

Mechanistic approaches for reactivation and elimination of latent HIV-1

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Abstract

Although HIV-1 can be adequately controlled by combination antiretroviral therapy (cART), it is still an incurable disease due in part to the latent reservoir that persists in resting CD4⁺ T cells (rCD4s). Latently infected rCD4s do not actively express HIV-1, allowing them to evade immune surveillance, but their ability to reinitiate viral expression maintains this barrier to a cure. Shock-and-kill, a prominent strategy for HIV-1 eradication, requires the reactivation of latent HIV-1 gene expression.

Global T cell activation is a well-characterized means of inducing HIV-1 transcription, but is considered too toxic for clinical applications. Here, we explore a strategy involving a combination of immune activation and the immunosuppressive mTOR inhibitor rapamycin. We show that rapamycin downregulates markers of toxicity, including pro-inflammatory cytokine release and cellular proliferation, induced by potent T cell activating agents. Using an *ex vivo* assay for HIV-1 mRNA in rCD4s from infected individuals on antiretroviral therapy, we demonstrate that despite this immunomodulatory effect, rapamycin does not affect HIV-1 gene expression induced by T cell activation. In contrast the immunosuppressant cyclosporin, a calcineurin inhibitor, robustly inhibits HIV-1 reactivation. Importantly, cytotoxic T lymphocyte (CTL) recognition and killing of infected cells is also not impaired by rapamycin treatment.

These findings are also being extended to HIV-positive individuals requiring solid organ transplant, which acts an *in vivo* immune activating stimulus without the need for additional intervention. A previous study has shown that a group of these patients who received rapamycin for immunosuppressive maintenance had lower levels of HIV-1 DNA in peripheral blood cells when compared with patients receiving a calcineurin inhibitor such as cyclosporin. This previous finding is in support of our findings, and suggests that rapamycin may allow for shock-and-kill *in vivo*. Here we compare pre- and post-

transplant reservoir size using a novel assay, the quantitative viral induction assay (QVIA), and discuss the surprising result of an increase in the size of the HIV-1 latent reservoir post-transplant.

Alternatively, compounds that may not elicit T cell activation phenotypes, and are not necessarily thought to act via any of the same pathways as T cell activation, may effectively stimulate HIV-1 transcription. Several latency reversing agents (LRAs) have activity in primary rCD4s isolated from patients on cART, including PKC agonists, HDAC inhibitors (HDACi), and bromodomain and extra-terminal (BET) bromodomain inhibitors. Of particular interest are HDACi, as there are several drugs in this class approved for clinical use. Although the activity of HDACi on HIV-1 expression is well documented; they act synergistically with other LRAs in primary cells and have high activity alone in several cell models for latency; these drugs have a broad scope of activities and their mechanism of action in this context is controversial. If a mechanism can be identified, a more targeted approach to reactivate latent HIV-1 may be developed. We therefore used a combination of gene expression and transcription factor array studies in the context of both primary and model cells to further understand how HDACi may be acting upon HIV-1 transcription. Using specific small molecule inhibitors we have identified a potential role for p21 in HDACi activity on latent HIV-1.

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Preface

I would first like to thank my advisor, Dr. Robert Siliciano. Bob sets an example for all of us through his kindness, modesty, and adherence to the most rigorous scientific methods. I truly appreciate the opportunities and freedom Bob has given me to work on the projects that I have been passionate about. Only with his support and guidance have I made it through the past 4 years of my thesis work. I would also like to thank Dr. Janet Siliciano, who is always looking out for us students. Janet is always there for advice in both lab and life. She has even on a few occasions stepped into an advisor role for me when Bob couldn't be there, for which I am grateful.

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Introduction

An estimated 35 million people worldwide are infected with HIV, yet a widely applicable cure strategy remains elusive. Recent case reports have suggested that cure of HIV infection is possible, renewing excitement for cure research efforts. Here, we describe those cases and discuss their relevance to the global HIV epidemic. We also review ongoing cure strategies that are transitioning from the lab to the clinic, and the assays and clinical assessments that can be used to evaluate cure interventions.

Infection with human immunodeficiency virus type 1 (HIV-1) is a continuing global health concern, with ~1.5 million AIDS-related deaths worldwide in 2013. Current combination antiretroviral therapy (cART) reduces viremia to undetectable levels, preventing further loss of CD4⁺ T cells and progression to AIDS. cART allows HIV-infected individuals to have near-normal life expectancy. (1) However, cART is not curative, and infected individuals must remain on therapy for life to maintain health. Here we review the need for a cure, the barriers to achieving this goal, exciting recent cases in which a cure has been achieved or nearly achieved, and strategies to make HIV-1 infection curable.

As therapy for HIV-1 infection has improved, the need for curative interventions, particularly those that pose risk to the patient, has been questioned. The benefits of a cure are best understood in the context of the global epidemic. According to the Centers for Disease Control and Prevention, only 36% of infected individuals worldwide are receiving cART. This is partly a financial issue, with lifetime cART estimated to cost \$379,668 in 2010 (<http://www.cdc.gov/hiv/prevention/ongoing/costeffectiveness/>). The majority of HIV-infected individuals worldwide live in low-income countries with more limited access to healthcare. Moreover, the number of people living with HIV-1 globally continues to increase, threatening to elevate the cost of HIV-1 healthcare to unsustainable levels. Although prevention strategies have allowed the number of new

HIV-1 infections per year in the United States to remain stable, the prevalence of HIV-1 is increasing because infected individuals live longer on cART. In addition, results from the Strategic Timing of Antiretroviral Treatment (START) trial have suggested that beginning cART as early as possible is best for long-term patient outcomes. (2) These findings will likely influence patient care, broadening the indication for these drugs to all HIV-infected individuals, a prospect that will clearly be unsustainable unless a cure is found.

At the level of the individual patient, there are also reasons why a cure, even one involving some risks, would be preferable to lifetime cART. Adverse long-term effects such as lipodystrophy are associated with some antiretroviral drugs. Drug toxicity can decrease adherence. (3) If a patient is poorly adherent or discontinues therapy without careful medical supervision, drug resistance can develop. Resistance makes it more difficult to successfully reinstate treatment later. (4) These problems contribute to the fact that only 75% infected individuals on treatment in the United States are able to maintain suppression of viral replication.

For patients able to adhere to cART, CD4⁺ T cell counts stabilize and increase, but the immune system never fully recovers. Even patients who have been on suppressive cART for years suffer from increased immune activation and inflammation, (5) which can lead to additional health problems including cardiovascular disease. It is not clear whether these abnormalities result from legacy effects of the period of unchecked viral replication before initiation of cART or from a low level of ongoing release of virus from stable reservoirs. Cure strategies would impact the latter but not the former.

Those infected with HIV-1 would also benefit from a cure for non-medical reasons. Along with the high cost of cART, there is the social stigma of being HIV+, as HIV-1 infection has historically been associated with drug use and high-risk sexual

activity. Therefore, although cART has greatly improved the lives of infected individuals, there are significant problems with life-long treatment. Clearly, a cure strategy would both benefit infected individuals and reduce the burden to the global health economy.

Due to a fundamental feature of HIV-1 biology, the infection has proven difficult to cure. HIV-1 stably integrates its genome into the DNA of the host cells it infects, predominantly CD4⁺ T lymphocytes. A small number of these infected cells enter a state of latent infection and persist indefinitely. Cells containing a latent HIV-1 genome can later resume active virus production. A single integrated provirus can produce a sufficient number of virions to perpetuate infection, and when therapy is discontinued, viremia quickly rebounds. (6) Thus, the persistence of latently infected cells is a major obstacle to cure. Cure strategies either aim to kill all infected cells or boost immunity to control infection. Efforts to produce a cure are accelerating with many different strategies currently being studied.

It is useful at the outset to define critical terms. Latency is a reversibly non-productive state of infection. Latent viral infection is characterized by the presence of infected cells that do not actively produce virus particles, but retain the ability to do so at some point in the future. A viral reservoir is a cell type that harbors replication-competent virus for long periods of time. (7) With respect to HIV-1 cure, the only relevant reservoirs are those that persist on a time scale of years in patients on optimal cART. This practical definition reflects the fact that cure strategies would only be implemented in patients who had suppression of active viral replication on cART for a substantial period of time. Another related term is compartment. This refers to an anatomical site of viral replication that has limited exchange of virus with other sites.

