $CD8^+$ effectors were divided into three categories on the basis of the distribution of the degree of inhibition observed in all the patients studied. T cells from some patients mediated either (i) a low degree of inhibition (defined as less than 35% inhibition), (ii) an intermediate degree of inhibition (defined as between 35 and 65% inhibition), or (iii) a high degree of inhibition (defined as greater than 65% inhibition). The ES $CD8^+$ T cells mediated a high degree of inhibition on days 5 and 7. $CD8^+$ T cells from CPs 1 to 4 (Table 1) were also found to

mediate high levels of inhibition, but the ES $CD8^+$ T cell response was found to be significantly superior on day 7 ($P < 0.02$; Fig. 2A). While coincubation of infected macrophages with $CD8^+$ T cells from ESs resulted in high levels of inhibition in all patients except ES5, the responses seen in CPs were much more heterogeneous, with the majority of patients having intermediate or low levels of inhibition (Fig. 2B). Low-level to no inhibition was seen in 16 out of 18 HDs at day 5 and 18 out of 18 HDs at day 7. Interestingly, when the $CD8^+$ effector response to MDM targets was compared to the response to $CD4^+$ T cell targets in ESs, a similar level of inhibition was observed on days 5 and 7 (Fig. 2C). The method by which $CD8^+$ T cells were isolated did not seem to change the suppressive ability, as purification of the cells by positive selection and negative selection resulted in similar levels of inhibition (Fig. 2D). Taken together, our data demonstrate that ES $CD8^+$ T cells effectively inhibit viral replication in MDMs, and this inhibition appears to be a correlate of protective immunity.

Although $CD4^+$ T cells are typically viewed as the targets of HIV infection, these cells can also act as effector cells and have been shown to be capable of suppressing infection in macrophages in the macaque model of elite suppression (30). Thus, we examined whether or not human ES $CD4^+$ T cells could mediate inhibition of viral replication in macrophages. On day 5 after infection, high levels of inhibition were seen in ESs 4, 6, 8, 9, and 31 as well as CPs 2, 5, 7, and 9, while an intermediate level of inhibition was seen in ESs 22 to 24 and CPs 1, 4, 6, and 8, making the distribution virtually identical ($P = 0.95$). At day 7, high levels of inhibition were seen in ESs 4, 6, 8, and 31 as well as CPs 1, 2, 5, 7, and 9 (Fig. 3A). The distribution of the degree of

inhibition was nearly identical in ESs and CPs on day 7, and high levels of inhibition were not seen in any HD (Fig. 3B).

We next compared the magnitude of the $CD4^+$ and $CD8^+$ T cell-mediated effector responses to target MDMs. The $CD8^+$ T cell-mediated inhibitory responses in ESs were significantly stronger than the $CD4^+$ T cell-mediated inhibitory responses ($P < 0.007$; Fig. 4). In contrast, no significant difference in the inhibitory responses mediated by $CD4^+$ and $CD8^+$ T cells was seen in CPs or HDs.

In order to clarify the mechanisms of inhibition mediated by the two different effector cells, we performed a variation of the inhibition assay where we used the transwell system to separate effector cells from infected target cells. In ESs, some inhibition of viral replication was still seen when effector $CD4^+$ T cells were not in direct contact with MDMs (Fig. 5A and B), suggesting that soluble factors were contributing to the control of viral replication. In contrast, no inhibition was seen when ES $CD8^+$ T cell effectors were physically separated from the infected target cells, suggesting that direct cell-to-cell contact was critical for the control of viral replication (Fig. 5C and D; $P = 0.015$). Similar results were obtained for CPs (data not shown).

Using a cytotoxicity assay, we sought to verify that the $CD8^+$ response against infected macrophages was indeed a result of cytolytic $CD8^+$ T cells. While there was a modest response in ES6 for unstimulated $CD4^+$ effectors as well as $CD4^+$ and $CD8^+$ T cell effectors that had been stimulated for 7 days with overlapping Gag peptides, the only strong cytotoxic responses were seen with Gag peptide-stimulated $CD8^+$ effectors for both ESs ($n = 6$) and CPs ($n = 5$) at 24 h after effector addition (Fig. 6A). The transwell system was again used to determine if this response was dependent upon cell-cell contact,

and we found that Gag peptide-stimulated $CD8^+$T cell effectors were incapable of killing infected macrophages when they were separated by transwells (Fig. 6B). For ES6, we also demonstrated that antibodies to class I blocked the $CD8^+$T cell-mediated killing (data not shown). Together, our results suggest that the vast majority of $CD4^+$ T cell effectors in the ESs and CPs that we studied were not able to kill infected macrophages after 24 h of coculture even when those cells were prestimulated with Gag peptides (Fig. 6B).

Because effector $CD4^+$ T cells are susceptible to infection, we hypothesized that they were infected with HIV-1 during the course of experimentation. Therefore, we determined what percentage of effector cells were infected by staining for intracellular Gag using flow cytometric analysis. On day 7 after infection, the $CD4^+$ T cell effectors from ESs, CPs, and HDs were found to be infected to similar degrees, with no significant difference seen at three different effector cell-to-target cell ratios (Fig. 7A). Furthermore, in ESs, a high level of infection of $CD4^+$ T cell effectors occurred by day 3 postinfection (Fig. 7B), which could potentially explain the lower level of inhibition seen at later time points in some patients (Fig. 3A). Infection of effector $CD4^+$T cells did not correlate with their ability to inhibit viral replication in macrophages in our combined cohort of ESs and CPs (Fig. 7C). While this lack of correlation held true for ESs when they were examined separately (Fig. 7D), there was a significant correlation between the degree of viral inhibition and the percentage of infected $CD4^+$ T cell effectors in CPs ($R^2 = 0.82$, $P = 0.034$; Fig. 7E).

DISCUSSION

Many lines of evidence suggest that the cause of elite suppression in some patients is a result of an efficient $CD8^+$ T cell response that prevents ongoing viral replication. This idea has long been supported by both functional studies examining the response and quality of ES $CD8^+$ T cells (2, 3, 5, 10, 36-40) and genome-wide association studies identifying major histocompatibility complex class I alleles (such as HLA-B*57 and HLA-B*27) (41-46). In addition, these protective HLA alleles have been shown to be overrepresented in multiple ES cohorts (4, 41, 47-52). While many studies have focused on the control of viral replication in $CD4^+$ T cells, a few studies have looked at the ability of human T cell clones to kill macrophages. As with $CD8^+$cytotoxic T cell clones and monocyte and dendritic cell targets (53), Nef-specific cytotoxic $CD4^+$T cells were found to be capable of efficiently killing both $CD4^+$ T cell and macrophage targets (28, 29). Similarly, in the macaque model of elite suppression, $CD4^+$ T cell clones were capable of suppressing the infection of macrophages (30). Given the importance of macrophages in the pathology of HIV infection (54), determining whether or not effector T cells can control viral replication in these cells is essential for the rational design of a vaccine. In this study, we demonstrate that unstimulated, primary $CD8^+$ T cell effectors from ESs are capable of effectively suppressing viral replication in macrophages.

Despite previous evidence to the contrary (55), macrophages from ESs seem to be able to be infected to the same degree as macrophages from patients on HAART and healthy donors. Similar results were obtained with and without spinoculation using the same replication-competent virus that was used in the prior study. While residual intracellular antiretroviral drugs may have resulted in low-level inhibition of HIV-1

replication in CP MDMs, this would not explain the lack of a significant difference in the replication kinetics between ES and HD MDMs. Macrophages in different activation states have been known to be differentially susceptible to infection (56, 57), so the disparity between our results and previous findings may be a result of the methods used to induce the differentiation of monocytes into macrophages.

In contrast to the macaque model of elite suppression, where SIV-specific $CD8^+$ T cell effectors were ineffective at inhibiting viral replication in macrophages (31), we show here that primary $CD8^+$ T cells from ESs efficiently inhibited virus production in MDMs. This suppression was cell contact mediated and probably the result of cytotoxic responses, as previously described (2, 5, 10, 58). The $CD8^+$ effector inhibitory response to HIV-1-infected macrophages was significantly more potent in ESs than HAART patients. However, a few individuals on HAART had primary, inhibitory $CD8^+$ T cell responses that were comparable to the responses seen in ESs. In contrast, there was a marked difference in the killing of infected macrophages between Gag peptide-stimulated $CD8^+$ T cells from ESs and stimulated $CD8^+$ T cells from patients on HAART. This difference is similar to the differences in the capacity of $CD8^+$ T cells from ESs versus those from CPs to eliminate HIV-infected $CD4^+$ T cells seen previously (5). Interestingly, $CD8^+$ T cells from some healthy donors induced a low level of inhibition of viral replication in autologous macrophages. This is contrast to the findings of our prior studies, where we saw no inhibition of viral replication in $CD4^+$ T cells by $CD8^+$ T cells from healthy donors (11, 35, 59). It is possible that this inhibition may represent an innate immune response or the development of an adaptive response during the 7-day period of coculture of infected macrophages and $CD8^+$ T cells. Taken together, while ESs clearly

have a superior inhibitory $CD8^+$ T cell response against HIV-1-infected macrophages, on average, it is possible that this phenotype alone is not sufficient to explain elite control in all patients.

In contrast to the responses seen with $CD8^+$ T cell effectors, there was no significant difference in the ability of primary $CD4^+$ T cell effectors from ESs and CPs to inhibit viral replication in macrophages. Within the ES group, the $CD4^+$ inhibitory response was also consistently inferior to the response mediated by $CD8^+$ effectors. Interestingly, some ESs and CPs had strong $CD4^+$ T cell inhibitory responses. This inhibition appeared to be mediated by both soluble factors and a cell contact-dependent mechanism. Potential soluble factors involved in the response include RANTES and macrophage inflammatory protein-1-alpha/beta, which inhibit the entry of CCR5-tropic viruses and have been associated with HIV-specific $CD4^+$ T cell responses (12, 60). With regard to the cell contact-mediated suppression, prior studies have shown that some $CD4^+$effectors may have cytotoxic activity against HIV-1-infected $CD4^+$ T cells (15, 27, 29, 30, 61–64). However, we saw very little $CD4^+$-mediated killing of infected macrophages over a 24-h period in this study. It is possible that $CD4^+$ T cells are capable of killing over a longer time frame, but, in general, the $CD4^+$ T cell effector response to macrophages does not appear to be a correlate of immunity in our cohort of patients.

Eight out of nine of the ESs studied here have the protective HLA B*27 and/or B*57 class I alleles (Table 1). In contrast, some ESs in other larger cohorts do not have these protective alleles or strong HIV-specific $CD8^+$ T cell responses (8, 49, 51, 65). It would be interesting to determine whether suppressive $CD4^+$ responses play a role in the control of viral replication in these patients. With that said, we show here that

$CD4^+$ effector cells are susceptible to infection, and this may limit the effectiveness of $CD4^+$ cytotoxic T cells. We observed a negative correlation between the susceptibility to infection and the suppressive capacity of effector $CD4^+$ T cells in CPs, and the decline in Gag-positive $CD4^+$ effector T cells over time could be due to the cytopathic effects of the virus. These data suggest that effector $CD4^+$ T cells could potentially have significant antiviral activity if they were engineered to be resistant to HIV-1 infection (66).

Our data are limited by the relatively low number of patients studied and the fact that we did not include viremic CPs in our analysis. The numbers of effector $CD8^+$ T cells decline over time in patients on HAART (67), so it possible that the lower level of $CD8^+$ T cell-mediated inhibition in CPs was due to the lower number of effector $CD8^+$ T cells in these patients. We tried to address this issue by stimulating $CD8^+$ T cells with Gag peptide prior to doing a cytotoxic T lymphocyte assay, but we still saw a marked difference in the killing of infected cells by effector cells in ESs and CPs. The results are consistent with those of a prior study that showed that ES $CD8^+$ T cells are more effective than CP $CD8^+$ T cells at killing infected $CD4^+$ T cells on a cell-per-cell basis (5).

In conclusion, primary, unstimulated $CD8^+$ T cells from ESs are capable of suppressing the macrophage production of replication-competent HIV-1 in a cell contact-mediated manner that is superior to the suppression mediated by $CD8^+$ T cells in patients on HAART. Macrophages are resistant to viral cytopathic effects and are the primary target cell in the central nervous system (53, 68). Thus, the induction of $CD8^+$ T cells that efficiently kill infected macrophages may be an important feature of an HIV-1 vaccine.

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III. Reactivation Kinetics of HIV-1 and Susceptibility of Reactivated Latently Infected CD4+ T Cells to HIV-1-Specific CD8+ T Cells

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INTRODUCTION

The goal of "shock and kill" HIV cure strategy is to selectively reactivate latent HIV-1 transcription without causing global T cell activation in order for the immune system to be able to recognize and eliminate latently infected cells (reviewed in 1 and 2). The presence of antiretroviral drugs during treatment with latency reversal agents will prevent reactivated virus from infecting other CD4+ T cells even if the kill component of the shock and kill strategy is not effective. However, subjects will eventually cease ART after treatment is deemed to be successful. As several recent cases have shown, residual latently infected cells may lead to a rebound in viremia and the re-establishment of a chronic HIV-1 infection (3-5) even when frequency of infected cells is lower than 1 in 150 million peripheral CD4+ T cells (3). Therefore, the ability of CD8+ T cells to kill newly-reactivated, latently-infected cells before the completion of the viral life cycle may be very important.

Through viral dynamics modeling combined with viral load data, the lifetime of an infected cell has been estimated using viral load data to be around 2 days from attachment of the virion to the death of the cell (6-9). Reactivated latently infected cells,

on the other hand, re-initiate the viral life cycle from proviral transcription onwards cutting down the time from activation to virus release. When activation of latently infected CD4+ T cells occurs, there is translocation of transcription factors such as NFkB and NFAT that allow for reactivation of the HIV-1 provirus (10-13). HIV-1 infected CD4+ T cells first produce the early proteins, Tat, Rev, and Nef, from a fully spliced HIV transcript (14-15). The production of Nef may complicate the ability of the CD8+ T cell response to eliminate infected cells by downregulating HLA-A and -B proteins (16-19). The timing and magnitude of this downregulation may adversely affect the ability of CD8+ T cells to adequately eliminate the latently infected CD4+ T cells.

To model the events needed for the rapid elimination of reactivated latently infected CD4+ T cells, we monitored the kinetics of transcription and virion release after the stimulation of primary CD4+ T cells from subjects on suppressive CART regimens. We used a primary CD4+ T cell model that involved nucleofection of cells with HIV-1 plasmids to determine the kinetics of protein production following proviral transcription in addition to HLA downregulation following viral protein expression. We then determined whether or not stimulated HIV-specific CD8+ T cells could eliminate infected autologous CD4+ cells before completion of the viral life cycle. Our results suggest that a successful therapeutic vaccine may need to induce effector CD8+ T cells that are capable of eliminating reactivated CD4+ T cells in a very short time frame.

METHODS

HIV+ and HIV- Donor Blood Samples. All blood was obtained from HIV+ subjects and HIV- donors with written and informed consent and handled as per Johns Hopkins University regulations. The chronic progressors were HIV-1 infected subjects who were started on suppressive ART regimens during chronic infection and had maintained viral loads of < 50 copies HIV-1 RNA/ml with no blips for a median of 4 years (range of 1 to 13 years). The viremic controllers maintained a median viral load of 295 copies/ml (range of 80 to 1133 copies/mL) without cART (Table).

Primary Resting Cell Isolation. PBMCs isolated from whole blood via Ficoll-Paque PLUS gradient centrifugation (GE Healthcare Life Sciences) underwent negative selection for CD4+ T cells using the MACS system (CD4 Isolation Kit, Miltenyi Biotec). The CD4+ T cells were further depleted CD25-, CD69-, and HLA-DR-expressing cells with Miltenyi microbeads (CD25 microbeads, CD69 Isolation Kit, and HLA-DR microbeads) for the isolation of the resting cells.

Outgrowth of Latent HIV-1. Resting CD4+ T cells were plated at a concentration of $5x10^6$ cells per mL with up to 3 replicates of each experimental condition in 12-well plates and incubated overnight in non-activating media (RPMI 1640, 10% FBS) prior to the time-zero supernatant sample. For the general time course (supernatant samples taken at 0, 6, and 24 hours and cells at 24 hours), $5x10^6$ resting CD4+ T cells were plated for each replicate. After the time-zero supernatant sample, all the cells save for the non-stimulated control wells were treated for 6 hours with PMA (50 ng/mL) and ionomycin (1 μM) in the presence of raltegravir (4 μM, RAL) and efavirenz (10 μM, EFV) to prevent new infection. For the longer time course, a supernatant sample was taken at the

conclusion of the 6 hours, and the resting CD4+ T cells were washed twice prior to re-plating at $5x10^6$ cells per 2 mL in fresh 12-well plates with raltegravir (4 μM, RAL) and efavirenz (10 μM, EFV) to prevent new infection. Subsequent supernatant samples were taken 24 hours. For each replicate at 24 hours, $100\text{-}500x10^3$ cells were removed for FACS analysis of activation, and the remaining $4.5\text{-}4.9x10^6$ CD4+ T Cells were placed into TRIzol (Life Technologies) for the isolation of intracellular viral RNA. For the time course examining upregulation of intracellular HIV-1 mRNA very early after stimulation (0, 1, 3, and 6 hours), $7.5x10^6$ resting CD4+ T cells were plated for each replicate and treated with PMA and ionomycin in the presence of raltegravir and efavirenz as above. At each time point, $7.4x10^6$ cells were harvested from each replicate and placed in TRIzol with $100x10^3$ cells were removed for FACS analysis of activation. Supernatant samples were likewise taken from the corresponding well for RNA isolation.

Intracellular and Supernatant RNA isolation. Intracellular RNAs were isolated via TRIzol (Life Technologies) at a ratio of $5\text{-}7.5x10^6$ CD4+ T cells per 1 mL of reagent and incubated on a Phase Lock Gel Heavy spin column (5Prime) for five minutes with GlycoBlue Coprecipitant (Life Technologies) per manufacturer's instructions to better facilitate RNA recovery. Chloroform was subsequently added to TRIzol at a 1:5 ratio and shaken well for at least 15 seconds to mix before centrifugation at 12,000 x*g* for 10 minutes at 4°C. The top layer of supernatant from the gel tube was transferred to a new tube with isopropanol at a 1:2 ratio and incubated for 10 minutes at room temperature to precipitate the RNA. The newly precipitated RNA was centrifugated at 12,000 x*g* for 10 minutes at 4°C, and the resulting pellet washed with 80% EtOH. After another centrifugation with the same settings, the RNA pellet was resuspended in molecular

biology-grade distilled water. Viral RNA was isolated from 250 µL supernatant samples with 750 µL TRIzol LS (Life Technologies) and the above protocol.