A latent reservoir (LR) for HIV-1 in resting memory CD4⁺ T cells was demonstrated in 1995. (8, 9) The majority of infected cells are activated CD4⁺ T cells, which die quickly due to viral cytopathic effects or host cytolytic mechanisms ($t_{1/2} = 6$

hours to 2 days). (10, 11) On rare occasions recently activated cells become infected during the transition from activated state to a resting memory state, which is non-permissive for viral gene expression. This allows long-term persistence of stably integrated proviruses in long lived memory T cells. (12) Early studies using a virus culture assay to quantify the frequency of latently infected cells demonstrated that this reservoir is extremely stable. (13) A recent study re-examined the half-life of the LR in CD4⁺ T cells in patients on newer, potentially more effective cART regimens. The authors estimated a mean reservoir half-life of 3.6 years, (14) the same as an original study completed 12 years previously, indicating that newer antiretroviral drugs do not increase the decay rate of the LR. This suggests that the stability of the LR is not due to incomplete suppression of viral replication. The finding that cART intensification does not cause additional reductions in the trace level of viremia that can be detected in treated patients using sensitive assays also supports this conclusion.(15, 16)

There are multiple definitions of HIV-1 cure, each being pursued with multiple strategies (Table 1). A sterilizing cure is defined as complete eradication of infectious forms of the virus from the body; that is, replication-competent provirus is no longer present. Another possibility is ART-free remission, a scenario in which the HIV-1 reservoir is not eliminated but is reduced enough to greatly decrease the chances of viral rebound. Modeling of infection dynamics has predicted that a 3- to 4-log decrease in the size of the LR is necessary to achieve ART-free remission of > 1 year. (17) A functional cure is thought to be a more attainable goal, as it does not require that the HIV-1 reservoir be reduced or eliminated. Instead, a functional cure allows for discontinuation of cART without rebound because the immune system is modified in some way so that it is able to control viral replication without therapy. (18, 19)

There is one example of a sterilizing cure. The 'Berlin patient' received an allogeneic hematopoietic stem cell transplant (HSCT) to treat acute myeloid leukemia.

This case was exceptional because an HLA-matched donor was found who was homozygous for the CCR5 delta32 mutation, which causes a deletion in the CCR5 coreceptor needed for HIV-1 entry. Hence, donor cells were resistant to infection. (20) Complete chimerism with the delta32/delta32 genotype was achieved 61 days post bone marrow transplant, meaning that the patient's immune system was almost entirely replaced by donor cells. The patient did not experience viral rebound even though cART was discontinued at the time of transplant, and HIV-1 DNA could not be detected in plasma or rectal mucosa throughout the followup period. (21) The Berlin patient is now considered cured, with no detectable HIV-1 present several years later. It is likely that the donor CCR5 delta32/delta32 genotype was crucial to the success of this case.

Efforts to reproduce this sterilizing cure in patients receiving allogeneic HSCT have resulted in ART-free remission, but no additional cures. Two recently described patients, referred to as the 'Boston patients,' (22) received transplants for malignancies from donors with wild-type alleles at the CCR5 locus. Although donor cells were fully susceptible to HIV-1 infection, it was hoped that if the patients maintained cART throughout the transplant period, donor cells would not become infected and host cells containing HIV-1 proviruses would be completely eliminated by graft versus host disease. The idea was to recapitulate the cure seen in the Berlin patient without need for an HLA-matched CCR5 delta32/delta32 donor. At 2-4 years after transplantation, neither patient had detectable HIV-1 DNA in peripheral blood mononuclear cells (PBMC) by PCR, and replication-competent HIV-1 was not recovered from co-cultures. The lack of measurable residual host cells and HIV-1 in these individuals suggested complete elimination of the LR. However, during closely monitored analytical treatment interruptions, the patients experienced viral rebound 3 and 8 months after discontinuing cART.(22)

It is unfortunately difficult to apply cure strategies used in the above cases to a typical patient with HIV-1 infection. Despite the promising results seen with some transplant cases, HSCT is only a realistic option for patients with a concurrent condition requiring this high risk procedure.

ART-free remission has also been achieved in a patient known as the 'Mississippi baby.' In this unusual case, a perinatally infected newborn with a high HIV-1 viral load on the day after delivery was immediately put on cART. Therapy was later discontinued against medical advice at 15 months, but surprisingly there was no rapid rebound. (23) It was hoped that because the child was started on therapy so early, a reservoir of infected memory CD4⁺ T cells might not have been established. However, the child presented with very high plasma HIV-1 at a routine appointment 26 months after discontinuing cART. (24) Subsequent cases of early treatment in perinatally infected infants have had similar results, with viral rebound after treatment interruption.(25)

Typically, when a patient discontinues cART, there is rapid rebound in viremia beginning within 6-15 days and peaking by 21 days post treatment interruption. (6) The patients mentioned achieved ART-free remission for longer periods of time, a finding with important implications for understanding obstacles to cure. In the Boston patients, little to no anti-HIV immunity was present at rebound, consistent with the idea that the donor-derived immune systems that developed in these patients had not experienced HIV-1 before and therefore had no HIV-specific immunological memory. (22) Therefore, the late rebounds after ART interruption were not due to immune control of viral replication and can only be explained by persistence of virus in a latent form. Modeling of the dynamics of the LR has shown that while a typical pool of latently infected cells can cause rebound within two to three weeks, a multi-log decrease in this pool can result in a delay from months to years. (17) HIV-1 re-emerges from a latent state when a CD4⁺

T cell harboring a latent provirus is reactivated by its cognate antigen or another activating stimulus. (26) When the total number of latently infected cells is smaller, at any given time re-emergence of replicating HIV-1 is statistically less likely.

A small subset of HIV-infected individuals known as elite controllers (ECs) have been viewed as a model for a functional cure, as they are able to maintain very low viral loads without therapy and do not progress to AIDS. It was initially thought that EC were infected with less virulent strains of HIV-1, but a study examining virus isolated from transmission pairs indicates that ECs have this phenotype even when infected with fully functional HIV-1 from a chronic progressor. (27) This and several other studies suggest that EC are infected with HIV-1 that is fit and virulent and that these individuals have unique immune control over HIV-1. (28, 29) This control may be related to increased CD8⁺ T cell responses(30) and in some patients to the presence of specific HLA class I alleles. (31) Although some HLA alleles are over-represented in ECs, these do not seem to automatically confer EC status, as patients with progressive disease may also possess these alleles. Because the factors contributing to immune control of HIV-1 in EC have not been fully characterized, it has proven difficult to develop a therapeutic method to recapitulate this state for a functional cure.

Here, we explore strategies to deplete the LR to achieve sterilizing cure or ART-free remission. Several strategies toward HIV-1 cure are currently being studied, including early cART, which improves long-term health outcomes for patients and may induce post-treatment control; vaccination, including preventative or therapeutic measures to control infection, and shock-and-kill, which targets the LR that persists long term in patients on effective cART. Here, we focus on this latter strategy in an attempt to reduce the burden of lifelong HIV-1 infection.

The shock-and-kill strategy for HIV-1 eradication aims to directly target latent HIV-1 in resting memory CD4⁺ T cells and eradicate all remaining replication-competent

HIV-1 from the body in patients on suppressive cART. (32) Since HIV-1 genes are not actively expressed in latently infected cells, the first step is to “shock” cells into reinitiating HIV-1 gene expression. When cells begin producing viral antigens, they can potentially be recognized and targeted by an immune response for the “kill” step.

Multiple classes of small molecule latency reversing agents (LRAs) have been identified to potentially initiate the “shock” step of this strategy. (33) Histone deacetylase inhibitors (HDACis), a class of drugs developed to treat certain cancers, are thought to act upon latent HIV-1 by modifying the chromatin state of the LTR promoter, (34) although the mechanism remains controversial. (35) HDACis such as vorinostat and romidepsin have been studied thoroughly *ex vivo* and brought into clinical trials with varying levels of success. (36-38) In clinical studies employing shock-and-kill, success in perturbing the LR is assessed by looking for transient increases in cell-associated HIV-1 RNA or viremia to indicate an initiation of HIV-1 gene expression. On a broader scale, success in decreasing the size of LR may be determined by measuring inducible virus.