Viral Quantification Assay. Viral RNA was quantified as described previously (20,21), the process of which is summarized as follows. Isolated intracellular and extracellular RNA were converted to cDNA with qScript cDNA SuperMix (Quanta Biosciences) per manufacturer's instructions (5' at 25°C, 30' at 42°C, 5' at 85°C, hold at 4°C). The cDNA was then used in a viral quantification assay, a highly sensitive real-time PCR assay that specifically measures HIV-1 mRNA transcripts previously described (20). Supernatant RNA samples were measured on a Roche LightCycler 480 Real-Time PCR thermocycler with TaqMan Fast Advanced Mastermix (Applied Biosystems) run as per manufacturer's instructions and the following primers: Forward (5′→3′) CAGATGCTGCATATAAGCAGCTG (9501–9523), Reverse (5′→3′) TTTTTTTTTTTTTTTTTTTTTTTTGAAGCAC (9629-poly A). The probe used is as follows: (5′→3′) FAM-CCTGTACTGGGTCTCTCTGG-MGB (9531–9550) (all nucleotide coordinates relative to HXB2 consensus sequence). Molecular standard curves were generated using serial dilutions of a TOPO plasmid containing the final 352 nucleotides of viral genomic RNA with 30 deoxyadenosines appended to the end.

CD8+ T Cell Suppression Assay. PBMCs isolated from whole blood via Ficoll-Paque PLUS gradient centrifugation (GE Healthcare Life Sciences) were stimulated with overlapping consensus Gag peptides (10 µg/mL) and IL-2 (10 units/mL) for 7 days. CD8+ T cells were isolated from the stimulated PBMCs by positive selection (CD8 Microbeads, Miltenyi Biotec; purity routinely greater than 95%) concurrently with the isolation of resting CD4+ T cells from whole blood described above ("Outgrowth of

Latent HIV-1"). The CD8+ cells were then cultured in RPMI 1640 with 10% FBS until the conclusion of the 6-hour stimulation of the CD4+ T cells with PMA and ionomycin. The CD8+ T cells were added in a 1:1 effector:target ratio to the CD4+ T cells after washing off the PMA and ionomycin from culture and incubated with entry inhibitors EFV and RAL. Subsequent supernatant samples were taken 24 hours. Viral release and production of intracellular viral RNA were determined in the same manner as described above.

FACS Analysis. Primary $CD4^+$ T cells examined for production of latent virus were stained with CD3-PacBlue, CD4-phycoerythrin (PE), CD8-allophycocyanin (APC)-H7, CD25-fluorescein isothiocyanate (FITC), CD69-BV605, and HLA-DR–peridinin chlorophyll protein (PerCP)–Cy5.5 (BD Biosciences) to confirm the endpoint activation state.

Statistical Methods. Statistical analyses performed were conducted using Wilcoxon matched-pairs signed rank test, the nonparametric alternative to the paired t-test. The nonparametric tests were used since many of the measurements evaluated were skewed and failed to meet the assumptions required for parametric tests. In addition, the matched-pairs tests were used since measurements were observed at different time points for the same subject.

RESULTS

Kinetics of Virion Release from Latently Infected CD4+ T Cells

Resting CD4+ T cells from ART-treated chronic progressors (CPs) were stimulated with PMA and ionomycin for 6 hours to induce reactivation of latent HIV and virion production. Stimulation was performed in the presence of the non-nucleoside reverse transcriptase inhibitor efavirenz (EFV) and the integrase inhibitor raltegravir (RAL), to prevent new infection events. HIV-1 mRNA was detected in 9 of 12 CPs examined. In 7 of these subjects, HIV-1 mRNA was detected in the culture supernatant as early as 6 hours after initiation of PMA and ionomycin treatment (Figure 1). The level of HIV-1 RNA detected in the supernatant from 0-6 hours and from 6-24 hours was significantly higher than the baseline level ($p = 0.01$ and $p = 0.011$, respectively); however, there was no statistical significance between the amount of mRNA detected in the supernatant at the 2 later time points (Figure 1).

Due to the detection of HIV-1 mRNA in culture supernatant as early as 6 hours, earlier time points were interrogated for upregulation of viral transcription and virion release in 8 CPs. Intracellular HIV-1 mRNA was significantly upregulated from baseline after 1 hour of PMA and ionomycin treatment ($p = 0.017$) and remained high through at least the next 5 hours (Figure 2). This upregulation has an average 4.96±1.42 fold increase from baseline to 1 hour of stimulation and an average 4.74±1.28 fold increase from baseline to 6 hours of stimulation. However, there was no significant increase in the level of mRNA at later time points compared to 1 hour post-stimulation. Low level HIV-1 mRNA was present in the supernatant (591-1863 copies) as early as 6 hours post initiation of PMA and ionomycin treatment in 3 of the these 5 CPs. Interestingly, very

low levels of HIV-1 mRNA (524 copies) was seen in culture supernatant at 3 hours after stimulation of CP10 CD4+ T cells (data not shown).

CD8+ T Cell-Mediated Elimination of Latently-Infected CD4+ T Cells

CD8+ T cells stimulated for 7 days with overlapping Gag peptides were co-cultured for 18 hours with resting patient CD4+ T cells pre-treated with PMA and ionomycin for 6 hours in the presence of RAL and EFV. Intracellular HIV mRNA was also quantified from non-stimulated resting CD4+ T cells and PMA and ionomycin-treated CD4+ T cells in the presence of RAL and EFV. Of the 7 CP examined, only CD8+ T cells from CP13 were capable of reducing the amount of intracellular HIV-1 mRNA by a log (14.4 fold decrease from stimulation alone; Figure 3a). However, modest elimination of CD4+ T cells expressing intracellular mRNA was also seen following co-culture with CD8+ T cells from CP16 (3.72 fold decrease) and CP18 (3.80 fold decrease). Overall, however, there was no significant difference in the amount of HIV-1 mRNA found in the stimulated CD4+ T cells versus the stimulated CD4+ T cells co-cultured with CD8+ effectors in chronic progressors ($p = 0.114$; Figure 3b).

Elite controllers or suppressors (ES) have more effective HIV-specific CD8+ T cell responses than CPs (24-29) however these subjects have very low frequencies of latently infected CD4+ T cells (30) and low levels of inducible cell associated HIV-1 mRNA (31). Therefore, we analyzed viremic controllers (VCs) as these subjects maintain low level viremia but have higher frequencies of latently infected cells than ES (32) and HIV-specific CD8+ T cell responses that are similar to the responses seen in ES (33). These subjects were chosen to further examine whether a cytotoxic T lymphocyte response would be capable of eliminating CD4+ T cells that had upregulated intracellular

HIV-1 mRNA ex vivo. As with the CPs, culture supernatant from stimulated CD4+ T cells isolated from VCs had detectable HIV-1 mRNA as early as 6 hours post initiation of PMA and ionomycin treatment (1497-13,883 copies/mL, data not shown). The presence of RAL and EFV in the culture medium ensures that only viral DNA that was integrated into the host genome was amplified. Co-culture of CD4+ T cells with autologous CD8+ T cells from subjects VC1 and VC12 resulted in levels of intracellular mRNA that were lower than the baseline seen in unstimulated CD4+ T cells (>10-fold reduction, Figure 4). However when all 4 VCs were analyzed together, CD8+ T cells did not have a significant effect on levels of intracellular HIV-1 mRNA.

DISCUSSION

HIV-1 cure strategies are currently focused on a way to eliminate the latent reservoir. The end goal of any of these cure strategies is to eventually take subjects off cART. During the curative strategies, the presence of cART will prevent $CD4^+$ T cells from becoming infected by residual virus. However, if any reservoirs of HIV-1 persist when cART is discontinued, a rebound in viremia is likely to eventually occur (3–5). For this reason, a $CD8^+$ T cell response capable of control must be developed in order to prevent residual latently infected $CD4^+$ T cells from reestablishing chronic infection. In this study, we sought to determine the kinetics of reactivation in latently infected cells in order to define the time frame in which an effective HIV-specific $CD8^+$ T cell must respond.

A single $CD4^+$ T cell is thought to produce enough virus to productively infect another 3 to 34 new cells per viral generation (34, 35). An effective way of preventing a rebound in viremia following a curative procedure would be to develop $CD8^+$ effectors that target infected cells before they are able to release enough virions to infect other $CD4^+$ T cells. Our studies suggest that virus is released from reactivated latently infected cells as soon as 6 h after stimulation (Fig. 1). In contrast, intracellular mRNA is upregulated as soon as 1 h after stimulation (Fig. 2). While this very quick time frame differs from the previous reports of a 2-day viral generation time (6–9), our study directly examines HIV-1 mRNA that is produced from reactivated latently infected cells and therefore does not include the viral entry, reverse transcription, and integration steps. Based on our findings, $CD8^+$ T cells would have at most a 6-h window to effectively kill reactivated latently infected $CD4^+$ T cells before viral spread occurred.

Coincubation of stimulated $CD4^+$ T cells with autologous $CD8^+$ T cells resulted in greater than 1-log-unit reduction in intracellular HIV-RNA in two of four viremic controllers and one of seven CPs on cART (Fig. 3A and 4). The low frequency of effective killing by CP $CD8^+$ T cells seen here may be partially due to the fact that the frequency of HIV-specific $CD8^+$ T cells decreases in subjects on cART (36), and 7 days of stimulation with HIV-1 peptides may not have resulted in adequate expansion of these cells (24, 37). The suboptimal T cell response is also in contrast to other recently published studies that looked at stimulated primary $CD8^+$ T cells or $CD8^+$ T cell lines (38, 39). The differences may be partially due to the fact that some experiments from the other studies used superinfection of $CD4^+$ T cells with autologous virus and measured a decrease in p24 production over several cycles of viral replication, whereas we looked at upregulation of endogenous viral mRNA over a 24-h period following stimulation in the presence of antiretroviral drugs. In one study, $CD8^+$ T cells were able to suppress virus release from the latent reservoir after treatment of $CD4^+$ T cells with latency reversal agents (39). Differences in $CD8^+$T cell stimulation and expansion and the fact that our study measured intracellular mRNA, whereas the other study measured extracellular RNA may partially explain the discordant results in the two models.

Our results may be limited by the fact that the PMA and ionomycin treatment we used to stimulate $CD4^+$ T cells may lead to kinetics of reactivation that are different from those seen *in vivo* for $CD4^+$ T cells, which become activated when their T cell receptors (TCRs) bind to their cognate antigen and receive a second activating signal from an antigen-presenting cell. However, the low frequency of latently infected $CD4^+$ T cells that recognize any given antigen necessitates the use of polyclonal T cell stimulation. It is

also possible that PMA and ionomycin may have had an effect on the $CD8^+$ T cell response as has been shown for the latency reversal drug vorinostat in *in vitro* studies (40), but not in *ex vivo* studies (39). While we removed culture supernatant containing the drugs before adding $CD8^+$ T cells, we cannot rule out the possibility that residual drugs may have had an effect on the effector T cell response. The measurement of mRNA rather than viral proteins may have also confounded our results, since some cells that express viral RNAs may not necessarily make proteins to be recognized by $CD8^+$ T cells (41). In spite of these limitations, our study represents a model that may approximate what occurs when a latently infected $CD4^+$ T cell becomes reactivated.

In summary, we designed experiments to determine the parameters necessary for an effective HIV-specific $CD8^+$ T cell response to reactivated latently infected $CD4^+$ T cells. We examined the reactivation kinetics after $CD4^+$ T cell stimulation with resting $CD4^+$ T cells from chronic progressors and viremic controllers and defined a 5-h window between transcription and the release of extracellular viral mRNA. This short time frame may represent a significant challenge for the adaptive immune response, since it will take time for naive and central memory $CD8^+$ T cells to differentiate into effector cells that are capable of inhibiting HIV-1 replication (42). In contrast, effector memory $CD8^+$ T cells are more effective at inhibiting viral replication at early time points (42), and a cytomegalovirus (CMV)-based vaccine that induced simian immunodeficiency virus (SIV)-specific effector memory $CD8^+$ T cells was effective at clearing latently infected cells in SIV-infected monkeys (43). Furthermore, studies have shown that stimulated $CD8^+$ T cells from some elite suppressors can kill productively infected $CD4^+$ T cells within an hour (27), and we demonstrate here that stimulated $CD8^+$ T cells from two of

four VCs and one CP are capable of eliminating $CD4^+$ T cells that upregulate HIV-1 mRNA following reactivation. Our data serve as an important proof-of-concept study that suggests that with effective therapeutic immunization, $CD8^+$ T cells from CPs may be capable of effectively killing reactivated latently infected $CD4^+$ T cells that may still persist following curative strategies. These $CD8^+$ T cells would thus be capable of preventing viral rebounds and the reestablishment of a chronic infection if curative strategies are not completely effective.

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IV. The Effect of Latency Reversal Agents on Primary CD8+ T Cells: Implications for Shock and Kill Strategies for HIV Eradication

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INTRODUCTION

Latently infected CD4+ T cells are the major barrier to HIV-1 cure efforts. The cells contain integrated proviruses that are transcriptionally silent and thus able to evade detection and clearance by the immune system. The shock-and-kill cure strategy seeks to first reactivate these latent viruses without causing global T cell activation followed by clearance of the reactivated cells by the immune system (reviewed in 1 and 2). Latency reactivating agents (LRAs) are drugs that induce HIV-1 transcription. Notable drug classes include PKC agonists and HDAC inhibitors (HDACi), which have been very effective in inducing HIV-1 transcription in cell lines (3-6). Unfortunately, in vitro experiments with primary resting CD4 T cells from patients on suppressive antiretroviral therapy (ART) regimens suggest that most individual LRAs are unable to induce substantive amounts of HIV-1 transcription with the notable exception of PKC agonists bryostatin-1-1 (7) and ingenol (8). However, LRA combinations in the same system are capable of inducing significant HIV-1 transcription (9-11).

The other half of the cure strategy deals with killing newly reactivated infected CD4+ T cells. Recent experiments suggest that reactivation from latency is not enough to

induce cell death (12), and therefore there may be a need for immune mediated eradication. Expanded CD8+ T cell lines were able to clear reactivated latently infected resting CD4+ T cells following exposure to the HDAC inhibitor, vorinostat (13). However primary CD8+ T cells from patients on suppressive ART regimens that were pre-stimulated with overlapping Gag peptides were unable to consistently reduce the amount of HIV-1 mRNA induced from autologous resting CD4+ T cells that were activated with PMA and ionomycin (14).

The combination of romidepsin and bryostatin-1 has been shown to be one of the best inducers of latent HIV-1 in primary CD4+ T cells (9). However, bryostatin-1 has been showed to be involved in the modulation of NFkB and NFAT (15) and romidepsin is known to affect the function of NK cells and CD8+ T cells (16-17). Other HDAC inhibitors have furthermore been known to induce Treg cells in vitro (18-19). The immunomodulatory activity of the two drug classes thought to be most promising in cure efforts therefore needs to be further studied in the context of CD8+ T cell elimination of reactivated latently infected CD4+ T cells.

In this study, we sought to determine the ability of HIV-specific CD8+ T cells from patients with progressive HIV-1 disease on ART (chronic progressors) to kill HIV-infected CD4+ T cells after treatment with LRAs. To elucidate the contribution of the drug treatments the HIV-specific response, suppression of infection was examined with elite suppressor CD8+ T cells that had been pre-treated with different LRAs, including an HDAC inhibitor, a bromodomain-containing protein 4 inhibitor, and multiple PKC agonists. Finally, we examined the mechanisms that may have contributed to the effects

of drug treatment on CD8+ T cell function. Our results have implications for the HIV-1 cure agenda.

METHODS

Donor blood samples. HIV-1 positive and HIV-1 negative blood samples were obtained from donors with written, informed consent and handled according to a Johns Hopkins University IRB approved protocol. The chronic progressors studied were HIV-1 positive individuals who were started on suppressive ART therapy during chronic infection and have a viral load of <20 copies of HIV RNA/mL. Elite suppressors are patients who have maintained undetectable viral loads without antiretroviral therapy. The clinical characteristics of the patients are summarized in Table 1.

Primary cell isolations. PBMCs were obtained from whole blood via Ficoll-Paque PLUS gradient centrifugation (GE Healthcare Life Sciences). PBMCs underwent negative selection for CD4+ T cells using the MACS system (CD4 Isolation Kit, Miltenyi Biotech). Resting CD4+ T cells were further isolated from the bulk population by depleting CD25+, CD69+, and HLA-DR+ cells (CD25 microbeads, CD69 Isolation Kit, and HLA-DR microbeads; Miltenyi Biotech). When applicable, CD8+ T cells were obtained via positive selection from PBMCs (CD8 microbeads, Miltenyi Biotech) prior to any negative selection performed in experiments described below.

Latency reactivation ex vivo and autologous suppression. Resting CD4+ T cells isolated from fresh blood samples from ART-suppressed individuals as described above ("Primary Cell Isolation") were plated in 12-well plates with 5×10^6 cells per replicate in non-stimulating media (RPMI 1640 + Glutamax, 10% FBS) and treated alone for six hours with the combination of bryostatin-1 (B, 10 nM; Sigma Aldrich) and romidepsin (R, 40 nM; Selleck Chemicals) in the presence of efavirenz (EFV, 10 μM) and raltegravir (RAL, 4 μM) to prevent new infection and more closely mimic in vivo conditions. CD8+

T cells were isolated at this time as described above from PBMCs that had been previously stimulated for seven days in the presence of 100 U IL-2/mL and overlapping consensus Gag and Nef peptides (10 μg/mL; AIDS Reagent Database). At the conclusion of the six hours, the pre-stimulated CD8+ T cells were co-cultured with the resting CD4+ T cells at a 1:1 effector:target ratio and concentration of 5×10^6 cells/mL for another 18 h in the presence of B/R, EFV, and RAL at the same concentration as the initial treatment. The CD8+ T cells were not washed prior to co-culture with resting CD4+ T cells. In a second set of experiments, the CD8+ and CD4+ T cells were co-cultured together for 24 h in the presence of B/R, EFV, and RAL. For each replicate, at the conclusion of the full 24 h, supernatant samples and the full 5×10^6 cell samples were harvested and placed in TRIzol LS and TRIzol (Life Technologies), respectively, for the isolation of supernatant and cell-associated RNA.