To date, no LRA has produced a substantial decrease in the size of the LR. One problem is that induction of HIV-1 gene expression is only half of the strategy. It is also important that the infected cells be eliminated after reversal of latency. Shan et al. have shown that CD8⁺ T cells from most patients on cART have only limited ability to kill infected cells after reversal of latency (39). They went on to describe a more successful approach in which CD8⁺ T cells were prestimulated with *gag* peptides to “prime” the response. These data suggest that some sort of vaccination may be necessary to activate an immune response for the “kill” step.

A sterilizing cure or prolonged ART-free remission will require a drastic decrease in the pool of latently infected CD4⁺ T cells. Latently infected cells are rare, with an average frequency of about 1 cell with replication-competent virus per 10⁶ resting CD4⁺ T cells. (9, 13) For this reason, measuring the LR is difficult. There is no available

clinical assay for the LR. It is important to have an accurate method to measure a change in size of the LR for patients undergoing cure interventions.

The gold standard for measuring the LR is the viral outgrowth assay (VOA), in which limiting dilutions of resting CD4+ T cells are stimulated with an activating mitogen in culture and the amount of infectious virus produced is measured by HIV-1 p24 antigen ELISA. (9, 40) This assay has the advantage of detecting only replication-competent virus, but has recently been shown to underestimate the size of the LR because some replication-competent proviruses are not induced after a single round of T cell activation in this assay (41). VOA is also requires a large number of cells and 2-3 weeks of cell culture, rendering it more expensive and time-consuming than PCR-based methods.

Multiple PCR-based methods have been developed to more easily measure the size of the LR. Detection of HIV-1 DNA in unfractionated PBMC or purified resting CD4+ T cells can be used to quantify the total number of cells carrying an HIV-1 provirus. (42, 43) A major caveat of this method that it detects defective proviruses, which do not pose a barrier to cure. This discrepancy can lead to an overestimation in the size of the LR by as much as 300-fold relative to estimates based on the VOA.(44)

PCR detection of induced HIV-1 RNA can also be used to measure the LR. This can be done with a T cell activation protocol similar to that used in the VOA, with the ultimate readout being RT-PCR detection of HIV-1 RNA instead of ELISA. (45) This method is less time-consuming than VOA and more accurate than proviral DNA detection, but may pick up some defective proviruses that can be transcribed but cannot produce infectious virus. Quantification of HIV-1 RNA transcripts can also be useful in identifying effective LRAs for shock-and-kill, as this method can measure HIV-1 expression changes in patient cells treated *ex vivo*. (46, 47)

Recent reports have renewed optimism for curing HIV-1 infection. Unfortunately, these cases do not represent the broad cure strategy necessary to impact the global HIV

epidemic as they cannot be applied to the vast majority of individuals living with HIV. Here, we evaluate broader strategies for HIV-1 cure by shock-and-kill, including the previously discarded but potent strategy of T cell activation, and the poorly understood mechanism of HDACis.

Chapter One: Revisiting global T cell activation in HIV-1 shock-and-kill

Introduction

HIV-1 persists in a latent reservoir in resting memory CD4⁺ T cells despite combination antiretroviral therapy (cART) (9, 13, 26). Latently infected cells represent a major barrier to eradication. Integrated proviruses in resting CD4⁺ T cells (rCD4s) are minimally transcribed, and thus this reservoir evades immune surveillance. A paradigm for HIV-1 cure (37) involves reactivation of proviral expression in latently infected cells to allow their elimination by immune mechanisms including CD8⁺ cytotoxic T lymphocytes (CTL) (32). Agents eliciting global T cell activation were used in the original detection of the reservoir (9, 26) and effectively reverse latency. However, in clinical settings they induce severe adverse reactions by causing systemic release of proinflammatory cytokines (48, 49). Although clinical trials using T cell activating agents such as anti-CD3 and IL-2 have demonstrated transient increases in viremia and changes in HIV-1 intracellular RNA levels (48), this approach has been abandoned in favor of latency reversing agents (LRAs) that induce HIV-1 transcription without cellular activation. While promising in cell line models for HIV-1 latency, no single LRA reactivates HIV-1 gene expression to levels induced by T cell activation in rCD4s derived from patient samples (47). The most effective single agents are PKC agonists, which affect a subset of the pathways stimulated by T cell activation (50). Thus, effective latency reversal may require some immune activation. This thesis explores the idea that immunomodulatory compounds in conjunction with T cell activation may limit toxicity while allowing maximal reactivation of latent HIV-1.

Adverse reactions elicited by the activation of large numbers of T cells are typically attributed to “cytokine storm” (48). A classic example is toxic shock syndrome, caused by a staphylococcal superantigen that activates all cells expressing a V β 2 T cell receptor (TCR) (about 10% of total T cells) (51). Additionally, agents that induce measurable *in vitro* lymphocyte proliferation have toxicity *in vivo*. This was dramatically

demonstrated by the CD28 superagonist TGN1412, for which other *in vitro* measures of immune toxicity failed to predict the widespread nonspecific immune activation observed *in vivo* (52).

We asked whether immunosuppressive agents could block the cytokine production and T cell proliferation induced by T cell activation without blocking HIV-1 latency reversal or subsequent killing of infected cells. Many immunosuppressive compounds affect upstream regulators of T cell activation pathways, and thus affect HIV-1 transcription due to similarities between the HIV-1 long terminal repeat (LTR) and the promoters of key genes upregulated by T cell activation including IL-2 (53). IL-2 signaling is crucial for proper activation of T cells. For instance, the widely used immunosuppressant cyclosporin inhibits the calcium-dependent phosphatase calcineurin, blocking nuclear translocation of the transcription factor NFAT, which acts on both the IL-2 promoter and the HIV-1 LTR (54). Cyclosporin inhibits HIV-1 expression induced by a T cell activating agent in a cell line model of latency (55). This makes cyclosporin a good control for expected immunosuppressive action on HIV-1 latency reversal.

To avoid inhibition of transcription factors that act upon the HIV-1 promoter, we explored immunosuppressants that do not affect signaling immediately downstream of TCR engagement. Rapamycin (sirolimus), an inhibitor of the mammalian target of rapamycin (mTOR), mediates immune suppression downstream of IL-2 signaling (56). mTOR integrates “signal 2” of T cell activation to facilitate metabolic changes needed to support cellular growth and proliferation. Rapamycin disrupts formation of mTOR complex 1 (mTORC1) which mediates these effects (56) (Figure 1A). Importantly, rapamycin does not affect mTOR complex 2 (mTORC2), which positively regulates PKC activation and downstream pathways in T cell activation (56, 57). It has been used in HIV-1 infected patients undergoing solid organ and bone marrow transplantation (58).

Rapamycin also protects mice from death after superantigen challenge (59). We hypothesized that rapamycin treatment would prevent the adverse effects of T cell activation without preventing upregulation of HIV-1 transcription.

Another consideration is the effect of immunosuppressive agents on CTLs. Histone deacetylase inhibitors (HDACis), an otherwise promising class of LRAs, have been shown to interfere with CTL killing, making this a prominent issue to address (60). Cyclosporin may also inhibit CTL function due to a dependence of CTL degranulation on calcium influx and downstream signaling (61). In contrast, rapamycin has a positive effect on the memory CD8⁺ T cell response to viral infection (62). We tested whether rapamycin interferes with CTL killing of infected cells in the context of HIV-1 shock-and-kill.

Materials and Methods

Human Subjects

Whole blood was obtained from both healthy donor and HIV-1 infected participants. All HIV-1 infected individuals enrolled were on a suppressive antiretroviral therapy regimen and maintained undetectable plasma HIV-1 RNA levels (<50 copies per mL) for at least 6 months prior to enrollment. Characteristics of HIV-1 infected donors are listed in Table 2.

Isolation of resting CD4⁺ T cells

Whole blood or leukapheresis blood samples were collected and isolated as described previously.⁷ Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation. Resting CD4⁺ T cells (CD4⁺, CD69⁻, CD25⁻, HLA-DR⁻) were enriched using magnetic microbeads through negative depletion (Miltenyi Biotec).

Drug treatment conditions

All treatments were performed in basic media (RPMI with 10% fetal bovine serum) unless otherwise noted. Drug concentrations were as follows: rapamycin 5.47 μM (5 $\mu\text{g/ml}$, rounded to 5 μM) or 547 nM (0.5 $\mu\text{g/ml}$, rounded to 500 nM) as indicated (Millipore, 553211), cyclosporin A 500 nM (Sigma, C3662), bryostatin-1 10 nM (Sigma, B7431), romidepsin 40 nM (Selleck Chemicals, S3020). All drug treatments, including vehicle alone, had a final DMSO concentration of $<.05\%$. $\alpha\text{CD3}/\alpha\text{CD28}$ treatment was set up as follows: 10 μL αCD3 antibody (BD Biosciences, 555366) plus 1 mL PBS each was added to wells on a 12 well plate and incubated at 37° C for 1.5 hours. After incubation, wells were washed twice with PBS to remove excess antibody. 1 μL αCD28 antibody (BD Biosciences, 555725) was added per 1 mL media at final treatment.