Isolation and quantification of cell-associated and supernatant HIV-1 mRNA. Cell-associated and supernatant RNA were isolated, and the HIV-1 mRNA present in those samples was then quantified as previously described (7, 9, 14). Supernatant RNA samples were measured on a Roche LightCycler 480 Real-Time PCR thermocycler with TaqMan Fast Advanced Mastermix (Applied Biosystems) run as per manufacturer's instructions and the following primers: Forward (5′ → 3′) CAGATGCTGCATATAAGCAGCTG (9501–9523), Reverse (5′ → 3′) TTTTTTTTTTTTTTTTTTTTTTTTGAAGCAC (9629-poly A). The probe used is as follows: (5′ → 3′) FAM-CCTGTACTGGGTCTCTCTGG-MGB (9531–9550) (all nucleotide coordinates relative to HXB2 consensus sequence). The molecular standard curves used for the quantification were generated using serial

dilutions of a TOPO plasmid containing the final 352 nucleotides of the HIV-1 genomic RNA with the addition of 30 deoxyadenosines on the 5′ end to mimic the poly-A tail.

Latency reactivation agents' effects on autologous suppression of ex vivo infection. Autologous bulk CD8+ and CD4+ T cells were freshly isolated from PBMCs from elite suppressors as described above ("Primary Cell Isolation") for a modified version of a previously described HIV suppression assay (20). CD8+ T cells were treated for six hours in non-stimulating media (RPMI 1640 + Glutamax, 10% FBS) with either nothing, DMSO, romidepsin (40 nM), JQ1 (1 μM; Sigma Aldrich), vorinostat (335 nM), panobinostat (30 nM), bryostatin-1 at three concentrations (10 nM, 1 nM, 0.1 nM), or prostratin at two concentrations (1 μM, 0.3 μM; Sigma Aldrich) alone or in the combinations of romidepsin and bryostatin-1, romidepsin and prostratin, or bryostatin-1 and JQ1 at those concentrations. Meanwhile, the bulk CD4+ T cells were spinoculated at 1200 ×*g* for two hours at 37 °C with HIV-$1_{NL4-3\ \Delta Env-GFP}$, a replication incompetent lab strain pseudovirus with *env* replaced with *gfp* and whose expression is controlled by the HIV promoter. At the conclusion of the six-hour drug treatments, the drug was washed from the CD8+ T cells before the cells were added in a 1:1 effector:target ratio to the spinoculated CD4+ T cells. The cells co-cultured in nonstimulating media (RPMI 1640 + Glutamax, 10% FBS) were incubated for three days prior to FACS analysis.

Bryostatin-1 treatment and its effects on cytokine production. PBMCs isolated from elite suppressors were plated at a concentration of 1×10^6 cells per mL in 48-well plates and treated for six hours with nothing, DMSO, romidepsin (40 nM), bryostatin-1 (10 nM), and the combination of romidepsin and bryostatin-1. After treatment, cells were washed and then re-plated as before in non-stimulating media (RPMI 1640 + Glutamax,

10% FBS). All samples were cultured with Golgi Plug and Golgi Stop (BD Biosciences) as per manufacturer's instructions and 1 µg/mL of anti-CD28 and anti-CD49d antibodies (NA/LE anti-CD28 clone CD28.2, anti-CD49d clone 9F10; BD Biosciences). The no-stimulation control had no additional treatment added, and the two stimulation conditions were incubated with 10 µg/mL overlapping consensus Gag peptides and 1 µg/mL anti-CD3 for stimulation (NA/LE anti-CD3 clone HIT3a; BD Biosciences), respectively.

General effects of latency reactivation agents on immune markers and cell death. PBMCs isolated from HIV-negative donor blood were plated at a concentration of 1×10^6 cells per mL in 48-well plates and treated for six hours with nothing, DMSO, romidepsin (40 nM), bryostatin-1 at three concentrations (10 nM, 1 nM, 0.1 nM), prostratin (0.3 µM), and the combination of romidepsin (40 nM) and bryostatin-1 (10 nM). The doses of these drugs were selected based on the concentrations needed to reverse latency either alone or in combination (9). 40 nM of romidepsin is below the concentration of the plasma levels achieved in patients treated with this drug for lymphoma (21). Plasma bryostatin-1 levels of close to 1 nM have been achieved in patients receiving the highest tolerated dose of the drug (22). Two sets of cultures were set aside for analysis by FACS at six hours post-treatment and 18 hours post-treatment. For the rest of the cultures, cells were washed after six hours of drug treatment prior to replating in fresh plates at the same concentration of cells in non-stimulating media (RPMI 1640 + Glutamax, 10% FBS) either in the presence or absence of 1 µg/mL anti-CD3/CD28 antibodies (NA/LE anti-CD3 clone HIT3a, anti-CD28 clone CD28.2; BD Biosciences) for an additional 1, 2, or 3 days before FACS analysis.

FACS analysis of suppression and immune markers. For the suppression experiments, samples were analyzed for infected CD4+ T cells by staining for CD3 (PacBlue, BD Biosciences), CD4 (BV605, Biolegend), and CD8 (APC-H7, BD Biosciences) and examining for the GFP+ (pseudovirus infected) cells. The amount of suppression was calculated by comparing the amount of infected CD4+ T cells with CD8+ T cell co-culture to those without effector cell co-culture (% Suppression = [1 − (% GFP+ CD4+ T cells cultured with CD8+ T cells) / (% GFP+ CD4+ T cells without effectors)] × 100%). Intracellular cytokine expression was determined with the following panel: CD3·PacBlue, CD4·BV605, CD8·APC-H7, CD69·APC (Biolegend), IL-2·PE (BD Biosciences), TNFα·PE-Cy7 (BD Biosciences), IFNγ·PerCP-Cy5.5 (BD Biosciences), and Perforin-FITC (Cell Sciences). Immune markers and cell death were examined in the HIV-negative donors' cells via three staining panels (Panel A: CD3·APC-Cy7 [Biolegend], CD4·BV605, CD8·APC [BD Biosciences], PD-1·FITC [Biolegend]; Panel B: CD3·PE [BD Biosciences], CD4·BV605, CD8·APC-H7, CD69·APC, 7-AAD [BD Biosciences], Annexin V·V450 [BD Biosciences]; Panel C: CD3·PacBlue, CD8·APC-H7, CD69·BV605, CD160·PE [Biolegend], TIM-3·PE-Cy7 [Biolegend], 2B4·APC [BD Biosciences]). CD69, the exhaustion markers, and Annexin V expression are shown as raw data, but expression of CD3 is compared via MFI ratio (MFI ratio = [MFI of marker in treatment]/[MFI of marker in no treatment]). All samples were run on a BD FACSCantoII flow cytometer and analyzed in FlowJo vX.0.7.

Statistics. Statistical analyses performed for HIV-1 RNA (Fig. 1) were conducted using Wilcoxon matched-pair signed-rank test, the nonparametric alternative to the paired *t*-test as previously described (14). Descriptive statistics for other experiments are presented as

means and standard deviations. Comparisons of treatment groups to the control (NT) were conducted using repeated measures ANOVA model with adjusted pair-wise comparisons to NT via Dunnett's correction. Strength of evidence, threshold *p*-values, will be presented as: ns (>0.05), * (<0.05), ** (<0.01), and *** (<0.001). Parametric methods were used due to the failure of nonparametric to detect significance for paired data with sample size less than six. All statistics and graphics were performed with GraphPad Prism 6.

RESULTS

Ex vivo reactivation of latently infected CD4+ T Cells and CD8+ effector-mediated elimination

Resting CD4+ T cells from six ART-suppressed HIV-1+ individuals were treated with 10 nM bryostatin-1 and 40 nM romidepsin (B/R) for twenty-four hours to reactivate latent HIV-1 proviruses. A subset of samples from each of the individuals was further co-cultured with autologous CD8+ T cells that had been previously cultured for 7 days in the presence of IL-2 and overlapping consensus Gag and Nef peptides to determine whether these immune effectors cells could eliminate latently infected cells. In a prior study we demonstrated that viral release occurred as early as 6 h after resting CD4+ T cell stimulation (14), so the CD8+ T cells were added at 6 h to minimize their exposure to the latency reversal agents. In all six individuals, cell-associated HIV mRNA was increased by a median of 12.72-fold due to B/R treatment as compared to resting CD4+ T cells cultured in the absence of drugs ($p < 0.0002$, Fig. 1B). However, the co-culture of B/R-treated resting CD4+ T cells with stimulated CD8+ T cells did not result in a significant decrease in the amount of HIV-1 mRNA, although three of the six individuals showed a trend towards a decrease in the amount of cell-associated HIV-1 mRNA (Fig. 1B). The effect of drugs and CD8+ T cells on the release of virus into culture supernatant was also examined. HIV-1 mRNA present in culture supernatant increased significantly from an undetectable baseline (500 copies/mL) following B/R treatment to a median of 5281.6 copies/ml ($p < 0.001$), and there was no significant difference seen when the B/R-treated CD4+ T cells were co-cultured with CD8+ T cells (Fig. 1C). Of the six individuals, only CP25 had a decrease in both cell-associated and supernatant HIV-1 mRNA due to co-

culture of B/R-treated resting CD4+ T cells with CD8+ T cells (Fig. 1C). We repeated the experiments with CD8+ T cells present at the time point 0 to ensure that the failure to eliminate reactivated CD4+ T cells was not due to early transcription and translation in the absence of the effector cells. However, there was still no significant decrease in either intracellular (Fig. 1D) or extracellular mRNA expression (Fig. 1E) when the stimulated CD4+ T cells were co-cultured with CD8+ T cells.

Elite suppressor CD8+ T cell responses after ex vivo treatment with latency reactivating agents

To elucidate whether or not the lack of an effective response to reactivated HIV-1 from latently infected CD4+ T cells in the chronic progressors was due to host factors or the drugs themselves we studied the effects of the drugs on HIV-specific CD8+ T cell responses. Elite suppressors were used for these studies, as they are known to have qualitatively superior HIV-specific CD8+ T cell responses as compared to the average chronic progressor (23-27). CD4+ T cells from ES were infected with replication incompetent HIV-$1_{NL4-3\ \Delta Env-GFP}$ pseudovirus and co-cultured with autologous CD8+ T cells that had been previously incubated with a variety of latency reversing agents (LRAs), as previously described (20, 28). DMSO, the vehicle for all the drugs, had no effect on the CD8+ T cell-mediated suppression as compared to no treatment (Fig. 2A–E). Romidepsin (40 nM throughout) alone and bryostatin-1 at either 10 nM or 1 nM significantly inhibited the ability of elite suppressor CD8 T cells to suppress infection as compared to untreated CD8+ T cells (Fig. 2A). The combination of romidepsin and all three concentrations of bryostatin-1 tested (10 nM and 1 nM significantly inhibited suppression as compared to untreated CD8+ T cells, and the combinations of romidepsin

and bryostatin-1 at either 10 nM or 1 nM were much more inhibitory than either drug alone, with an 81.8% and 84.0% reduction in the amount of suppression seen for the combination of bryostatin at 10 nM and romidepsin from each drug alone, respectively, and 72.6% and 70.8% reduction for the combination of bryostatin at 10 nM and romidepsin from each alone.

In order to determine whether the inhibition of suppression is unique to bryostatin-1 or is a feature of PKC agonists, CD8+ T cells from elite suppressors were also treated with prostratin at either 1 μM or 0.3 μM alone or in conjunction with romidepsin. As opposed to bryostatin-1, prostratin alone at either concentration had no significant effect upon the suppressive capacity of the CD8+ T cells (Fig. 2B). The combination of prostratin at 1 μM and 0.3 μM with romidepsin significantly inhibited the suppression of infection ($p < 0.0001$), but this inhibition was very similar to the inhibition seen by romidepsin alone (Fig. 2B).

The effect of JQ1, a bromodomain-containing protein 4 inhibitor, was also examined alone and in conjunction with bryostatin-1 or prostratin as these combinations of drugs have also been shown to be effective at reversing latency in vitro (9). Treatment of CD8+ T cells with JQ1 (1 μM) alone resulted in a slight, nonsignificant decrease in CD8+ T cell suppression (Fig. 2C). A significant decrease in CD8+ T cell-mediated suppression was seen when JQ1 was given in combination with bryostatin-1 at 10 nM and 1 nM ($p < 0.001$ for both), but this was not significantly different from the inhibition seen with bryostatin-1 alone at either concentration (Fig. 2C). In contrast, the combination of prostratin and JQ-1 did not have a significant effect on the CD8+ T cell function (Fig. 2D).

In order to determine whether romidepsin's effect on CD8+ T cell suppressive activity was unique to this drug or a feature of all HDAC inhibitors, we compared the effects of romidepsin vorionostat and panobinostat on ES CD8+ T cell function. The concentrations used were based on concentrations that had been shown to be effective in prior studies (9, 29) and were similar to levels that have been achieved in vivo (21, 29-31). While romidepsin and panobinostat had significant inhibitory effects on ES CD8+ T cells, vorinostat did not cause significant suppression (Fig. 2E). These results are similar to results obtained with HIV-specific CD8+ T cell clones (17).

CD8+ T cell cytokine production after LRA treatment

We next sought to find a mechanism for the inhibition of suppression due to bryostatin-1 treatment and first examined the ability of elite suppressor HIV-specific CD8+ T cells to produce cytokines after a 6-hour treatment with bryostatin-1 and romidepsin followed by 12-h of stimulation with either Gag peptides or anti-CD3 and CD28 monoclonal antibodies. Bryostatin-1 treatment alone tended to cause an increase in the percentage of cells that produced TNF-α, both TNF-α and IFN-γ, and IL-2 following anti-CD3/CD28 stimulation (for IL-2, $p < 0.05$; Fig. 3B). In contrast, the B/R combination caused an increase in the percentage of cytokine-producing CD8+ T cells at baseline (IFN-γ, TNF-α, and IFN-γ and TNF-α), and following stimulation with Gag peptides (TNF-α; $p < 0.05$) and anti-CD3/CD28 monoclonal antibodies (TNF-α, TNF-α and IFN-γ, and IL-2; for IL-2 only,$p < 0.05$). Thus it appears that a decrease in cytokine expression was not the mechanism of suppression of CD8+ T cell antiviral activity.

PKC agonist effects upon cell death and exhaustion in T cells

To further elucidate the mechanisms by which the LRAs affected CD8+ T cell function, CD8+ T cells from HIV-negative donors were interrogated for the amount of cell death induced by drug treatment as determined by Annexin V expression. For both 6-hour and 18-hour treatments with LRAs, the combination B/R treatment induced a trend towards a higher amount of cell death than cells treated with DMSO (not shown). To examine the effects of the LRAs on cell viability over time, cells were treated for six hours before being washed and cultured for an additional three days in the absence of stimulation. For this time course, 10 nM bryostatin-1-treated CD8+ T cells had a trend towards more cell death on day 1 and significantly more on day 2 ($p < 0.05$). A trend towards higher cell death was likewise observed in B/*R*-treated CD8+ T cells (Fig. 4B). To model the effect of the LRAs on activated CD8+ T cells, CD8+ T cells treated with 10 nM bryostatin-1 and B/R for six hours were stimulated with anti-CD3/CD28 antibodies for an additional two days. Cells treated with anti-CD3/CD28 generally had increased cell death compared to CD8+ T cells that did not receive this treatment, but only bryostatin-1 and B/R treatment of anti-CD3 activated cells caused a significant increase in cell death compared to antibody treatment alone as determined by annexin V expression (Day 1, bryostatin-1: $p < 0.05$; Day 2, B/R: $p < 0.05$; Fig. 4B) and supported by trends with annexin V and 7-AAD co-expression.

We also examined the expression of the exhaustion markers PD-1, Tim-3, 2B4, and CD160 following 6 h of treatment with the different drugs as a potential cause of the observed inhibition of CD8+ T cell responses. Bryostatin-1 at 10 nM and B/R induced modest but significant increases in PD-1, TIM-3 and 2B4 expression at different time points over a 3 day time period (example of PD-1 gating shown in Supplementary Fig.

1A; Fig. 5A–C). In order to determine the effects of the LRAs on activated cells, we looked at the expression of these exhaustion markers on cells that were exposed to drugs for 6 h and then stimulated with CD3 and CD28 specific antibodies. PD-1, CD160 and 2B4 expression levels were significantly increased in activated cells that were treated with bryostatin-1 or B/R on day 2 whereas romidepsin tended to increase CD160 expression on the activated cells at the same time point (Fig. 6A–C). Furthermore, bryostatin-1 also significantly PD-1 expression in the stimulated cells on day 2 as measured by ratio of the mean fluorescence intensity of the treated cells to that of the untreated cells ($p < 0.05$; Supplementary Fig. 1).

In order to determine whether the expression of the exhaustion markers was just a marker of T cell activation we looked at CD69 expression on CD8+ T cells treated with LRAs. Bryostatin-1 treatment has been shown to induce CD69 on resting CD4+ T cells (9), and we found very high levels of this early activation marker on CD8+ T cells that were treated with 10 nM and 1 nM bryostatin-1, B/R, and prostratin (Fig. 7). PD-1, 2B4 and TIM-3 expression was not upregulated on prostratin-treated cells, implying that the expression of these exhaustion markers was not just a reflection of partial activation although further experiments are needed to verify this.

PKC agonist-induced modulation of TCR-related markers

PKC agonists such as PMA have been known to downregulate CD3 (32), which has the potential to inhibit T cell responses. In order to determine whether LRA treatment had an effect on CD3 expression, we examined the change in the mean fluorescence intensity (MFI) of this marker. CD8+ T cells treated with bryostatin-1 at 10 nM and 1 nM and B/R had a significant decrease in CD3 expression after six hours of treatment

(bryostatin-1: $p < 0.01$ at 10 nM, $p < 0.05$ at 1 nM; B/R: $p < 0.01$; Fig. 8A). However, after 18 h of treatment, bryostatin-1 at 10 nM and 1 nM, the B/R combination, and prostratin alone cause significant decreases in CD3 expression compared to untreated cells ($p < 0.0001$ for 10 nM bryostatin-1 and B/R; $p < 0.001$ for bryostatin-1 at 1 nM and prostratin; Fig. 8A). For CD8+ T cells treated for six hours and then cultured for a day in the absence of treatment, bryostatin-1 at 10 nM and 1 nM as well as B/R continued to cause a significant decrease in CD3 expression (bryostatin-1: $p < 0.01$, $p < 0.05$, respectively; B/R: $p < 0.001$; Fig. 8B). Unlike bryostatin-1 treatment alone, however, the effect of B/R on CD3 expression maintained at day 1 (Fig. 8A,B). Overall, while bryostatin-1 has an effect on CD3 expression alone, the combination of bryostatin-1 with romidepsin causes both a more severe and longer-lasting phenotype.