Quantification of HIV-1 mRNA transcripts

All drug treatments were performed in 1 mL media total with duplicate treatments of 5×10^6 cells each. RNA isolation and cDNA synthesis were performed as previously described⁷. Real-time PCR was then performed in triplicate using Taqman Fast Advanced MasterMix (Life Technologies, 4444556) on an ViiA7 Real-Time PCR System (Life Technologies). Primers used were specific for correctly terminated polyadenylated HIV-1 mRNA, as described by Shan et al. (46).

Supernatant cytokine analysis

Cytokine release was quantified directly from supernatants of cells used for viral RNA quantification. Supernatants were collected after 24 hours of drug treatment, when cells were used separately for intracellular RNA measurement. Samples were kept at -80 until use and then analyzed according to a previously described protocol (2). Supernatant cytokines were measured using two kits: CBA Human Th1/Th2/Th17 kit (BD

Biosciences, 551811) to quantify 7 cytokines in total, shown are the 4 pro-inflammatory cytokines induced to highest levels by α CD3/ α CD28 stimulation (IL-2, TNF α , IFN γ , IL-6); or Human Soluble Protein Master Buffer Kit (BD Biosciences, 558264) with MIP-1 α , MIP-1 β , IL-8, MCP-1, and IL-1 β Flex Sets (BD 558325, 558288, 558277, 558287, and 558279 respectively).

Cell proliferation assay

Cellular proliferation was quantified using CellTrace CFSE (Invitrogen, C34554) in PBMC derived from healthy donor blood, according to manufacturer's protocol. Briefly, cells were stained for 5 minutes at room temperature with CFSE at a final concentration of 5 μ M. Cells were washed to remove excess stain, and then treatments set up. After treatment, cells were resuspended in PBS and analyzed with a FACSCanto II (BD Biosciences). Treatments were set up as follows: 5 million cells per condition, in duplicate, for 24 hours at drug concentrations described above.

Cell death assay

Cell death was determined in healthy donor PBMC after 24-hour treatment of 5×10^6 cells per condition using the Zombie Aqua Fixable Viability Kit (Biolegend, 423101) according to the manufacturer's standard cell staining protocol. Briefly, cells were stained post-treatment using 1 μ L stain per 1×10^6 cells in 100 μ L PBS at room temperature for 15 minutes. Cells were then washed and dead cells quantified on a FACSCanto II.

Measurement of cell surface markers

Surface marker expression was determined in healthy donor PBMC 24-hour after treatment of 1 million cells per condition. Stains used were: PD-1 PE (BD 560795), CD3 Pacific Blue (BD 558117), CD4 FITC (BD 555346), CD69 APC-Cy7 (BD 556656), and

CD25 APC (BD 555434) according to manufacturer's protocols. Cells were analyzed by flow cytometry using a FACSCanto II.

CTL coculture

Whole blood samples from HIV-1 infected individuals were collected and processed as described previously (3). Briefly, PBMCs were isolated from whole blood and isolated to CD4⁺ T cells or prestimulated to prime a CD8⁺ response. Prestimulated PBMC were cultured with IL-2 (10 U/ml) with or without a mixture of 129 Gag peptides (80 ng/ml for each) (NIH AIDS Reagent Program) for 6 days. Rapamycin (500 uM) was added to some PBMC cultures during the last 3 days of prestimulation without media change. CD4⁺ T cells were cultured for 3 days, activated as described in cytokine enriched media for an additional 3 days, then infected with NL4-3-Δenv-GFP reporter virus (NIH AIDS Reagent Program). Cells remained in cytokine-enriched media for 1 hour before washing, replating in basic media, and addition of rapamycin to some cell populations as indicated. On the day of coculture, CD8⁺ T cells were isolated from prestimulated PBMC by negative selection (Miltenyi Biotec, 130-096-49). At coculture rapamycin (500 nM) or cyclosporin (500 nM) were added to some wells as indicated. Cells were cocultured for 3 days at a 1:1 effector:target (E:T) ratio before staining with CD8 APC (Biolegend 300912) and CD3 Pacific Blue (Biolegend 300330) according to the manufacturer's protocol. Samples were read on a FACSCanto II.

Results

To determine whether rapamycin affects HIV-1 transcription elicited by T cell activation, we treated purified rCD4s from infected individuals on suppressive cART with latency reversing stimuli and quantitated changes in HIV-1 gene expression. This system more closely approximates the in vivo response than do latency models involving

transformed cell lines or primary cells infected in vitro (47). Because the in vivo frequency of latently infected cells is low, large numbers of cells must be evaluated with sensitive assays for HIV-1 RNA. We treated aliquots of 5×10^6 purified rCD4s with T cell activating stimuli in the presence of varying concentrations of immunosuppressants to determine the concentrations that blocked cytokine production without cellular toxicity. After 24 hours, intracellular HIV-1 mRNA was measured by qRT-PCR using primers that detect all correctly polyadenylated HIV-1 transcripts (47). At concentrations as low as 50 nM, cyclosporin inhibited HIV-1 induction by α CD3/ α CD28 (Figure 1B) and IL-2 release (Figure 1C). In sharp contrast, rapamycin did not produce a dose-dependent inhibition of reactivation of HIV-1 from latency even at concentrations as high as 5 μ M, despite inhibiting IL-2 production at lower concentrations (Figure 1B, 1C). Neither drug affected cell viability in the concentration range tested (Figure 1D).

Using dose-response data from the above experiments and published studies (55, 59), we compared the effects of cyclosporin and rapamycin in cells from patients on cART by treating 5×10^6 cells per condition with vehicle alone (DMSO), α CD3/ α CD28 alone, or α CD3/ α CD28 plus rapamycin or cyclosporin. HIV-1 mRNA levels increased significantly after treatment with α CD3/ α CD28 alone for 24 hours ($p=0.048$, Figure 2A). To account for substantial interpatient baseline variability, the data were normalized to DMSO control (Figure 3A). We observed no significant difference in HIV-1 RNA induction by α CD3/ α CD28 with or without co-treatment with 5 μ M rapamycin. However, cyclosporin significantly suppressed α CD3/ α CD28-induced HIV-1 mRNA induction even at a 10-fold lower concentration (500 nM) ($p=0.003$). To rule out effects of immunosuppressants on baseline HIV-1 transcription, we treated cells with vehicle alone (DMSO), rapamycin, cyclosporin, or α CD3/ α CD28 for 24 hours. α CD3/ α CD28

significantly increased HIV-1 mRNA transcripts ($p=0.008$), while rapamycin or cyclosporin had no effect (Figure 2B).

We also examined supernatants from the cultures described in Figure 3A for pro-inflammatory cytokines. IL-2, TNF α , and IFN γ were induced at high levels in rCD4s, whereas other cytokines tested were not substantially produced after α CD3/ α CD28 treatment. Co-treatment with α CD3/ α CD28 and rapamycin significantly decreased release of IL-2, TNF α , and IFN γ compared with α CD3/ α CD28 alone in these rCD4s ($p=0.0009$, 0.0002 , and 0.019 respectively; Figure 3B-D) Cyclosporin treatment also significantly decreased production of these cytokines (IL-2, $p=0.001$; TNF α , $p=0.012$; IFN γ , $p=0.029$). Co-treatment also inhibited production of IL-2, MCP-1, MIP-1 α , IL-1 β , MIP- β , IFN γ , TNF α , and IL6 by unfractionated PBMC (Figure 4A). Rapamycin inhibited MIP-1 α production by over 50% and all other measured cytokines by over 80% (Figure 4B). This effect was not due to cellular toxicity (Figure 5A).