DISCUSSION

Current HIV-1 cure strategies seek to eliminate the latent reservoir by specifically activating HIV-1 so the immune system can clear the latently infected cells. The most promising latency reactivating agent (LRA) regimens in vitro thus far appear to be combinations of HDAC inhibitors (HDACi) and PKC agonists (9). However, some of these drugs have previously been suggested to have immunomodulatory effects (17; reviewed in 33-34). In this study, we confirm that the combination of bryostatin-1 and romidepsin is effective in reversing latency, however we found that even following stimulation with high concentrations of IL-2 and Gag and Nef consensus peptides, CD8+ T cells from fully suppressed chronic progressors were unable to reduce the amount of HIV-1 mRNA associated with CD4+ T cells or prevent release of virions from these cells in the context of bryostatin-1 and romidepsin treatment. One limitation of our study is that we did not measure actual viral protein production or antigen presentation following latency reversal. It is possible that some of the mRNA we measured is defective and did not lead to the synthesis of functional proteins that could be recognized by CD8+ T cells. Another possibility is that the effects of the LRAs may be short lived in vivo since HIV-specific CD8+ T cells from patients treated with vorinostat (13) and romidepsin (35) appeared to be functional in ex vivo studies, but even a short term effect could be important for viral clearance. Another limitation is the relatively small number of patients studied in this manuscript.

In a prior study, autologous ex vivo expanded virus-specific cytototoxic T lymphocytes, but not unexpanded CD8+ T cells, from HIV infected patients were able to significantly reduce the number of latently infected cells following reversal with

vorinostat (13). The discrepancy between that study and our findings could potentially be explained by the fact that vorinostat has much less of an effect of on CD8+ T cells than did romidepsin, bryostatin-1 and the combination of the 2 drugs. Chronic progressors' primary CD8+ T cells are generally not effective in controlling HIV-1 replication (reviewed in 36), which may also partially account for observed results. In order to determine other potential causes, we examined the effect of four separate LRAs alone and in combination on the HIV-specific CD8+ T cell response of elite suppressors.

Elite suppressors are HIV-positive individuals who have viral loads of <50 copies of HIV-1 RNA per milliliter in the absence of antiretroviral therapy (37). These individuals are known to have qualitatively superior HIV-specific CD8+ T cell responses than chronic progressors (23-27). We performed suppression assays with cells from four elite suppressors and found that untreated CD8+ T cells were able to suppress infection effectively but that treatment with romidepsin (40 nM) or bryostatin-1 (10 nM) alone significantly inhibited this suppression by nearly 50%. The combination of the two LRAs at those concentrations fully ablated the suppression in two individuals and otherwise significantly reduced the average suppression by 90% suggesting that the two drugs may have an additive, negative effect (Fig. 2A). Interestingly, prostratin, another PKC agonist did not have an inhibitory effect on CD8+ T cell mediated suppression, suggesting that the effect on CD8+ T cell function may not be a feature of the entire class of drugs. HDAC inhibitors such as romidepsin and panobinostat have been shown to selectively cause the death of activated cells (17), and PKC agonists such as bryostatin-1, prostratin, and PMA are known to cause partial activation in T cells (38-40), with bryostatin-1 specifically acting as a TLR-4 ligand (41). We therefore combined

romidepsin with prostratin, which also induced partial activation as determined by CD69 expression, to determine if the additive negative effect seen when bryostatin-1 and romidepsin were combined was a general effect of adding an HDAC inhibitor to a PKC agonist. Interestingly, the combination had an equal amount of suppression as romidepsin alone (Fig. 2B). JQ1 (1 μM), a bromodomain-containing protein 4 inhibitor, also had minimal effect on CD8+ T cell mediated suppression and did not appear to have an additive inhibitory effect when it was combined with bryostatin-1. The combination of prostratin and JQ1 has also recently shown to be effective (9) and these drugs together did not have an adverse effect on CD8+ T cell function.

In order to determine the mechanism for the inhibition of suppression seen with romidepsin, bryostatin-1, and the combination of the two, we examined the LRA treatment-dependent toxicity and induction of exhaustion on CD8+ T cells. Bryostatin-1 treatment at 10 nM causes an increase in cell death and PD-1 expression, and combined bryostatin-1 and romidepsin treatment more closely mimics the effects of bryostatin-1 treatment as compared to romidepsin treatment, suggesting that the phenomena are bryostatin-1-mediated. The increased cell death and exhaustion shortly after treatment with 10 nM bryostatin-1 likely contributes to the CD8+ T cell dysfunction in the suppression assays, given that CD8+ T cell killing of HIV-1 infected CD4+ T cells can occur within an hour (26).

PKC agonists are known to downregulate CD3 (32) as well as CD4 (42-45). Downregulation of CD3-TCR complexes as well as the coreceptors CD4 and CD8 may inhibit the ability of effector T cells to respond to their cognate antigen, leading us to examine the expression of these markers in T cells. We found that bryostatin-1 treatment

caused transient downregulation of CD3 in unstimulated CD8+ T cells, but treatment with bryostatin-1 and romidepsin prolonged this effect. Even a transient effect may be important because we have previously shown that virion release from latently infected CD4+ T cells may occur as early as 6 h after activation (14), and therefore a quick immune response will be needed to eliminate reactivated CD4+ T cells.

In summary, we have shown that the HDACi/PKC agonist LRA combination of bryostatin-1 and romidepsin causes marked inhibition of the HIV-specific CD8+ T cell response, as do both drugs alone. The inhibition of the T cell response by bryostatin-1 may be due an increase in T cell death and exhaustion marker expression as well as a downregulation of CD3, resulting in a decreased ability of T cells to respond to stimuli. The combination of romidepsin with bryostatin-1 furthermore causes a more severe and possibly longer-lasting downregulation phenotype, potentially contributing to the more severe inhibition of the T cell response. Any HIV-1 cure strategy involving LRA-based reactivation will likely depend upon the immune response to eliminate any latently infected cells, but given the range of negative effects the LRAs have upon the immune response alone and in combination, each potential LRA therapy should be examined for its broad effects on adaptive immunity before use in the context of HIV-1 cure. Combinations of latency reversal agents such as prostratin and JQ1 that together do not have significant effects on HIV-specific immune responses may be the most effective candidates for the shock and kill approach to HIV-1 eradication.

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V. CONCLUSION

While current treatment for HIV-1 extends the lives of millions around the world, the need for both a cure and a vaccine remain. The current strategy for eradication would require the use of latency reactivating agents (LRAs) to reactivate the latent HIV-1 present in CD4+ T cells. However, despite the fact that many LRAs in combination are capable of robustly inducing HIV mRNA expression (1), the effects of the LRAs upon the adaptive immune system vary from causing no change to complete ablation of the suppressive capacity of the CD8+ T cell response of elite suppressors (2). The mechanism behind the inhibition of the CD8+ T cell response caused by bryostatin-1 appears to be an increase in cell death, the downregulation of CD3, and the increase in expression of exhaustion markers (2). HDAC inhibitors alone have also been seen to cause CD8+ T cell effector dysfunction in vitro (3), and the combination of bryostatin-1 and romidepsin causes a detrimental effect upon the CD8+ T cell response that was worse than either drug alone (2). Despite the fact that the combination of bryostatin-1 and romidepsin induce reactivation in resting CD4+ T cells, the prevention of an effective CD8+ T cell response due to the drug treatment undermines the premise of the "shock and kill" strategy.

While multiple HDAC inhibitors and bryostatin-1 had detrimental effects upon the CD8+ T cell response, not all LRAs alone or in combination do (2). The combination of JQ1 and prostratin appear to keep the "kill" component of "shock and kill" intact (2). However, the CD8+ T cell response is not always capable of eliminating reactivated primary latently infected CD4+ T cells in all HIV-positive individuals, even when PMA and ionomycin are used to induce HIV mRNA expression and are removed from culture

prior to the addition of effectors (4). Very few of the chronic progressors examined and only half of the viremic controllers exhibited this capacity (4), but a therapeutic vaccine that induced a CD8+ T cell response like what was seen in with the CMV-based SIV vaccine (5) might be able to properly boost the T cell responses of those individuals whose responses were inadequate.

Any infection that remains within other cellular reservoirs, such as macrophages, might also be taken care of by a therapeutic vaccine. The CD8+ T cell response of elite suppressors can kill infected monocyte-derived macrophages (MDMs), and CD4+ T cells are also capable of suppressing infection of the MDMs (6). Macrophages are found in many tissues, including the brain (7), and although they may be of less concern when HIV-positive individuals are treated with cART, the ability of the adaptive immune response to eliminate any infected macrophages would be a concern for both post-cure, assuming complete eradication is unlikely, as well as for any T-cell based vaccine. Given that CD4+ T cells that come in physical contact with infected macrophages are efficiently infected (6), the killing of the macrophages would be necessary to prevent any reseeding of the latent reservoir post any curative strategies that are unable to induce a sterilizing cure before cART cessation.

An immeasurable amount of progress has been made in the treatment of HIV-1 infection since its discovery in 1981 (8). With the advent of cART in the mid-nineties, HIV-1 infection went from incurable and almost always fatal to a chronic condition (9). In the twenty years since, only one individual has ever been cured of HIV-1 (10), and no vaccine yet exists. The "shock and kill" cure strategy has promise, but to succeed, it will likely need to be used in conjunction with some sort of therapeutic vaccine or

immunomodulatory treatment. Any single LRA or combination thereof should be examined for their effects upon the adaptive immune system before being used clinically in order to minimize risk to the patients and maximize the likelihood that both the "shock" and the "kill" will be able to cause a sterilizing cure.

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V. FIGURES AND TABLES

CHAPTER II

Table 1. Clinical characteristics of study patients.

Patient	Protective HLA allele	Date (yr) HIV infection diagnosed	Current $CD4^+$ T cell count (no. of cells/μl)	Current viral load (no. of copies/ml)	Date (yr) treatment initiated	Pre-ART[a] $CD4^+$ T cell count (no. of cells/μl)	Pre-ART viral load (no. of copies/ml)
ES4	None[b]	1996	477	<20			
ES5	B*57	1992	630	<20			
ES6	B*57	1992	675	<20			
ES8	B*57	1998	698	101[c]			
ES9	B*27, B*57	1999	816	<20			
ES22	B*57	2009	981	<20			
ES23	B*57	1985	708	<20			
ES24	B*57	2009	1,368	<20			
ES31	B*27, 5801	2008	960	<20			
CP1	B*57	2012	653	<20	2013	494	27,380
CP2	ND[d]	1997	804	<20	1998	190	NA[e]
CP3	ND	2005	1,042	<20	2005	NA	NA
CP4	ND	1986	720	<20	2010	NA	NA
CP5	ND	1993	423	<20	2000	15	>750,000
CP6	B*57	1998	462	<20	2001	12	300,000
CP7	B*57	1999	500	<20	1999	NA	NA
CP8	B*57	1988	1,310	<20	2006	177	111,089
CP9	ND	1995	820	<20	1996	35	NA
CP10	B*57	2007	409	<20	2007	18	297,092

[a]ART, antiretroviral therapy.

[b]The patient is HLA-B*08/44 positive.

[c]The patient usually has a viral load of <75 copies/ml in the branched DNA assay.

[d]ND, not determined.

[e]NA, not available.

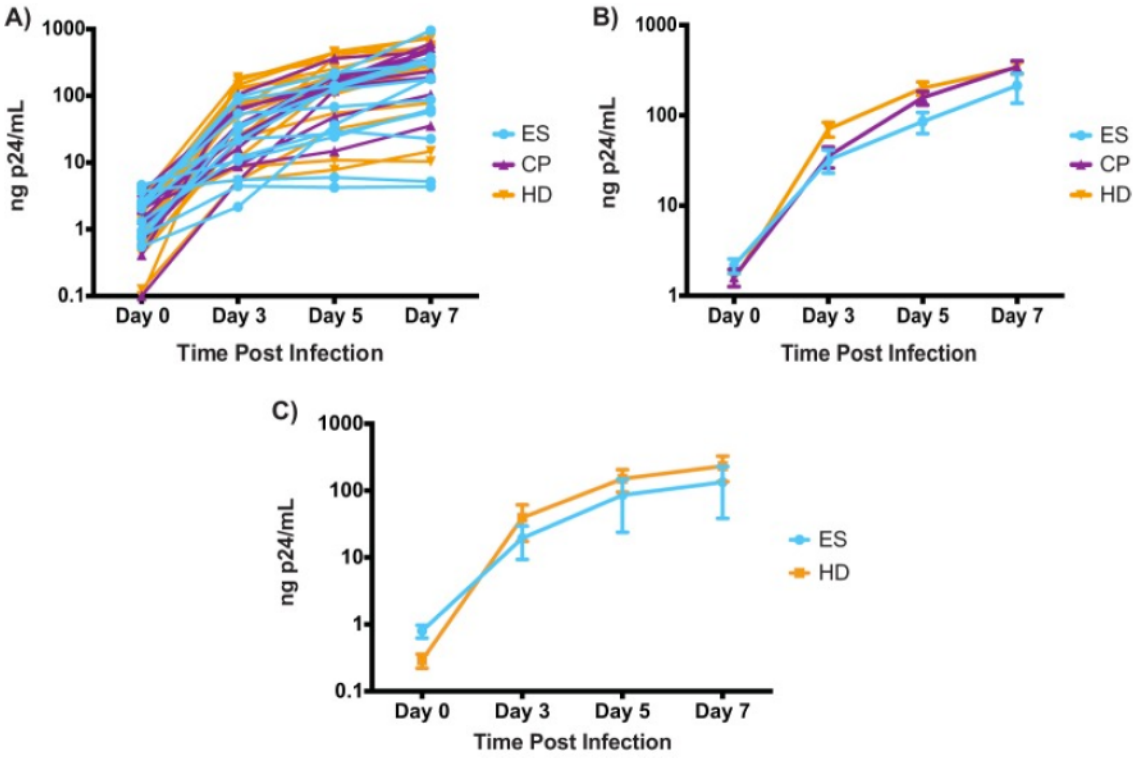


Figure 1. ES macrophages are infected as well as HD and CP macrophages. (A) Individual HIV-1 growth curves for ESs (n = 12), CPs (n = 11), and HDs (n = 19) whose MDMs were infected by spinoculation of HIV_{BaL}; (B) average ± standard error virus production by MDMs from the aforementioned groups; (C) HIV-1 replication in MDMs from ESs (n = 4) and HDs (n = 4) after infection without spinoculation.

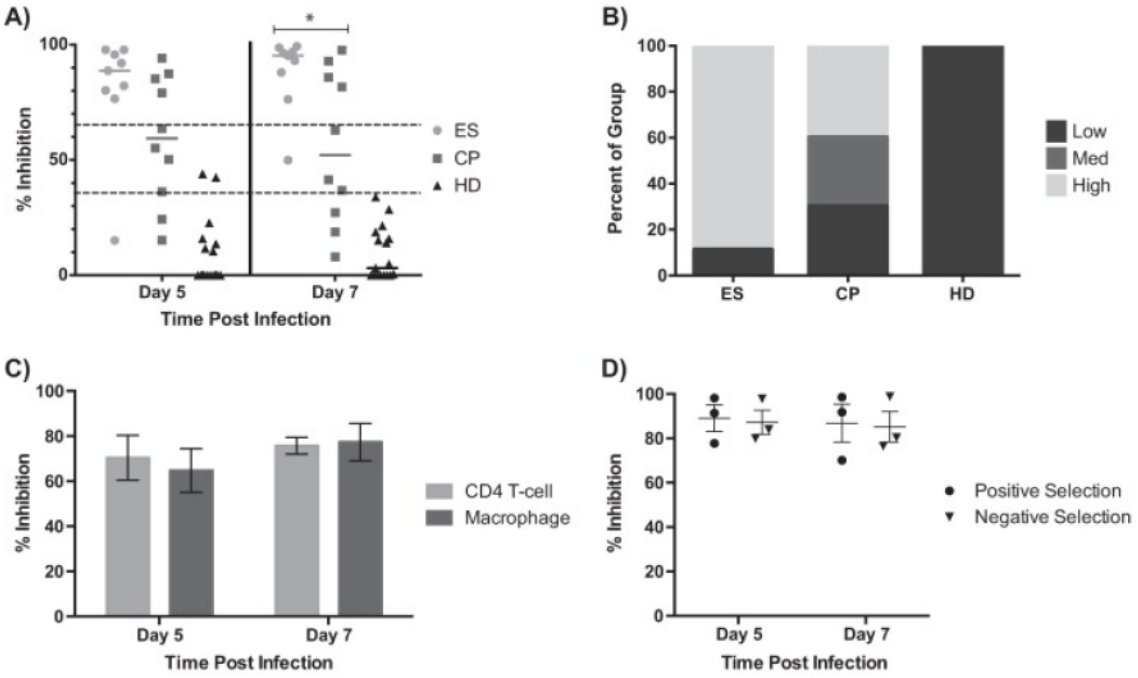


Figure 2. $CD8^+$ T cells from ESs are more effective than $CD8^+$ T cells from CPs at inhibiting viral replication in macrophages. MDMs were infected with 500 ng HIV_{BaL} per 10^6 cells and cultured with $CD8^+$ T cells in a 1:2 ratio, and the percent inhibition was calculated by measuring the p24 content of the supernatant via ELISA. (A) Inhibition of viral production on days 5 and 7 for ES (n = 9), CP (n = 10), and HD (n = 18) MDMs, with the medians indicated by horizontal bars; (B) distribution of the inhibitory responses in each subset on day 7; (C) CD8-mediated inhibition of virus production by $CD4^+$ T cell and macrophage targets (n = 3 [cells from ESs 6, 22, and 24]); (D) the inhibitory abilities of $CD8^+$ T cells when isolated via positive and negative selection are equal (n = 3 [cells from ESs 5, 8, and 9]; data are averages ± standard errors). *, $P < 0.05$.

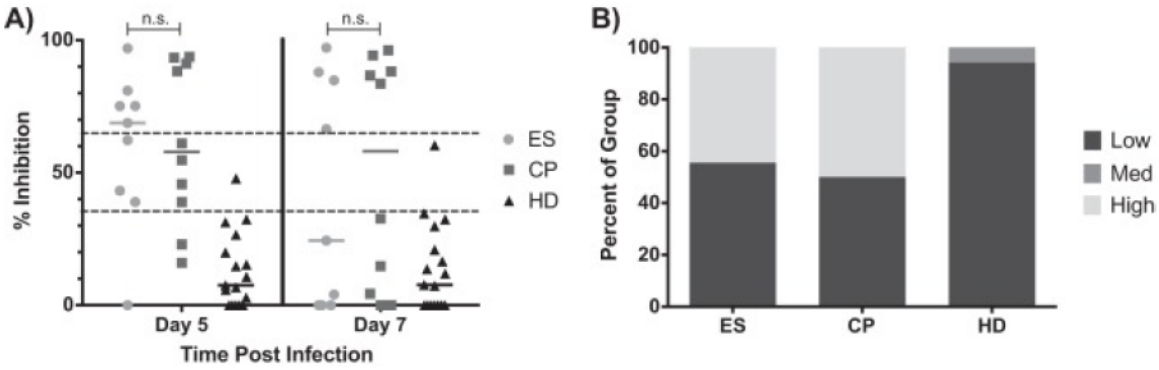


Figure 3. $CD4^+$ T cells from ESs are not more effective than $CD4^+$ T cells from CPs at inhibiting viral replication in macrophages. MDMs were infected with 500 ng HIV_{BaL} per 10^6 cells and cultured with $CD4^+$ T cells in a 1:2 ratio, and the percent inhibition was calculated by measuring the p24 content of the supernatant via ELISA. (A) Inhibition of viral production on days 5 and 7 for ES (n = 9), CP (n = 10), and HD (n = 18) MDMs, with the medians indicated by horizontal bars; (B) distribution of the inhibitory responses in each subset on day 7. n.s., nonsignificant.

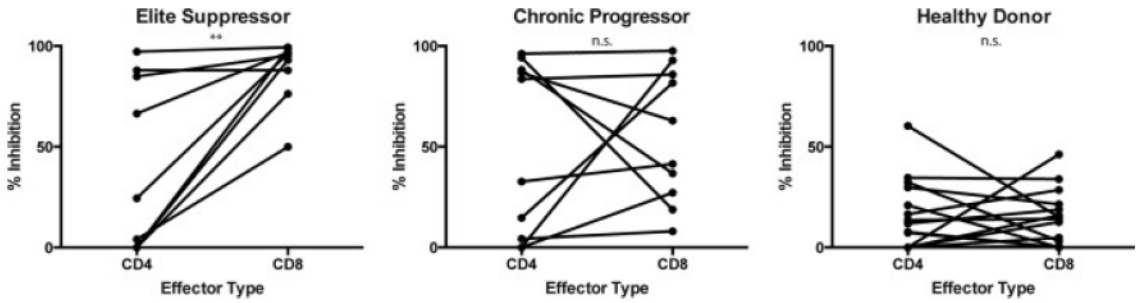


Figure 4. $CD8^+$ T cells from ESs are more effective than $CD4^+$ T cells at inhibiting viral replication in macrophages. MDMs were infected with 500 ng HIV_{BaL} per 10^6 cells and cultured with $CD4^+$ or $CD8^+$ T cells in a 1:2 ratio. Infection was measured by p24 ELISA for ES ($n = 9$), CP ($n = 10$), and HD ($n = 18$) MDMs. **, $P < 0.01$; n.s., nonsignificant.

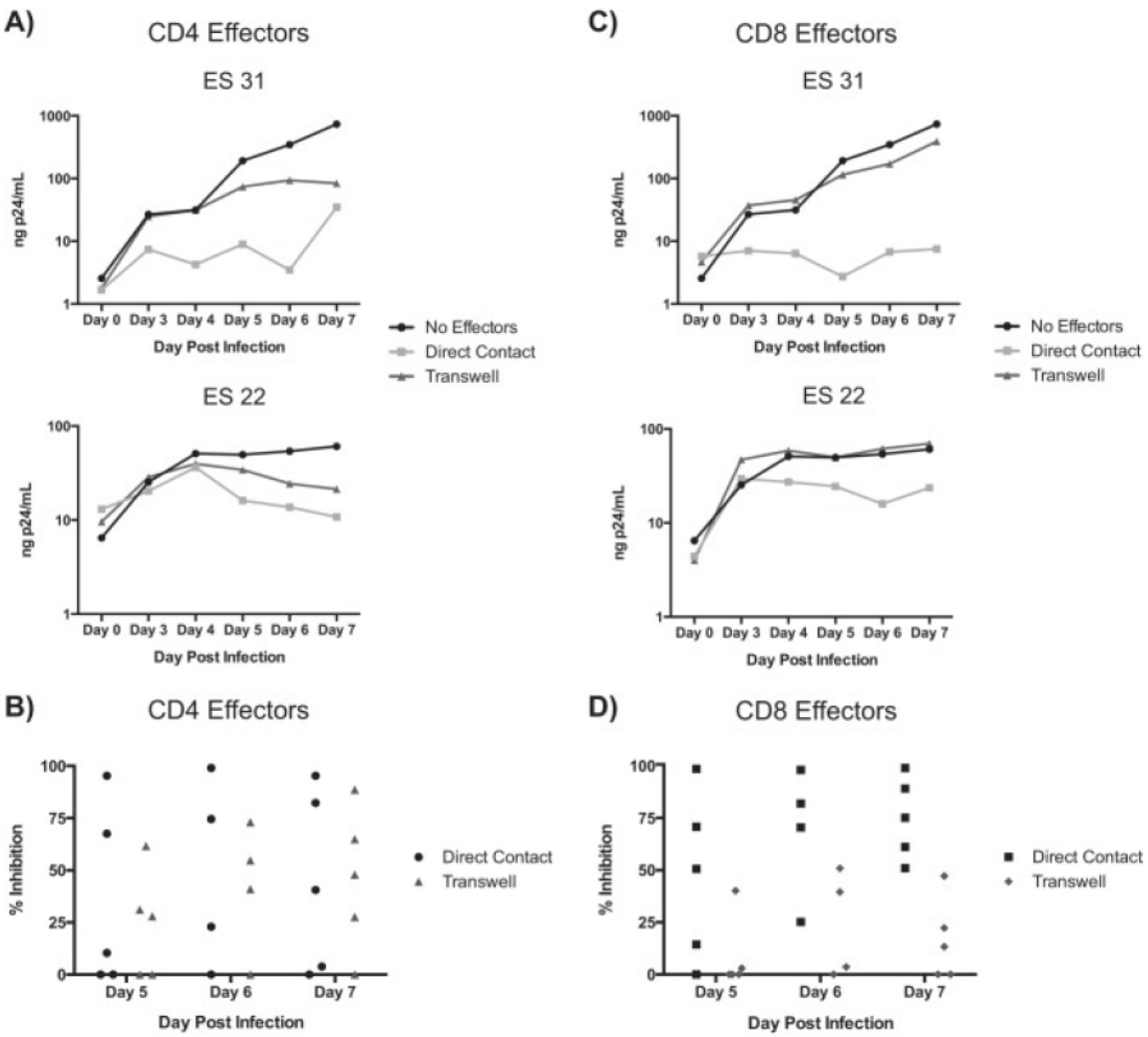


Figure 5. $CD4^+$ T cell-mediated inhibition of viral production is partially soluble factor mediated, while $CD8^+$ T cell-mediated inhibition is contact dependent. MDMs infected with HIV_{BaL} were cocultured with $CD4^+$ or $CD8^+$ T cells in a 1:1 effector cell/target cell ratio either directly or with separation in transwells. Viral production was measured by p24 ELISA. (A) $CD4^+$ effector-mediated inhibition of virus production by two ES individuals, with the $CD4^+$ response shown; (B) $CD4^+$ effector-mediated inhibition (n = 5); (C) $CD8^+$ effector-mediated inhibition of virus production by two ES individuals, with the $CD4^+$ response shown; (D) $CD8^+$ effector-mediated inhibition (n = 5).

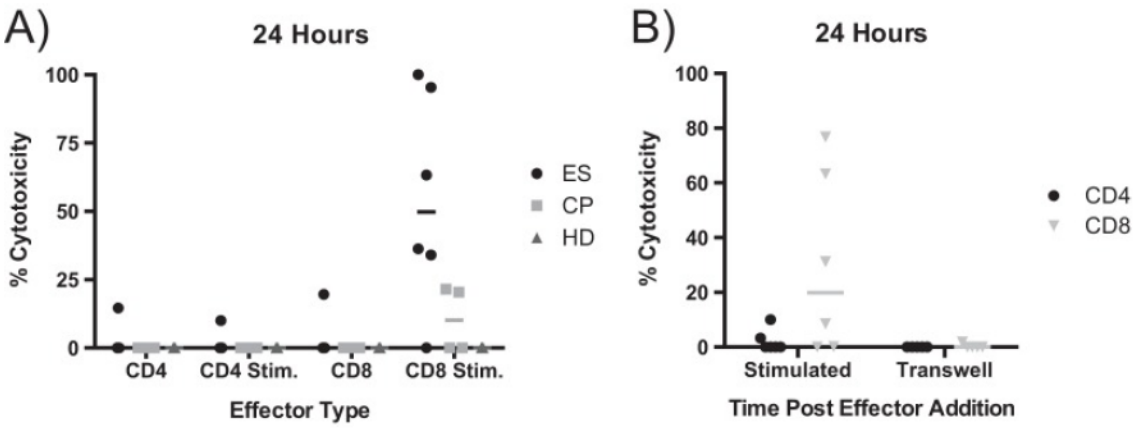


Figure 6. Stimulated ES $CD8^+$ T cells kill infected macrophages. Stimulated and unstimulated effector cells were added to cultures of MDMs that had been infected with HIV_{BaL} a week earlier. Target cell killing was determined with the Cytotox-96 nonradioactive cytotoxicity assay. (A) Comparison of the killing ability of primary and stimulated (Stim.) effector cells from ESs (n = 6), CPs (n = 5), and HDs (n = 5) 24 h after effector cell addition; (B) comparison of the ability of CD4 and CD8 effector cells from ESs to kill infected MDMs (n = 5).

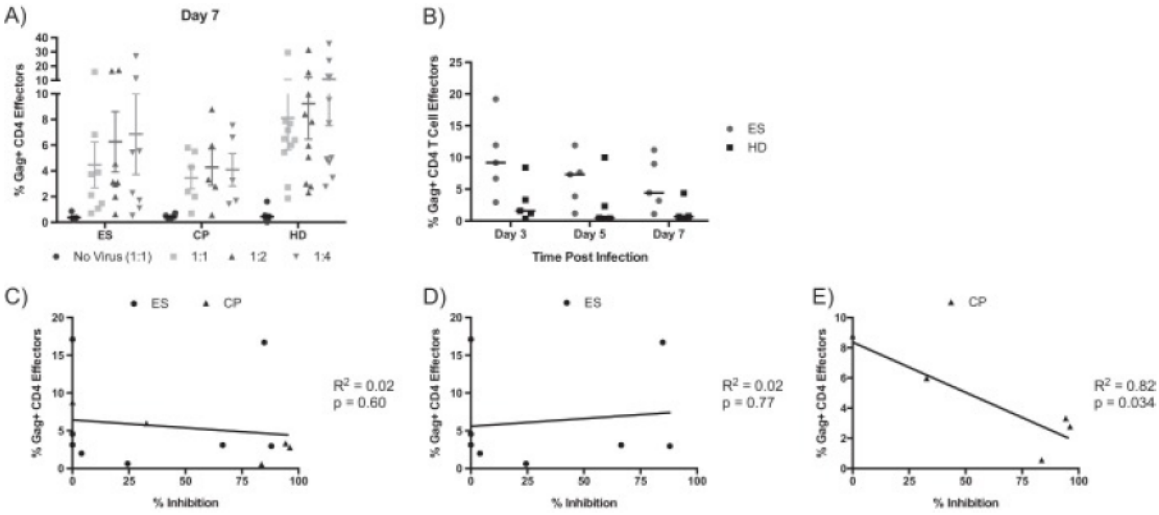


Figure 7. $CD4^+$ effectors from ESs, CPs, and HDs are infected by target MDMs. MDMs infected with HIV_{BaL} were cocultured with $CD4^+$ T cells at 1:1, 1:2, and 1:4 effector cell/target cell (E:T) ratios immediately after spinoculation. (A) $CD4^+$ effector infection on day 7 determined via intracellular Gag positivity for 8 ESs, 6 CPs, and 10 HDs (data are averages ± standard errors); (B) infection of $CD4^+$ T cell effectors from ESs (n = 5) and HDs (n = 5) over time, with medians indicated as horizontal bars; (C) correlation between infection of $CD4^+$ cells and their ability to inhibit infection at a 1:2 ratio for the 8 ESs and 5 CPs on day 7; (D) correlation between infection of $CD4^+$ cells and their ability to inhibit infection at a 1:2 ratio for the 8 ESs alone; (E) correlation between infection of $CD4^+$ cells and their ability to inhibit infection at a 1:2 ratio for the 5 CPs alone.

CHAPTER III

Table 1. Clinical information on the subjects in this study

Subject[a]	CD4 count	HIV RNA (copies/ml)	Length of time (yr) on suppressive cART regimen[b]	Current cART regimen[c]
CP9	991	<20	8	TDF, FTC, ELV/c
CP10	424	<20	7	RAL, EFV
CP11	1,001	<20	7	TDF, FTC, DRV/r
CP12	802	<20	2	3TC, RAL, EFV
CP13	744	<20	2	TDF, FTC, ELV/c
CP15	896	<20	7	TDF, FTC, ATV/r
CP16	946	<20	4	3TC, ABC, ATV/r
CP17	671	<20	4	TDF, FTC, DRV/r
CP18	1,084	<20	4	TDF, FTC, EFV
CP20	405	<20	8	TDF, FTC, ATV/r
CP21	509	<20	1	TDF, FTC, RPV

Subject[a]	CD4 count	HIV RNA (copies/ml)	Length of time (yr) on suppressive cART regimen[b]	Current cART regimen[c]
CP22	1,210	<20	4	TDF, FTC, DRV/r
CP23	448	<20	1	ABC, 3TC, ATV
CP24	657	<20	1	TDF, FTC, RAL
CP25	418	<20	4	TDF, FTC, RAL
CP26	964	<20	8	TDF, FTC, RAL
CP27	725	<20	6	TDF, FTC, ELV/c
CP28	722	<20	4	TDF, FTC, EFV
CP29	715	<20	5	TDF, FTC, EFV
CP30	471	<20	13	TDF, FTC, EFV
VC1	1,190	80	NA	NA
VC10	441	335	NA	NA
VC11	407	255	NA	NA

Subject[a]	CD4 count	HIV RNA (copies/ml)	Length of time (yr) on suppressive cART regimen[b]	Current cART regimen[c]
VC12	1,254	1,133	NA	NA

[a]The subjects were chronic progressors (CPs) and viremic controllers (VCs).
[b]NA, not applicable.
[c]Nucleoside reverse transcriptase inhibitors (NRTI) tenofovir (TFV), emricitabine (FTC), lamivudine (3TC), and abacavir (ABC) were used. Nonnucleoside reverse transcriptase inhibitors (NNRTI) efavirenz (EFV) and rilpivarine (RPV) were used. Integrase inhibitors elvitegravir (ELV) and raltegravir (RAL) and protease inhibitors darunavir (DRV), atazanavir (ATV), and ritonavir (r) were used. The boosting agent cobicistat (c) was used. NA, not applicable.

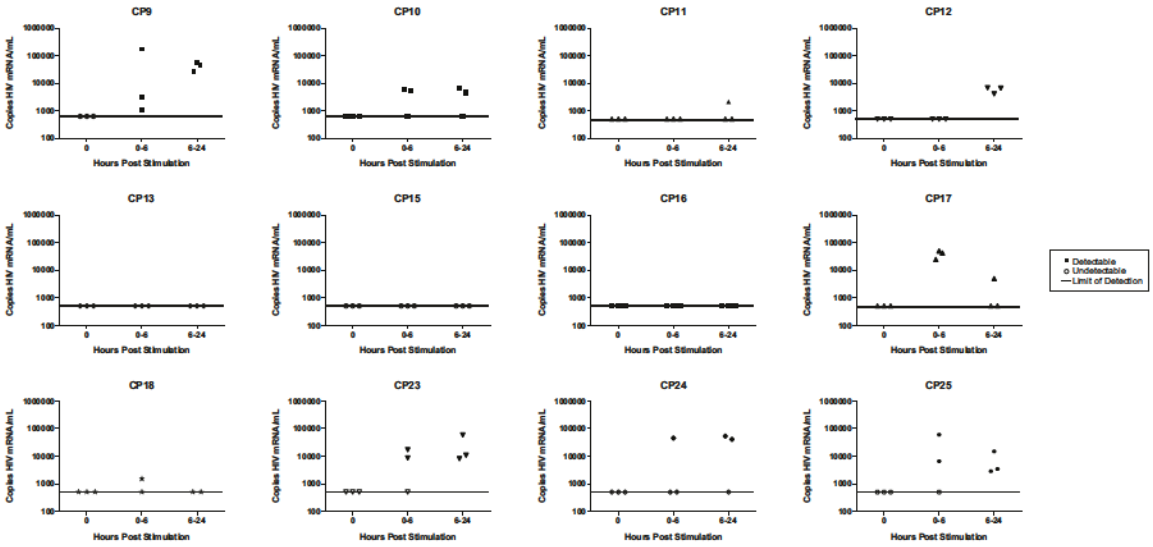
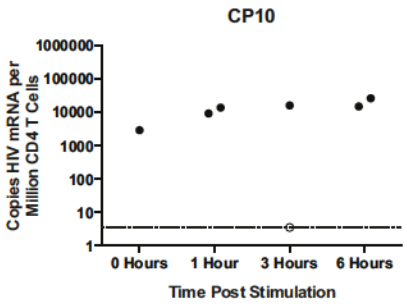
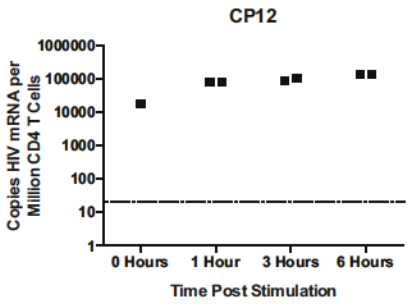
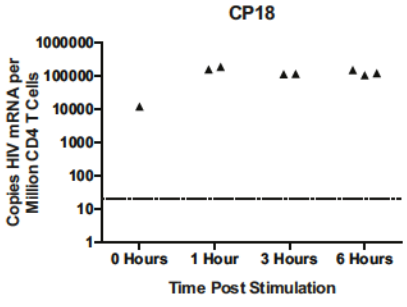
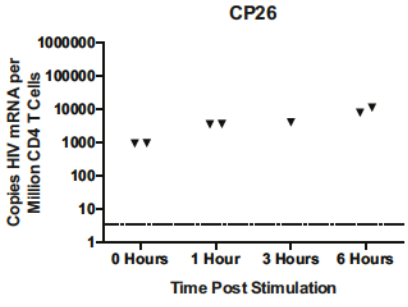


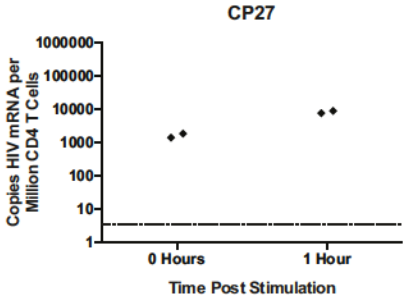
Figure 1. Resting $CD4^+$ T cells from HIV+ individuals produce virions in supernatant by 6 h posttreatment with PMA and ionomycin. Primary, resting $CD4^+$ T cells from chronic progressors were treated with PMA and ionomycin for 6 h in the presence of raltegravir and efavirenz throughout. RNA isolated from culture supernatant was converted to cDNA, and HIV-1 mRNA was quantified via quantitative PCR (qPCR). Quantification of extracellular HIV-1 mRNA in culture supernatant at baseline, 0 to 6, and 6 to 24 h is shown for each donor. Solid symbols represent detectable RNA, and open symbols represent undetectable RNA levels. The dashed line represents the lower limit of detection (500 copies per ml).