We then measured the effect of each treatment on cell proliferation, another potential indicator of clinical toxicity, using a CFSE assay with healthy donor PBMC 5×10^6 cells per condition were treated with DMSO alone, α CD3/ α CD28, or α CD3/ α CD28 and rapamycin or cyclosporin for 24 hours (Figure 5B). α CD3/ α CD28 stimulation caused significant proliferation ($p=0.014$) that was substantially inhibited by rapamycin and cyclosporin ($p=0.012$, 0.018 , respectively) (Figure 3E). α CD3/ α CD28 treatment strongly induced activation marker expression on PBMC from infected individuals after 24 hours. Co-treatment with rapamycin decreased expression of CD25 and PD-1 ($p=0.024$ and 0.016), as did cyclosporin (CD25, $p=0.009$ and PD-1, $p=0.004$) (Figure 5C), suggesting that immunosuppressant treatment can downregulate chronic inflammation and T cell exhaustion, respectively. Together, these data demonstrate that rapamycin decouples latency reversal and cytokine production, allowing maximal HIV-1 induction while

preventing toxic consequences of T cell activation. In contrast, cyclosporin was not suitable due to inhibition of HIV-1 gene expression.

To measure the potential discrepancy between specific inhibition of mTORC1 by rapamycin and pan-mTOR inhibition, we also tested mTOR kinase inhibitors Torin1 and pp242. mTOR kinase inhibitors downregulate formation of both mTORC1 and mTORC2. mTORC2 has a role in the PKC pathway and therefore may interfere with T cell activation pathways (56). We found that both of these pan-mTOR inhibitors displayed dose-response inhibition of α CD3/ α CD28 stimulated HIV-1 expression. pp242 displayed significant inhibition of HIV-1 expression, while Torin1 treatment showed a trend toward inhibition (Figure 6A-B).

We next tested the effect of rapamycin on a class of LRAs that may induce some level of immune activation: PKC agonists with and without HDACi (10,11). Rapamycin did not affect HIV-1 mRNA induction by the PKC agonist bryostatin-1, the HDACi romidepsin, or the combination of these (Figure 7A). Shown as fold change, it becomes clear that only a modest increase in HIV-1 mRNA was seen with bryostatin-1 or romidepsin alone compared to a more substantial induction by the combination (Figure 7B), consistent with previous reports(47, 63). We also tested the release of cytokines suggested to be associated with bryostatin-1 administration in vivo(64). Similar to the robust inhibition of PBMC cytokine release induced by α CD3/ α CD28 treatment seen above, IL-8 and MIP-1 α were substantially (>50%) reduced by addition of rapamycin, whereas IL-1 β , IL-6, and TNF α were not significantly inhibited (Figure 7C). In contrast to global immune activation by stimuli including α CD3/ α CD28 or PMA/ionomycin, it is unclear whether cytokine release is related to in vivo toxicity of bryostatin-1. However, these results demonstrate the potential for rapamycin to have a wide applicability to different latency reversing approaches.

To further assess the feasibility of rapamycin use in cure strategies, we tested whether it affected CTL killing of infected CD4⁺ T cells using a previously described coculture system (detailed setup in Figure 8) (39). Activated CD4⁺ T cells from infected donors were super-infected with an HIV-1 reporter virus expressing GFP (NL4-3-Δenv-GFP) and then cocultured at a 1:1 effector to target (E:T) ratio with autologous CD8⁺ T cells that had been pre-stimulated with a Gag peptide mixture and IL-2 for 6 days. After 3 days of coculture, cells were stained with APC-CD8 to exclude CD8⁺ T cells and more accurately measure GFP⁺ CD4⁺ T cells (gating strategy shown in Figure 5D).

For each patient, a decrease in GFP⁺ CD4⁺ T cells was observed when infected CD4⁺ T cells were cocultured with pre-stimulated CD8⁺ T cells, compared with infected CD4⁺ T cells cultured alone. CD8⁺ T cells pretreated with IL-2 alone caused a less substantial reduction in GFP⁺ cells. Treating the infected CD4⁺ T cells with rapamycin for 24 hours prior to coculture did not prevent CTL killing, showing that epitope presentation by infected CD4s was not affected (Figure 9A). We next tested whether rapamycin inhibited CTL killing of targets presenting HIV-1 epitopes using the above coculture system. Coculture with CD8⁺ T cells prestimulated with IL-2 plus Gag peptides caused significant elimination of GFP⁺ CD4⁺ T cells, compared with CD8⁺ T cells prestimulated with IL-2 alone ($p=0.047$). Rapamycin added at the time of coculture did not affect CTL-mediated killing of infected CD4s, whereas cyclosporin had the expected inhibitory effect ($p=0.027$) (Figure 9B). In addition, pre-treatment of CD8⁺ T cells with rapamycin for 3 days before coculture had no effect on the killing of infected cells (Figure 3B).

Discussion

Overall, our results show that rapamycin inhibits cytokine release and cellular proliferation but does not affect HIV-1 latency reversal or CTL mediated killing of HIV-1

infected cells. Our CTL data are consistent with previous reports of the effect of these immunosuppressants on CTLs (61, 62). Rapamycin has also been suggested as a therapeutic in HIV-1 infection, as it downregulates CCR5 and may effectively inhibit viral entry of R5 tropic viruses (65). Importantly, pan-mTOR inhibitors downregulate HIV-1 expression, suggesting the importance of targeting only mTORC1. We found that cyclosporin was not suitable for HIV-1 shock-and-kill due to its inhibition of HIV-1 expression and CTL activity.

In the search for effective approaches for reactivating latent HIV-1, T cell activation continues to be the standard for maximal transcriptional activation of latent proviruses. However, compounds that elicit T cell activation markers are often overlooked in favor of less effective LRAs. To avoid this compromise, this project has explored a latency reversal strategy that allows signaling downstream of TCR stimulation to effectively activate HIV-1 gene expression while controlling toxicity by co-treatment with the mTOR inhibitor rapamycin. We hypothesize that previously discarded strategies eliciting T cell activation could allow for effective reversal of HIV-1 latency in infected individuals on effective cART who are also treated with mTOR inhibiting drugs such as rapamycin. These findings may allow an expansion in the repertoire of clinically relevant LRAs currently being investigated.

**Chapter 2: In vivo administration of immunosuppressants to
HIV-infected individuals undergoing solid organ transplantation**

Introduction

Recently, the field of HIV cure research has turned to transplantation as an area of interest. The only patient ever cured of HIV-1 infection, the “Berlin patient”, underwent allogeneic hematopoietic stem cell transplant (HSCT) to treat a malignancy. The donor was homozygous for the CCR5 delta32 mutation, meaning that lymphocytes derived from this tissue are resistant to HIV-1 infection due to a deletion in the CCR5 coreceptor needed for HIV-1 entry (21). Although a full, sterilizing cure has not been since achieved using this strategy, there is still interest in studying HIV-infected individuals requiring HSCT to learn more about how we can achieve cure.

A recent report by Stock et al. (58) presented HIV-1 LR data in patients who underwent solid organ transplantation. This type of transplantation is inherently different, in that the immune system is not reconstituted post-transplant, and so this approach has not gained the same type of interest in the field. Regardless, the study found that there may have been a change in the size of the LR based on the immunosuppressant that patients received. They reported that HIV-1 infected kidney transplant recipients who received rapamycin (sirolimus) for immunosuppression had lower levels of proviral DNA in PBMCs compared to HIV-1 infected transplant recipients who received cyclosporin or tacrolimus, both calcineurin inhibitors. HIV-1 LR measurements were only measured post-transplant; without baseline data, it is difficult to conclude whether there was an actual change in the size of the LR. Additionally, PCR for HIV-1 proviral DNA picks up many defective HIV-1 proviruses, which make up a large proportion of the LR. Though this is not the most sensitive or accurate method of measuring the LR, the study interestingly still noted a significant difference that appeared to be immunosuppressant dependent.

Although this method has its caveats, it suggests a reduction in the size of the HIV-1 LR in response to rapamycin treatment. The authors suggest that this change may

be due to a reduction in chronic immune activation or inflammation characteristic of HIV-1 infection. It is thought that this inflammation may drive homeostatic proliferation of infected cells and perpetuate the LR (66). Alternatively, we propose that the high levels of immune activation induced by transplantation (67) could reactivate expression of latent HIV-1. Our previous data indicate that HIV-1 transcription could occur in patients on rapamycin, allowing elimination of latently infected cells through viral cytopathic effects or cell mediated cytotoxicity, leaving rapamycin recipients with a long-term reduction in HIV-1 DNA levels. In contrast, HIV-1 gene expression would be inhibited in individuals receiving calcineurin inhibitors including cyclosporin or tacrolimus, minimizing this potential effect. Our hypothesis explains a difference in the two classes of immunosuppressants; if chronic immune activation has a role, it is unclear that mTOR inhibition would display more substantial inhibition of activation than calcineurin inhibitors would.