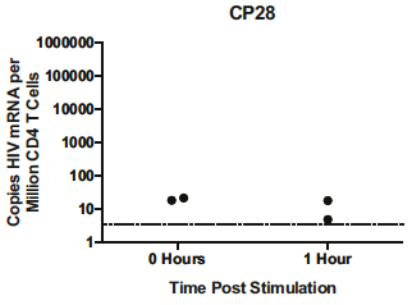

CP10
Copies HIV mRNA per Million CD4 T Cells
1000000
100000
10000
1000
100
10
1
0 Hours
1 Hour
3 Hours
6 Hours
Time Post Stimulation

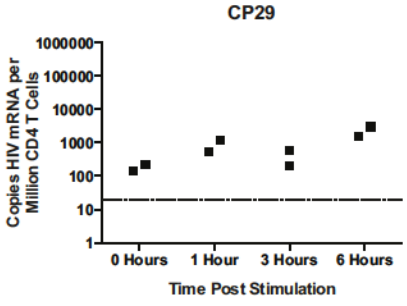

CP12
Copies HIV mRNA per Million CD4 T Cells
1000000
100000
10000
1000
100
10
1
0 Hours
1 Hour
3 Hours
6 Hours
Time Post Stimulation

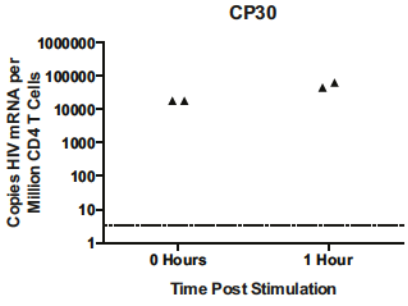

CP18
Copies HIV mRNA per Million CD4 T Cells
1000000
100000
10000
1000
100
10
1
0 Hours
1 Hour
3 Hours
6 Hours
Time Post Stimulation


CP26
Copies HIV mRNA per Million CD4 T Cells
1000000
100000
10000
1000
100
10
1
0 Hours
1 Hour
3 Hours
6 Hours
Time Post Stimulation


CP27
Copies HIV mRNA per Million CD4 T Cells
1000000
100000
10000
1000
100
10
1
0 Hours
1 Hour
Time Post Stimulation


CP28
Copies HIV mRNA per Million CD4 T Cells
1000000
100000
10000
1000
100
10
1
0 Hours
1 Hour
Time Post Stimulation


CP29
Copies HIV mRNA per Million CD4 T Cells
1000000
100000
10000
1000
100
10
1
0 Hours
1 Hour
3 Hours
6 Hours
Time Post Stimulation


CP30
Copies HIV mRNA per Million CD4 T Cells
1000000
100000
10000
1000
100
10
1
0 Hours
1 Hour
Time Post Stimulation

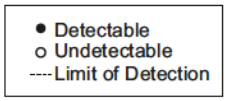

Detectable
Undetectable
Limit of Detection

Figure 2. HIV-1 mRNA is upregulated in primary resting $CD4^+$ T cells 1 h poststimulation with PMA and ionomycin. Primary, resting $CD4^+$ T cells were treated with PMA and ionomycin in the presence of raltegravir and efavirenz for 6 h with supernatant and cellular samples taken before and 1, 3, and 6 h after initiation of stimulation. Intracellular and supernatant RNA was isolated with TRIzol and converted to cDNA, of which the HIV-1 mRNA was then quantified via qPCR. Intracellular HIV-1 mRNA is upregulated *in vitro* after 1 h of PMA and ionomycin treatment. Solid symbols represent detectable RNA, and open symbols represent undetectable RNA levels. The dashed line represents the limit of detection (3.33 to 16.89 copies per million cells).

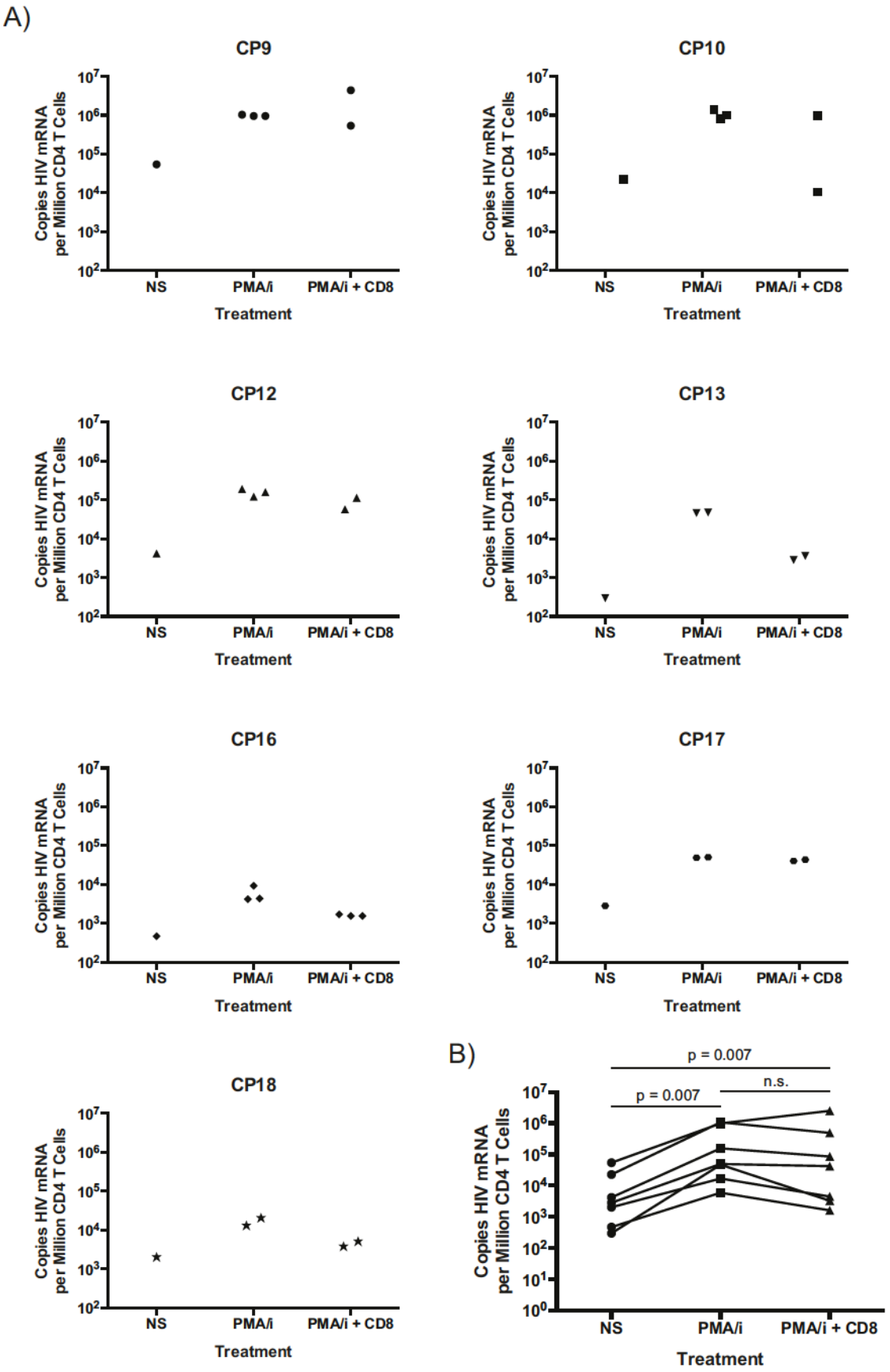

A)
CP9
CP10
CP12
CP13
CP16
CP17
CP18
Copies HIV mRNA per Million CD4 T Cells
NS
PMA/i
PMA/i + CD8
Treatment
B)
p = 0.007
p = 0.007
n.s.

Figure 3. Autologous, Gag-stimulated $CD8^+$ T cells are not effective in reducing intracellular HIV-1 mRNA in $CD4^+$ T cells from chronic progressors. Primary, resting $CD4^+$ T cells were treated with PMA and ionomycin in the presence of raltegravir and efavirenz for 6 h before coculture with $CD8^+$ T cells prestimulated for a week with overlapping Gag peptides for 18 h. (A) Intracellular HIV-1 mRNA at 24 h after initiation of stimulation. (B) Median intracellular HIV-1 mRNA in cells with three different treatments with statistics corresponding to the entire data set. The three treatments were no stimulation (NS) (control), PMA and ionomycin treatment only (PMA/i), and PMA and ionomycin treatment with $CD8^+$ T cell coculture (PMA/i + CD8). Solid symbols represent detectable RNA. n.s., not significant.

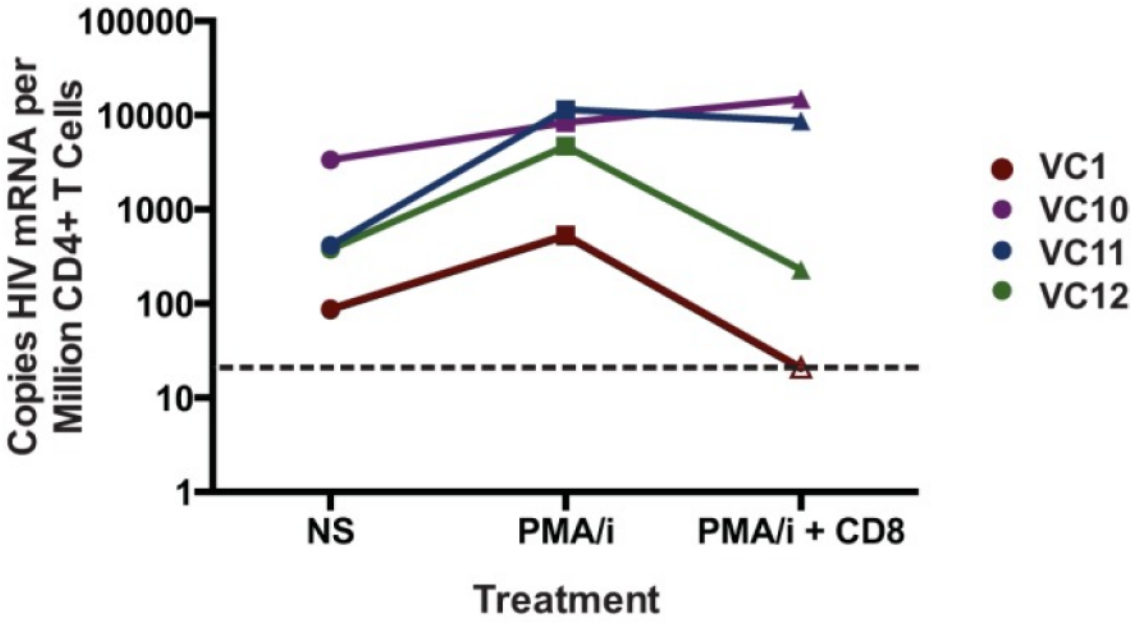


Figure 4. Autologous, Gag-stimulated $CD8^+$ T cells are capable of reducing intracellular HIV-1 mRNA in $CD4^+$ T cells from viremic controllers. Primary, resting $CD4^+$ T cells were treated with PMA and ionomycin in the presence of raltegravir and efavirenz for 6 h before coculture for 18 h with $CD8^+$ T cells prestimulated for a week with overlapping Gag peptides. Intracellular HIV-1 mRNA was measured by qPCR at 24 h after initiation of stimulation for each individual. NS = No stimulation. PMA/i = PMA and ionomycin treatment only. PMA/i + CD8 = PMA and ionomycin treatment and $CD8^+$ T cell coculture. Solid symbols represent detectable RNA, and unfilled symbols represent undetectable RNA levels. The dashed line represents the limit of detection (20.4 copies per million cells).

CHAPTER IV

Table 1. Clinical characteristics of the chronic progressors studied.

Subject	Current CD4+ T cell count	Nadir CD4+ T cell count	Time on suppressive regimen	Current regiment	HLA-A	HLA-B
CP8	424	18	8 years	3TC, RAL EFV	1, 68	57, 58
CP9	991	190	8 years	TDF, FTC, DRV/c	34, 68	58, 81
CP11	1032	177	8 years	TDF, FTC, DRV/r	2, 11	25, 57
CP 14	646	12	4 years	DRV/r, DTG		
CP 16	921	203	5 years	3TC, ABC, DTG	29, 20	42, 81
CP25	584	NA	4 years	TDF, FTC, RAL	3, 30	8, 42
ES 3	1149	NA	NA	NA	25, 68	51, 57
ES 6	601	NA	NA	NA	23	15, 57
ES 9	798	NA	NA	NA	2, 30	27, 57
ES 22	1033	NA	NA	NA	30, 31	15, 57
ES 24	1742	NA	NA	NA	24, 30	7, 57
ES 31	1236	NA	NA	NA	3	27, 58

3TC: lamivudine, ABC: abacavir, FTC: emtricitabine, TDF: tenofovir, DTG: dolutegravir, EFV: efavirenz, RAL: raltegravir, DRV/c: cobicistat boosted darunavir, DRV/r: ritonavir boosted darunavir. NA: Not applicable.

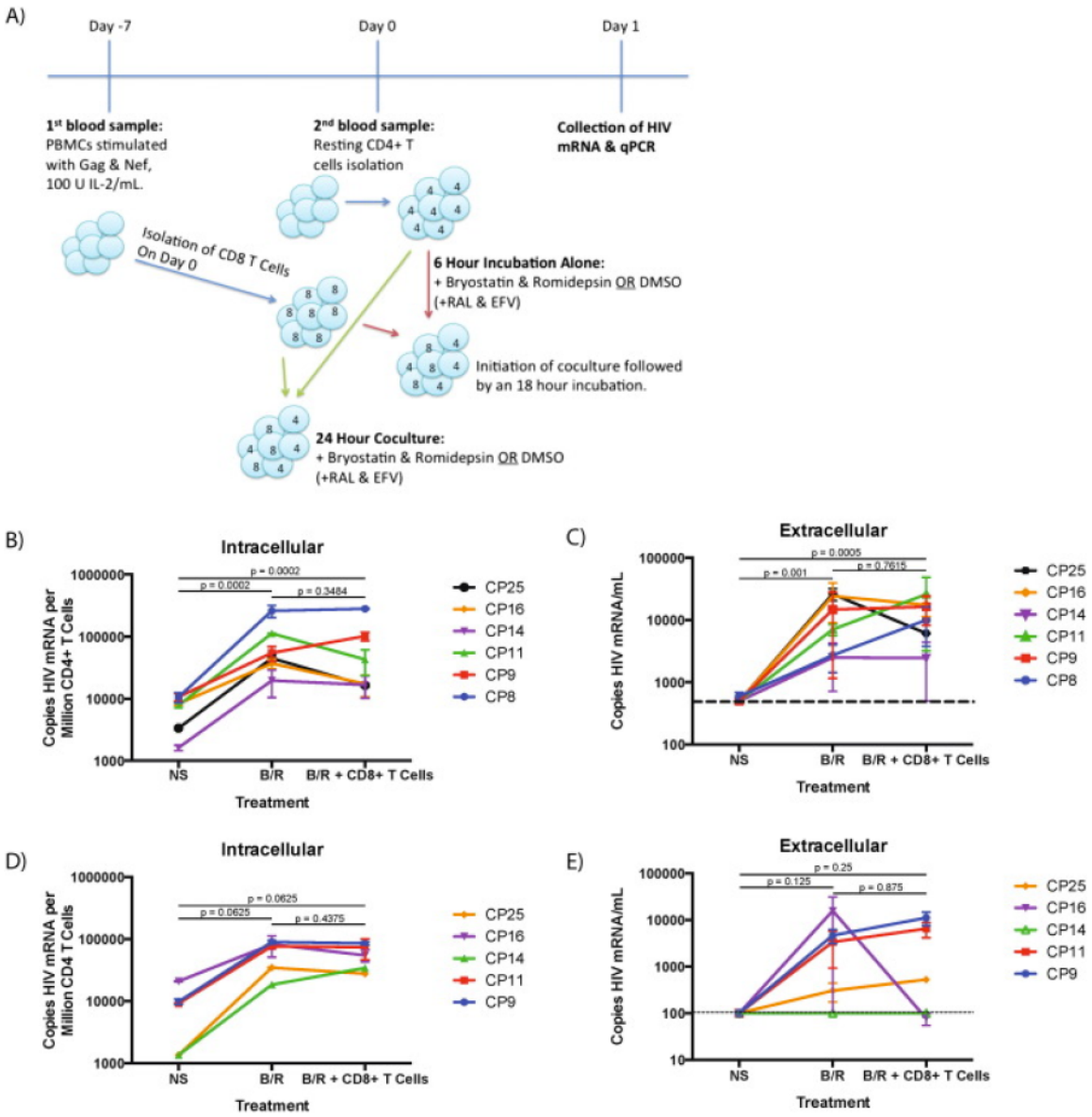


Figure 1. Nef- and Gag-stimulated CD8+ T cells from chronic progressors are not capable of eliminating newly-reactivated autologous CD4+ T cells following bryostatin-1/romidepsin-treatment. A) Schematic of methods. Blue arrows indicate methods common to both experiments, red arrows indicate methods for Fig. 1B and C, and green arrows indicate methods for Fig. 1D and E. B) Level of cell-associated HIV-1 mRNA seen with no stimulation (NS), treatment with bryostatin-1/romidepsin (B/R) or treatment with B/R and co-culture with CD8+ T cells for 18 h. No significance is indicated by n.s., and the listed *p*-value indicates the level of significance as determined by a Wilcoxon

signed rank test for significance. C) Level of HIV-1 mRNA present in culture supernatant for 6-hour drug treatment followed by 18-hour CD8+ T cell co-culture. Dotted line indicates the level of detection (500 copies HIV mRNA/mL). The listed *p*-value indicates the level of significance as determined by a Wilcoxon signed rank test for significance. D) Cell-associated HIV mRNA from 24-hour drug treatment and CD8+ T cell co-culture with resting CD4+ T cells. The listed *p*-value indicates the level of significance as determined by a Wilcoxon signed rank test for significance. E) Supernatant HIV mRNA from 24-hour drug treatment and CD8+ T cell co-culture with resting CD4+ T cells. The listed *p*-value indicates the level of significance as determined by a Wilcoxon signed rank test for significance. Dotted line indicates the level of detection (100 copies HIV mRNA/mL). Mean ± standard error of 1–3 replicates is shown for each individual.

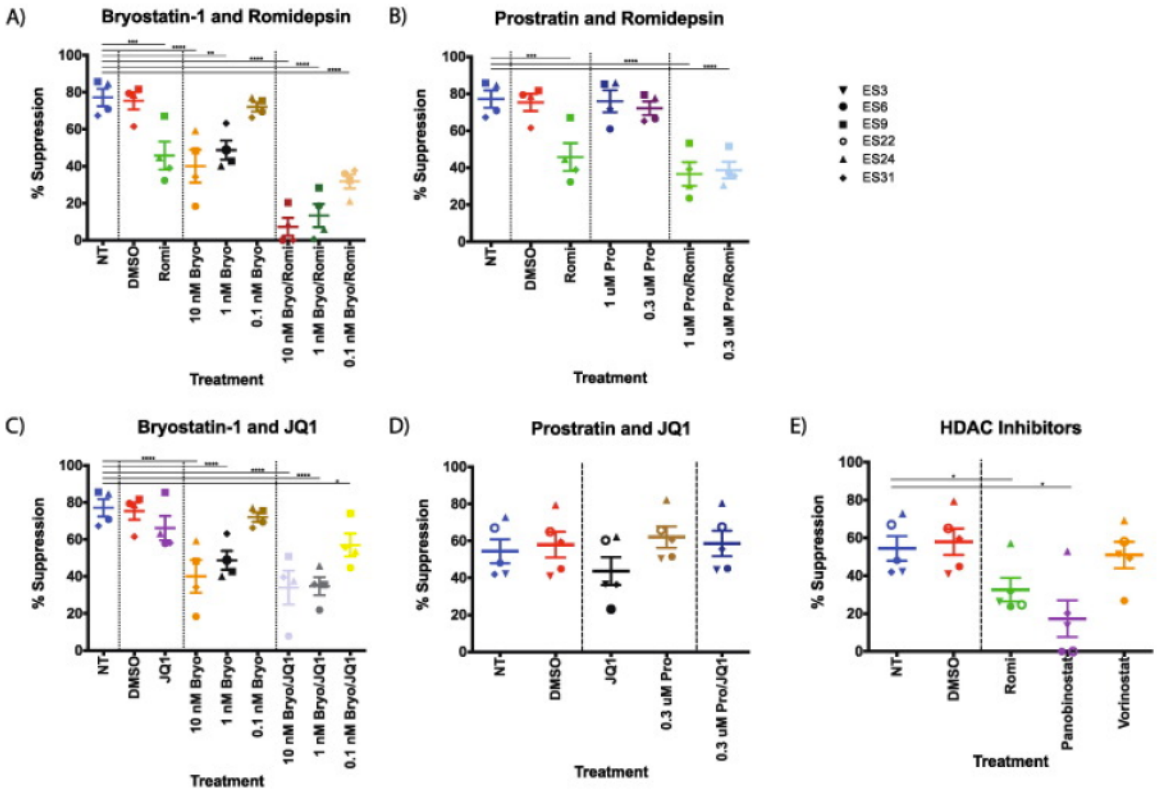


Figure 2. Elite suppressor CD8+ T cell responses are inhibited by bryostatin-1 and romidepsin alone and in combination. CD8+ T cells from 4 elite suppressors were preincubated with the indicated LRAs for six hours prior to the addition to autologous CD4+ T cells infected with lab strain HIV-1 pseudovirus in a 1:1 effector:target ratio and the percent suppression of viral replication was determined. Triplicates were performed and the mean values are shown for each individual. For panels D and E, data from 2 separate experiments with cells from ES6, ES22, and ES24 were averaged, and data from an additional elite suppressor was included. A) Comparison of bryostatin-1 and romidepsin treatments. B) Comparison of prostratin and romidepsin treatments. C) Comparison of bryostatin-1 and JQ1 treatments. D) Comparison of prostratin and JQ1 treatment. E) Comparison of romidepsin and other HDAC inhibitors. One-way repeated measures ANOVAs were used to determine significance for each of the two sets of

experiments. Symbols directly above treatments indicate differences from NT, no treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$.

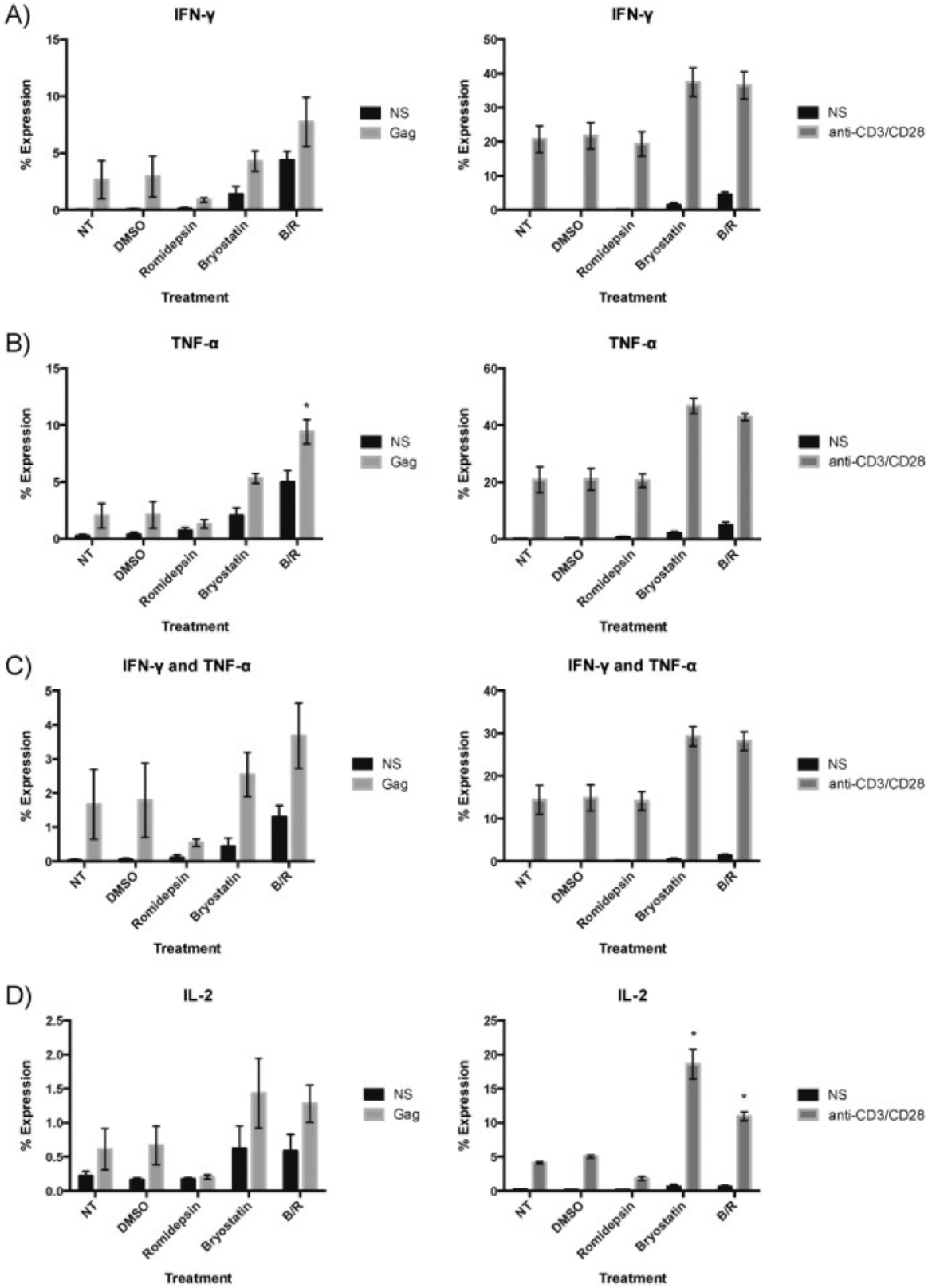


Figure 3. Production of IFN-γ, TNF-α, and IL-2 by CD8+ T cells increases with bryostatin-1 treatment. IFN-γ (A), TNF-α (B), simultaneous IFN-γ and TNF-α (C), and

IL-2 (D) production in unstimulated (NS), Gag-peptide stimulated, and anti-CD3/CD28 stimulated CD8+ T cells is shown for each of three elite suppressors with the mean ± standard error. Significance was determined via a series of one-way repeated measures ANOVAs examining each of the twelve conditions separately, and level of significance indicated above a bar is in comparison to no treatment from the same stimulation. * $p <$ 0.05.

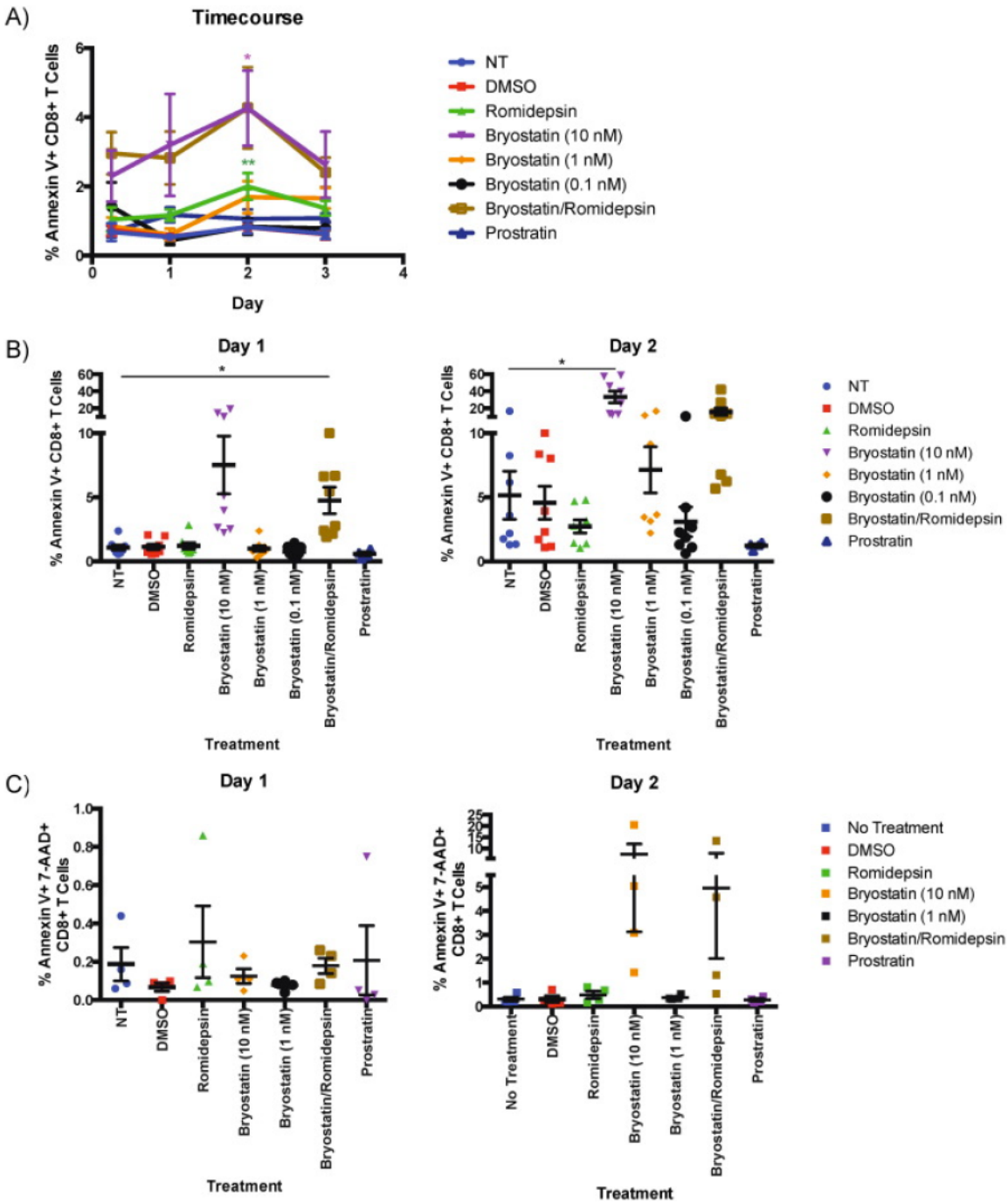


Figure 4. Bryostatin-1 and bryostatin-1/romidepsin combination treatments cause an increase in CD8+ T cell death. Eight HIV negative donors' PBMCs were treated with LRAs and examined for annexin V expression on CD8+ T cells. A) Annexin V expression in CD8+ T cells treated with drug for 6 h and then cultured for three days post-treatment in non-stimulating media. B) Annexin V expression in CD8+ T cells

treated with drug for 6 h and then stimulated with anti-CD3/CD28 for either one or two days afterward. C) Annexin V and 7-AAD expression in CD8+ T cells treated with drug for 6 h and then stimulated with anti-CD3/CD28 for either one or two days afterward. One-way repeated measures ANOVAs were used to calculate significance, and the level of significance indicated is in comparison to the no treatment condition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

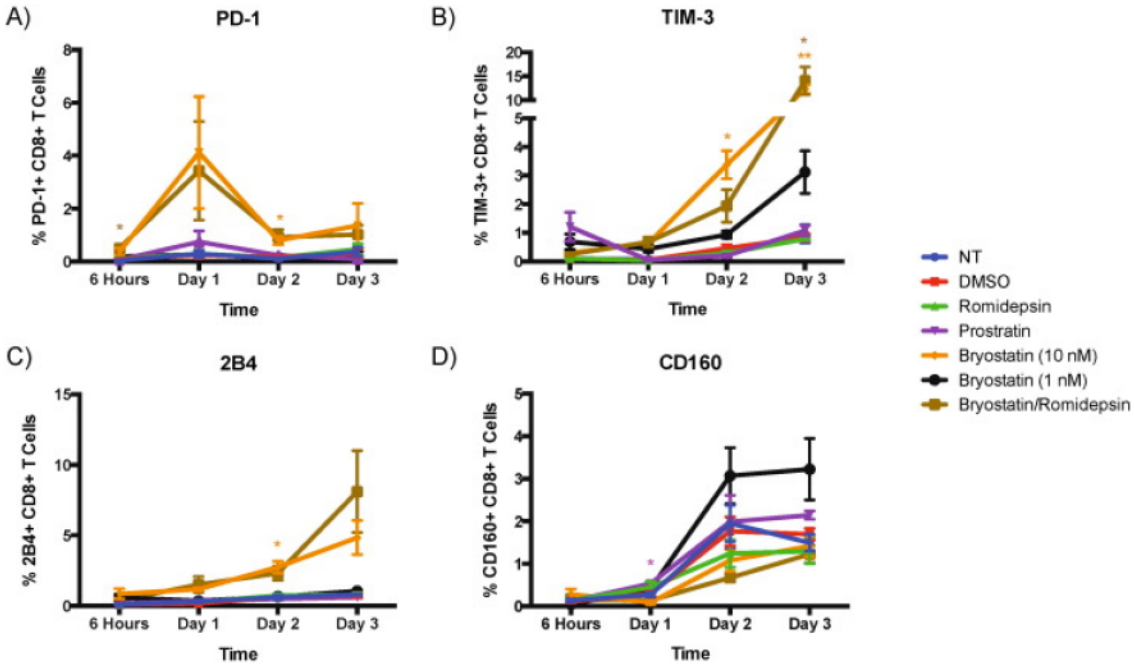


Figure 5. Bryostatin-1 treatment induces an increase in exhaustion marker expression in unstimulated CD8+ T cells. Eight HIV-negative donors' PBMCs were treated with LRAs and examined for PD-1 expression on CD8+ T cells, and four donors' PBMCs were examined for TIM-3, 2B4, and CD160 expression. A) PD-1 expression in CD8+ T cells treated with drug for 6 h and then incubated in non-stimulating media for an additional three days. B) TIM-3 expression. C) 2B4 expression. D) CD160 expression. The level of significance indicated is in comparison to no treatment. * $p < 0.05$, ** $p < 0.01$.