This clinical finding (58) suggests that our above *ex vivo* results may be applicable to HIV-1 infected patients requiring solid organ transplant. We therefore chose to expand on these combined findings by measuring the size of the LR before and after transplantation, to determine whether there is in fact a reduction in the size of the LR in patients receiving rapamycin. In order to get an accurate estimate of the size of the LR, we developed a novel assay using a working knowledge of the landscape of the HIV-1 latent reservoir and currently available assays. We would also like to compare hypotheses and determine whether perturbations of the LR are due to immune activation induced by organ transplantation, or a reduction in chronic immune activation due to immunosuppressant treatment as proposed by this other group. To specifically rule out the other group's hypothesis, we plan to include measurements of plasma and cellular activation markers before and after transplantation.

As the landscape of the LR becomes more fully understood, it has also become clear that our ability to accurately measure the LR and to quantify changes after an intervention is quite a difficult task. Although only replication competent proviruses able to produce infectious viral particles are of importance in a cure strategy, these only compromise a small percent of total integrated HIV-1 DNA in rCD4s (41, 68). This is in part due to the high mutation rate of HIV-1 reverse transcriptase, which causes the proportion of defective proviruses to increase over time (68). These defective proviruses further complicate our ability to accurately measure the LR because they have different levels of defects: although by definition, none can produce infectious virus, they may still undergo transcription, translation and viral peptide production, and even assembly of viral particles. Some of these defective proviruses can also be recognized by CTLs and targeted during an intervention, further complicating LR measurements (69). This makes it difficult to establish a sensitive assay to distinguish intact and defective provirus.

As explained above, there are three basic types of assays to measure the LR: viral outgrowth, PCR for HIV-1 DNA, or PCR for inducible HIV-1 RNA. The quantitative viral outgrowth assay (QVOA) is considered to be the most vigorous of these assays, as it detects only replication-competent virus (45). It does, however, have some caveats, including a requirement of a large blood sample and its time-consuming nature. The most serious fallback of this assay may be its small dynamic range; it estimates the majority of patients to have 1 infectious unit per million cells (IUPM), while the true number of intact proviruses per million may be 30-40 times larger (41, 68). Additionally, IUPM estimates from QVOA do not correlate with the number of intact proviruses, meaning that QVOA may not be an accurate measurement of latently infected cells that are a barrier to cure. For these reasons, we consider QVOA to be a minimal estimate of the LR. After an intervention, it may not be the most sensitive way to measure a change

in the LR due to its small dynamic range and inability to pick up many cells harboring an intact provirus.

If QVOA is the minimal estimate, then measurement of HIV-1 DNA is the maximal estimate of the size of the LR. This method quantifies all cells carrying an HIV-1 provirus regardless of defective status, as long as primers are compatible with the sequence. This estimate is up to 300-fold higher than that predicted by QVOA (44). Measuring changes in the LR using this method is also not optimal, because it includes many proviruses that will not be eliminated during an intervention. In between these two estimates of the LR is PCR for induced HIV-1 RNA. This method restricts detection to only transcription-competent HIV-1 proviruses. Interestingly, new data has shown that many defective proviruses capable of transcription produce only low levels of HIV-1 RNA (69), meaning that use of qPCR may enable a cutoff to increase likelihood of detecting intact proviruses. Other defective proviruses capable of higher levels of transcription approaching that of intact proviruses are more likely to produce viral peptides, making them potential targets in a “shock-and-kill” strategy (69). Therefore, use of qPCR for inducible HIV-1 may enable a more accurate estimate of the LR than other methods. Although it does not detect only proviruses that pose a barrier to cure, currently available assays can be altered to primarily detect viruses that may be targeted during intervention.

Limiting dilution assays for inducible virus are currently available to estimate the size of the HIV-1 LR. Of particular note is TILDA, the *tat/rev* induced limiting dilution assay, which has generated interest due to its small required sample size and minimal time requirement. In TILDA, cells are plated at limiting dilution and stimulated for 12 hours with PMA and ionomycin for maximal induction of HIV-1 RNA. *Tat/rev* nested primer sets for multiply spliced RNA are then used for detection of induced proviruses to reduce read-through of RNA transcripts (70). The number of “positive” wells at each

dilution can then be used to calculate an estimate of the number of infected cells per million. One major caveat of this assay is the use of nested PCR for amplification. This PCR method may be necessary due to a combination of the low number of cells used in the assay and the lack of an RNA isolation step as a part of the protocol. However, nested PCR washes out any potential difference in qPCR readouts, which may be important due to the likelihood that defective, transcription competent proviruses will produce low levels of RNA (69). An ideal assay would allow distinction of low or high levels of viral transcription in each well in order to get a better idea of which proviruses are likely to be eliminated during an intervention, and are truly a barrier to cure. We sought to improve on this assay by using a different primer set and PCR protocol, using a cycle threshold cutoff that would give us a more accurate idea of which proviruses were induced to produce high levels of HIV-1 RNA.

With knowledge of the landscape of the LR expanding, we felt that the field is approaching an assay to more accurately measure the size of the latent reservoir than currently available limiting dilution induction assays. An ideal assay should distinguish between intact and defective proviruses, but need not necessarily exempt transcription or translation competent viruses that can be targeted during a cure intervention. We developed the Quantitative Viral Induction Assay (QVIA) to better fit these needs than any assays described to date. By using smaller dilutions and more wells than QVOA, it has a much higher dynamic range, making QVIA more sensitive to changes in infected cells over time. Here we discuss preliminary data distinguishing a qPCR cutoff for induced proviruses, intra-assay variability of QVIA, and assessment of clinical samples over time using QVIA.

Materials and Methods

Quantitative Viral Induction Assay (QVIA)

From PBMC, isolate CD4+ T cells by negative selection. From this population, isolate resting CD4+ T cells using CD69, CD25, and HLA-DR negative selection (CD4, CD69, CD25, HLA-DR negative isolation kits: Miltenyi 130-096-533, 130-092-355, 130-092-983, 130-046-101 respectively). Resuspend 4 million resting CD4+ T cells in 3.6 ml media (RPMI + 10% FBS). Plate cells on a 96 well round-bottomed plate at serial dilution (1:2 dilution from A-H), First, pipette 180 μ l original suspension to columns 1-10 in row A. Add 1.8 ml fresh media to remaining cells to dilute 1:2 for next row, and repeat through row H. This will yield 200K cells in row A, 100K cells in row B, etc. Keep columns 11-12 free. (80 wells total). Should have 180 μ L total / well to leave space for 10x drug. Prepare drugs at 10x, including DMSO control. Add DMSO only to column 1; PMA and ionomycin to columns 2-10 (PMA 50 ng/ml, ionomycin 1 μ M). Treat cells for 18 hours.

Prepare cells for lysis: pellet cells in 96 well plate (1400 RPM for 5 minutes). Wash cells with 100 μ l/well PBS, pellet again. Add 150 μ L Lysis/Binding Buffer (Dynabeads mRNA Direct Purification kit: Thermo Fisher Scientific 61012) to cell pellet and pipette to lyse. Option to wrap plate in parafilm and foil and freeze at -80C until ready to proceed.

To isolate RNA using Dynabeads mRNA Direct Purification kit: Resuspend beads thoroughly by vortexing. Transfer beads (10 μ l beads per well) to microfuge tube and place on magnet (30 sec or until solution is clear). Remove supernatant and resuspend beads in equivalent volume of fresh Lysis/Binding Buffer. Move cell lysate to a PCR plate (200 μ l well size). Add washed beads to cell lysate: 10 μ l/well. Incubate with continuous mixing for 3-5 minutes at RT to bind mRNA to beads (longer if viscous). Place plate on magnet for 2 minutes, then remove supernatant (10 minutes if viscous).

Wash beads 2-3 times with Washing Buffer A (150uL per well per wash). Wash beads 2 times with Washing Buffer B (150uL per well per wash). Elute in 5-10ul water. RNA amples can be stored at -20C.