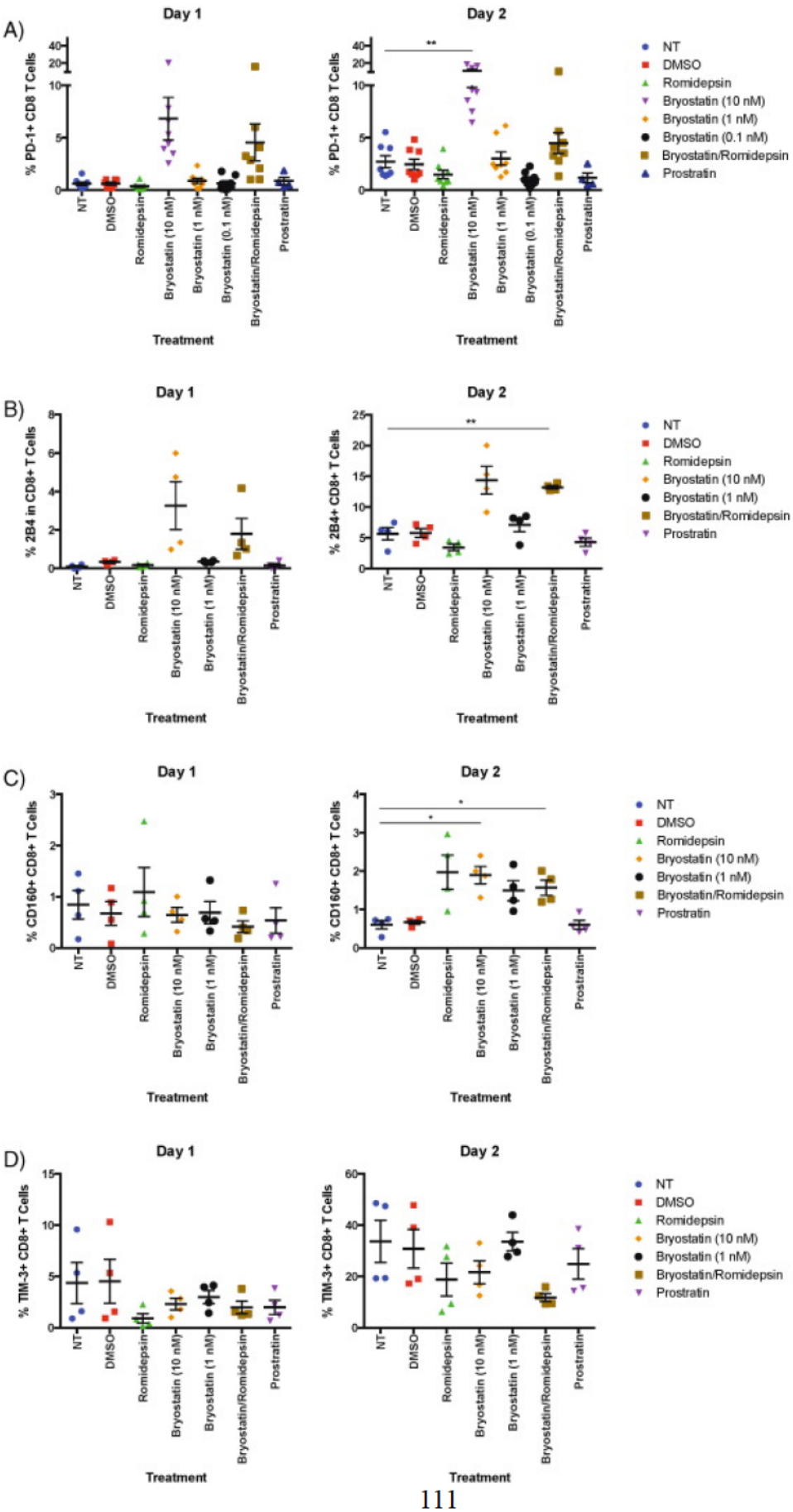

A)
Day 1
Day 2
% PD-1+ CD8 T Cells
Treatment
**
B)
% 2B4 in CD8+ T Cells
% 2B4+ CD8+ T Cells
C)
% CD160+ CD8+ T Cells
*
D)
% TIM-3+ CD8+ T Cells
NT
DMSO
Romidepsin
Bryostatin (10 nM)
Bryostatin (1 nM)
Bryostatin (0.1 nM)
Bryostatin/Romidepsin
Prostratin

Figure 6. Bryostatin-1 treatment induces an increase in exhaustion marker expression in stimulated CD8+ T cells. Eight HIV-negative donors' PBMCs were treated with LRAs and examined for PD-1 expression on CD8+ T cells, and four donors' PBMCs were examined for TIM-3, 2B4, and CD160 expression. A) PD-1 expression in CD8+ T cells treated with drug for 6 h and then incubated with anti-CD3/CD28 antibodies for an additional two days. B) 2B4 expression. C) CD160 expression. D) TIM-3 expression. The level of significance indicated is in comparison to no treatment. * $p < 0.05$, ** $p < 0.01$.

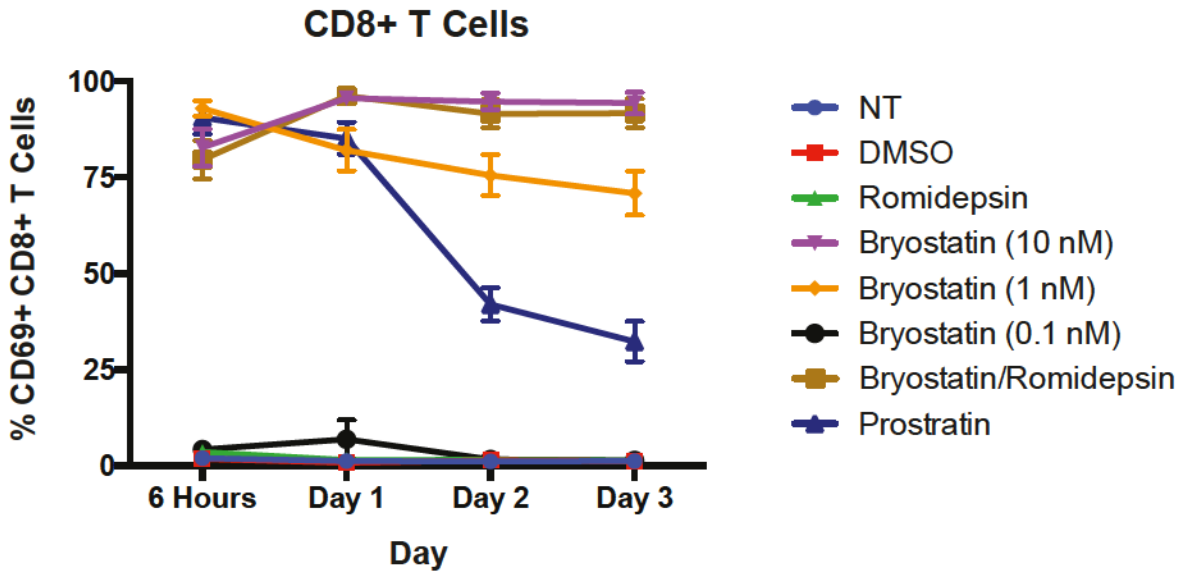


Figure 7. CD69 expression is upregulated due to treatment with PKC agonists. Eight HIV-negative donors' PBMCs were treated with LRAs for 6 h before being washed and then cultured in non-stimulating media for up to three days and examined for CD69 expression on CD8+ T cells. Mean expression ± standard error is indicated for each treatment. Bryostatin-1 treatment at 10 nM and 1 nM and bryostatin-1 (10 nM)/romidepsin treatment all significantly upregulate CD69 for all four timepoints ($p < 0.0001$), as did prostratin treatment ($p < 0.001$, $p < 0.001$, $p < 0.01$, and $p < 0.05$ at the respective timepoints) as calculated by multiple one-way repeated measures ANOVAs.

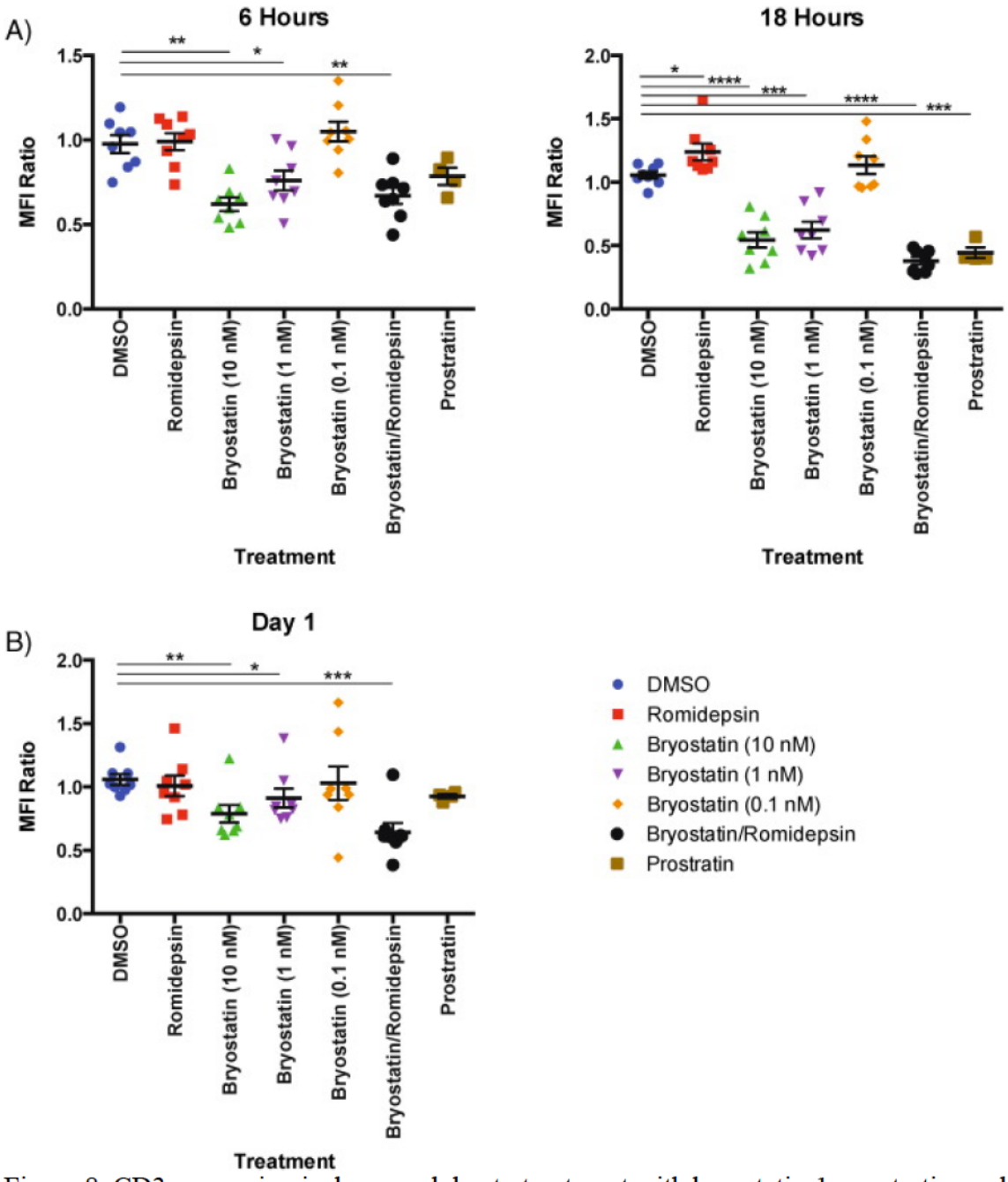
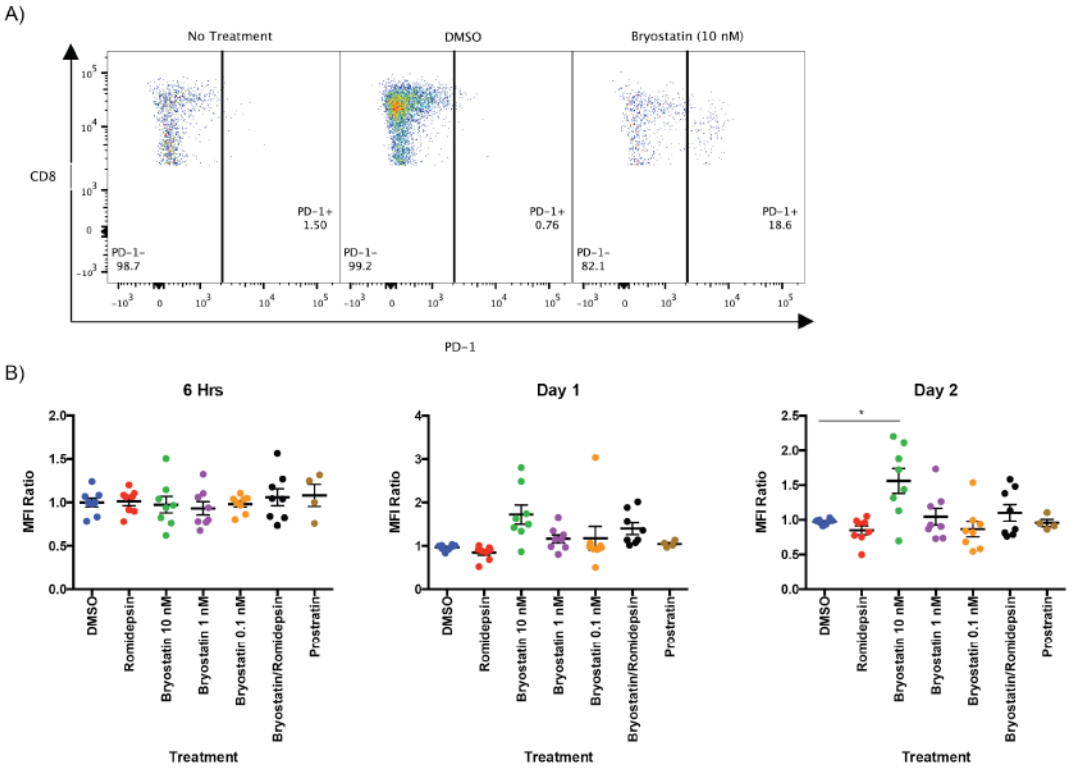


Figure 8. CD3 expression is decreased due to treatment with bryostatin-1, prostratin, and the combination of bryostatin-1/romidepsin. Eight HIV-negative donors' PBMCs were treated with LRAs for at least 6 h and examined for CD3 expression on CD8+ T cells. A) Mean fluorescence intensity (MFI) of CD3 expression in CD8+ T cells treated with drug for 6 or 18 h normalized to no treatment. One-way repeated measures ANOVAs were used to calculate significance for 6-hour and 18-hour treatments separately. B) Mean

fluorescence intensity (MFI) of CD3 expression in CD8+ T cells treated with drug for 6 h and then incubated in non-stimulating media for one day afterward normalized to no treatment. A one-way repeated measures ANOVA was used to calculate significance. The level of significance indicated is in comparison to vehicle (DMSO). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



Supplementary Figure 1. Bulk CD8+ T cell expression of PD-1 is upregulated as measured by mean fluorescence intensity (MFI). Eight HIV-negative donor PBMCs were treated with LRAs for six hours prior to washing and further culture in the presence of anti-CD3/CD28 antibodies. A) Gating schematic for PD-1 as measured in Figs. 5 and 6. B) Expression of PD-1 as calculated instead by the MFI of PD-1 in treated cells divided by the MFI of PD-1 in untreated cells. * $p<0.05$.

VII. CURRICULUM VITAE

CURRICULUM VITAE for Ph.D. CANDIDATES

The Johns Hopkins University School of Medicine

Victoria Walker-Sperling 6 May 2016

Educational History:

Ph.D. Expected	2016	Immunology Training Program Mentor: Dr. Joel Blankson	Johns Hopkins School of Medicine
B.A.	2011	Biological Sciences	Wellesley College

Other Professional Experience:

Summer Internship	2010	Lab of Dr. David Rudrauf, University of Iowa Hospitals and Clinics	

Academic and Other Honors:

2007	National Merit Scholarship Program Commended Student	
2011	Cum Laude	Wellesley College
2011	Honorary Membership in Sigma Xi	Wellesley College
2011	Fiske Prize in Biology	Wellesley College
2015	Young Investigator Scholarship	Conference on Retroviruses and Opportunistic Infections
2016	Young Investigator Scholarship	Conference on Retroviruses and Opportunistic Infections

Publications, peer reviewed:

- **Walker-Sperling VE**, Pohlmeyer CW, Blankson JN. (2016) The Effect of Latency Reversal Agents on Primary CD8+ T Cells: Implications for Shock and Kill Strategies for HIV Eradication. EBioMedicine. doi: 10.1016/j.ebiom.2016.04.019
- Durand CM, Buckheit RW 3rd, Salgado M, Pohlmeyer CW, **Walker-Sperling VE**, Hegarty RW, Ambinder RF, Blankson JN. (2015) A Human Immunodeficiency Virus Controller With a Large Population of CD4(+)CD8(+) Double-Positive T Cells. Open Forum Infect Dis. 2(2):ofv039. doi: 10.1093/ofid/ofv039.
- **Walker-Sperling VE**, Cohen VJ, Tarwater PM, Blankson JN. (2015) Reactivation Kinetics of HIV-1 and Susceptibility of Reactivated Latently Infected CD4+ T Cells to HIV-1-Specific CD8+ T Cells. J Virol. 89(18):9631-8. doi: 10.1128/JVI.01454-15.
- Metcalf Pate KA, Pohlmeyer CW, **Walker-Sperling VE**, Foote JB, Najarro KM, Cryer CG, Salgado M, Gama L, Engle EL, Shirk EN, Queen SE, Chioma S, Vermillion MS, Bullock B, Li M, Lyons CE, Adams RJ, Zink MC, Clements JE, Mankowski JL, Blankson JN. (2015) A Murine Viral Outgrowth Assay to Detect Residual HIV Type 1 in Patients With Undetectable Viral Loads. J Infect Dis. 212(9):1387-96. doi: 10.1093/infdis/jiv230.
- **Walker-Sperling VE**, Buckheit RW 3rd, Blankson JN. (2014) Comparative analysis of the capacity of elite suppressor CD4+ and CD8+ T cells to inhibit

HIV-1 replication in monocyte-derived macrophages. J Virol. 88(17):9789-98. doi: 10.1128/JVI.00860-14.

- Mattila HR, Rios D, **Walker-Sperling VE**, Roeselers G, Newton IL. (2012) Characterization of the active microbiotas associated with honey bees reveals healthier and broader communities when colonies are genetically diverse. PLoS One. 7(3):e32962. doi: 10.1371/journal.pone.0032962.

Posters, Abstracts, Etc.:

- **Walker-Sperling VE**, Pohlmeyer CW, Blankson JN. (2016) Effect of Primary CD8 T Cells on Romidepsin and Bryostatin-Treated CD4 T Cells. Conference on Retroviruses and Opportunistic Infections, Boston, MA, 22-25 February 2016.
- Pohlmeyer CW, Bullen CK, Laird G, Martin AR, **Walker-Sperling VE**, Chioma SU, Siliciano RF, Blankson JN. (2015) Measurements of Viral Transcription in Elite Suppressor CD4+ T Cells. Conference on Retroviruses and Opportunistic Infections, Seattle, WA, 23-26 February 2015.
- **Walker-Sperling VE**, Cohen VJ, Blankson JN. (2015) Kinetics of HIV-1 gene expression following reactivation in a primary cell model of latency. Conference on Retroviruses and Opportunistic Infections, Seattle, WA, 23-26 February 2015.
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Service and Leadership:

2015-2016 Aided in training rotating Ph.D. students and lab technician.