To make cDNA: (qScript Flex cDNA Kit: Quanta Biosciences 95049-100) Keep all components (except enzyme) on ice. Assuming 10ul samples) Prepare random primer: mix 3ul water + 2 ul random primer / each well. Add 5 ul to each well, mix. Incubate 5 min at 65C, snap chill on ice. Prepare reaction mix: 4ul 5x reaction mix + 1ul RT. Add 5ul / each well. Incubate 10 min @ 25C, 45 min @ 42C, 5 min @ 85C, hold @ 4C. Samples can be stored at -20C.

Measure induced proviruses per million (IPPM) by qPCR: Prepare qPCR reaction mix: 100ul forward primer, 100ul reverse primer, 200ul probe, 1ml FastAdvanced Mastermix (Taqman FastAdvanced Mastermix: Life Technologies 4444964; primers published (46): Applied Biosciences ViiA7). Add 14ul qPCR reaction mix to every well on a 96-well, 100ul qPCR plate. Pipette 6ul cDNA sample to columns 1-10 (corresponding to experimental setup). Pipette standard DNA (in duplicate) to columns 11-12 at the following copy numbers: 60000, 6000, 600, 300, 150, 60, 30. Remaining 2 wells should be no template control. Run according to fast plate protocol for 50 cycles. IPPM can be calculated using positive wells measured.

Results

We first performed assay optimization and characterization to better understand the IPPM readout of QVIA. QVIA requires only 4×10^6 CD4⁺ T cells or rCD4s and takes 2 days from start to finish (Figure 10A). The negative assay frequency is 0.19 induced cells per million (IPPM) with a measured frequency range of 0.29 to 1,510.22 IPPM. After each qPCR run, a cutoff for “positive” wells can be established by a characteristic gap in Ct (cycle threshold) values: anything below this gap is considered “negative”.

(Figure 10B-C). Negative wells with low signal can be included in a measurement of PPM (proviruses per million), which provides more information about inter-patient variability in the LR.

We have also begun data collection to assess inter- and intra- assay variability of QVIA. To assess intra-assay variability, we isolated bulk CD4⁺ T cells or rCD4s from cART-suppressed individuals and set up QVIA plates from the same blood draw, separately. All later steps including lysis, RNA isolation, and cDNA synthesis were also completed separately from each other. Our preliminary data indicate that IPPM measurements from the same blood draw come out virtually identical (Figure 11A-B). To measure our inter-assay variability, we have collected samples from patients at different timepoints. The IPPM readouts from these QVIA setups can be compared to measure variability over time.

Next, to measure the HIV-1 LR in patients pre- and post-intervention, we set up QVIAs for IPPM measurements in transplant patient samples. All patients were on suppressive cART before they received kidney or liver transplantation from HIV+ or HIV- donors. 60 ml whole blood was obtained pre-transplant (baseline), and at 13 weeks and 26 weeks post-transplant for all organ recipients. During processing, plasma samples and small amounts ($2-3 \times 10^6$) of PBMC were saved for later analysis of immune activation or exhaustion markers. Cells were isolated to bulk CD4⁺ and QVIA setup performed on this cell set. Any cells in excess of 4×10^6 CD4⁺ T cells were frozen down. We found that IPPM measurements significantly increased from baseline to 13 weeks post-transplant ($p=0.007$, Figure 12A, Table 3). We measured a maximum 10-fold increase in one patient from baseline to 13 weeks and a mean increase of 10.088. Overall, the mean baseline IPPM = 7.689, mean 13-week IPPM = 15.255, mean 26-week IPPM = 13.593. Only 1 out of 8 patients measured had a decreased IPPM post-

transplant, from a low value of 1.473 at baseline to 0.0193 (undetectable) at both 13 and 26 weeks.

To get an idea for what may cause the increased IPPM, it must first be defined whether the measured increase is due to a difference in the number of infected cells, or increased “inducibility” of latent proviruses. We can get an idea for this difference by looking at the total wells in which RNA was detected in this assay, including those considered “negative” in the IPPM readout. This measurement of total proviruses per million is termed PPM. We found that there was a close relationship between IPPM and PPM, with a relative increase in PPM that matched IPPM increases in most cases (Figure 12B). These data represent a relative increase of low copy number wells that matched that of positive wells, and seems to suggest a total increase in the number of infected cells from these samples. We also measured for viral “blips”, when the viral load comes briefly above the clinical limit of detection. Although samples were not obtained for all patients at every timepoint, blips were measured in 3 out of 8 of the patients studied in the days immediately post-transplantation (Figure 12C).

Discussion

Our original hypothesis was for a role of rapamycin treatment in decreased HIV-1 LR size post solid organ transplant. Instead, we saw the surprising result of increased IPPM in most patients at 13 weeks post-transplant. This increase must be better characterized: although we saw an increase in the total number of wells containing a provirus with any detectable RNA signal, this does not definitively mean that the number of infected cells has increased at this timepoint. It is possible that the overall ability of latent proviruses to express any RNA increased. This could be explained by an overall change in immune activation status in these individuals. The next step is to quantify HIV-1 proviral DNA at each timepoint using frozen PBMCs from these samples. These

patients also need continued followup to measure changes in the LR at 26 months and at later times, to characterize whether the trend at 13 weeks holds. There are not enough data points to assess a role of immunosuppressant treatment at this time, but it will be interesting to see whether this influences is a difference in LR modulation.

Detection of blips in viral load during this process is potentially consistent with our original hypothesis. During latency reversing interventions, blips are often looked for because they represent an increase in viral expression during “shock” in shock-and-kill. Although samples were not collected every day post-transplant for every patient, we were still able to measure blips above the clinical limit of detection in 3 out of 8 of the patients. This number represents a much more frequent measurement of blips than is typically seen in a clinical setting, where patients are adherent to cART. The next step for these samples will be to sequence virus from these blips to characterize what is being reactivated and compare these sequences to what is activated in QVIA at different timepoints.

To further our knowledge of the landscape of the latent reservoir, it is important to also consider other compartments harboring latently infected cells, including the lymph nodes. Lymph nodes have been suggested as “sanctuary” locations, where cells may not be exposed to high enough levels of cART to prevent viral replication (71). HIV-1 RNA in the lymph nodes versus peripheral blood have only been quantified previously in untreated patients, and no difference was measured (72). It is important to the field to do an updated study characterizing potential viral evolution and differences in the proportion of infected cells in the lymph nodes compared with peripheral blood in patients on suppressive ART. Lymph node mononuclear cells (LNMCs) were collected from patients during transplantation, and are comparable to baseline blood samples. We can compare QVIA readouts and subsequent viral sequencing data in from PBMC and LNMC in order to get a more complete, unprecedented scope of the LR.

Characterization of QVIA is still in preliminary stages. We are currently working to sequence mRNA from positive wells. Our current hypothesis is that wells with higher copy number, considered to be “positive wells”, have a higher probability of expressing intact or translation-competent provirus than do low copy number wells below the qPCR cutoff. It is likely that wells with lower RNA expression have 5' deletions, since our primers detect at the far 3' end of HIV-1 mRNA. If this pattern holds true, this aspect of QVIA could make it more appealing for use than other limiting dilution PCR based methods to measure the LR. We must also continue to assess the inter- and intra- assay variability of QVIA in order to establish it as a consistent, accurate assay.

QVIA can also be used to quantify the number of cells that respond to a stimulus, and at what level on an individual cell basis. In most *ex vivo* assays, the response of rCD4s derived from HIV-1 infected individuals is measured by pooling large numbers of cells prior to stimulation (47). This setup increases the probability of including an equal number of latently infected cells in each treatment condition in order to minimize variation. Although this strategy for comparing the latency reversing potential of agents is effective on an overall quantitative scale, it does not give any information about whether higher amounts of HIV-1 RNA in response to a stimulus represent higher amounts of transcription, or a larger quantity of cells responding. QVIA can easily be set up with stimuli other than PMA and ionomycin in order to compare strategies and measure this discrepancy. This data would give us more information about the potential efficacy of various LRAs: the best latency reversing agents would maximize number of cells with induced HIV-1 expression in order to increase the potential to eliminate infected cells.

We would also like to know whether IUPM readouts from QVOA correlate with QVIA IPPM measurements. These two assays are measuring different aspects of the LR: QVOA only picks up intact, replication competent proviruses expressed in response

to PHA stimulation. QVIA measures all latent proviruses capable of high levels of transcription. Although the former represents the true barrier to a cure for HIV-1 infection, it also fails to measure many intact, potentially infectious viruses. Conversely, QVIA will measure some proviruses that are not a true barrier to cure, but will still represent a population of proviruses enriched for those that will be targeted during a cure intervention. Thus, we propose that QVIA will still give an accurate idea of changes in the LR during an intervention, but may not correlate with QVOA readouts. It might be more informative to measure a correlation of IPPM values with the true frequency of intact proviruses.

Chapter Three: Elucidating the mechanism of HIV-1 latency reversal by histone deacetylase inhibitors (HDACi)

Introduction

Although T cell activation produces the most robust reactivation of latent HIV-1 expression of any studied technique, the field has also explored several other more targeted methods for activating HIV-1 expression. Classes of small molecules that can effectively activate HIV-1 transcription in latently infected cells from HIV-1 infected patients have been identified and are candidates for clinical use (33). Before many of these compounds were tested using primary cells, they were identified in cellular models designed to recapitulate latency. However, not only do many of these compounds activate latency more robustly in models than in primary cells, it also became apparent that many other compounds identified as “hits” in model cell screens did not have any effect in either primary cells or in patients (47). Although this phenomenon has been demonstrated using multiple compounds, there has been little work questioning the discrepancy between these systems. Here we identify pathways varying between primary rCD4 and our lab’s bcl-2 transduced cell model of latency at baseline and in response to LRA stimulation to study additional unknown blocks to HIV-1 transcription in HIV-1 latency.

Of the identified latency reversing agents (LRAs), many have a proposed mechanism of action by which they specifically reactivate latently HIV-1 expression. However, these mechanisms remain somewhat controversial and require further investigation. Histone deacetylase inhibitors (HDACis) are a class of LRAs with a vague mechanism unsupported by empirical evidence associated with their activity on HIV-1. HDACis are a broad class of drugs with relevance to certain cancer types. Several, including vorinostat, panobinostat, and romidepsin have FDA approval to treat lymphoma and myeloma diagnoses. They do have activity on histones, inhibiting their deacetylation and the increasing overall transcription state in a cell. HDACis have several activities that are thought to be relevant to their cancer killing potential, including

direct non-histone related activity on transcription factors, structural proteins, and chaperones; and activity on cell cycle, apoptosis, and DNA damage pathways directly downstream of histone activity (73). HDACi activity on latent HIV-1 reactivation has been classically attributed to a simple, direct activity of these drugs on histones associated with the HIV-1 LTR promoter (74). It is thought that HDACi allow for a more permissive transcriptional state, promoting HIV-1 gene expression.

A study examining the latency reversal activity of the HDACi vorinostat in HIV-infected individuals exemplifies the lack of support for this mechanism (75). In this study, several HIV-1 infected individuals on suppressive cART were given vorinostat to test its *in vivo* latency reversal and potential activity on the latent reservoir. In this trial, they collected samples from patients at several timepoints, and measured various activities of vorinostat including changes in histone acetylation state and HIV-1 cell-associated RNA. Although the authors assume that increases in histone acetylation caused by the direct HDACi activity of vorinostat is allowing for more permissive transcription, and therefore higher levels of HIV-1 RNA expression, their own data has a chronological contradiction: they show that histones in lymphocytes have peak acetylation between 8-24 hours after vorinostat treatment, declining after 7-14 days. However, peak measurements in cell-associated HIV-1 RNA were not measured in most (18/19) patients until at least 7 days after vorinostat administration, and as much as 84 days in some patients (75). Because this increase in HIV-1 expression seems to continue long after a decline in histone acetylation status, it seems that the latency reversing activity of HDACi may be explained by one of the many other activities of this class of drug.

Here we perform gene expression and transcription factor screens, identifying “hits” by comparing baseline expression and differences in response to stimulation with HDACi in our model system and in primary cells. After performing preliminary experiments using a combination of LRAs and specific pathway inhibitors, we identified

p21 as a continuing pathway of interest, and continue our focus on a role for p21. Although HDACi do not have a well-characterized mechanism of action as LRAs, they are known to have substantial activity upregulating p21 gene expression. In fact, in the above study where patients were administered vorinostat, CDKN1A/p21 was one of the genes that was shown to be overexpressed, as a proof-of-concept that the treatment had the expected effect (75).

p21 is a small protein that promotes G1 arrest in response to many stimuli including cellular stress, DNA damage, and cytokine signaling. Transcription of the p21 gene is directly downstream of p53 signaling (76). The p21 protein is known to have a role during initial HIV-1 infection, when it associates with Vpr to induce cell cycle arrest (77). p21 expression has not been implicated in HIV-1 infectivity, but because it is specifically overexpressed during infection, it is possible that it has a role in facilitating HIV-1 expression to perpetuate infection. If this is true, it would logically follow that upregulation of p21 during a latency reversal strategy would upregulate HIV-1 gene expression.

We therefore followed up on p21 by studying the effects of p21 modulators on the activity of HDACi on both HIV-1 expression and on p21 protein expression. We indirectly attempted to downregulate p21 activity by upregulating p27, another protein involved in cell cycle regulation that typically has reciprocal activity from p21. We also looked for an effect of these modulators on the PKC agonist bryostatin, which displays synergy with HDACi: if p21 is important in the HIV-1 latency reversal potential of HDACi, bryostatin treated cells may also respond to p21 up- or down-regulation.

Materials and Methods

Isolation of resting CD4⁺ T cells

Whole blood samples were collected and isolated as described previously (47). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation. CD4⁺ T cells were enriched using magnetic microbeads through negative depletion (Miltenyi Biotec). Where indicated an additional enrichment was performed to isolate resting CD4⁺ T cells (CD69⁻, CD25⁻, HLA-DR⁻).

bcl-2 transduced cell model for HIV-1 latency

Model cells were produced as previously described (78). Briefly, primary CD4⁺ T cells were isolated from healthy HIV- donors, activated, and transduced with a lentiviral construct containing a constitutively active bcl-2 gene. These cells were cultured for 4 weeks to enrich for immortalized cells, then again activated, expanded, and infected with a single-round infection lentiviral vector with GFP driven by the HIV-1 promoter. Cells were cultured for 6 weeks to allow a return to resting state so cells may become “latent”, as defined by GFP⁻ status that may revert to GFP⁺ upon stimulation.

qPCR array

The qPCR array data was collected using the Human Stress & Toxicity PathwayFinder RT² Profiler PCR Array (SABiosciences) according to the manufacturer’s protocol. Briefly, RNA was isolated from samples and cDNA made according to an above protocol. Total cDNA was then mixed with the RT² qPCR mastermix, and added to a 96-well plate that already contained primers a given target in each well. qPCR was then performed on an AB ViiA7 instrument, and analyzed using the manufacturer’s data analysis spreadsheet to identify hits.

Transcription factor profiling array

To assess transcription factors that were differentially regulated by different cell types at baseline and upon stimulation, we used TF Activation Profiling Plate Array II (Signosis) according to the manufacturer's protocol. Briefly, specific DNA probes bound to biotin are combined with nuclear extract from the sample. After incubation the sample is column separated to remove free probe, and then probes with bound activated transcription factors can hybridize to wells containing specific complementary DNA to each probe in a 96-well plate. The sample is then incubated with streptavidin-HRP, a chemiluminescent substrate, and read with a luminometer. This kit allows for comparison of 96 transcription factors between conditions.

Drug treatments

All treatments were performed in basic media (RPMI with 10% fetal bovine serum) unless otherwise noted. Drug concentrations were as follows: romidepsin 40 nM (Selleck Chemicals, S3020), panobinostat 30 nM (Selleck Chemicals, LBH589), bryostatin 10 nM (Sigma-Aldrich, B7431), disulfiram 500 nM (Sigma-Aldrich, D2950000). Inhibitors were used at their known IC50 values: UC2288 (EMD Millipore, 5328130001), SKPin C1 (R&D Systems, 4817). All drug treatments, including vehicle alone, had a final DMSO concentration of <.05%.

Measurement of intracellular p21 protein levels

Cells were fixed and permeated using Cytofix/cytoperm kit (BD Biosciences, 554714) before intracellular staining for FACS analysis. Briefly, cells were washed then resuspended in 100µl Fix solution per well, and incubated for 20 minutes at 4°C protected from light. After this incubation, supernatants were removed and cells were resuspended in 1x perm wash solution with a 1:200 dilution of the 1° rabbit anti-p21 antibody (Abcam, ab109520). Cells were incubated for 30 minutes at 4°C with the

primary antibody. After this incubation, the 2° goat anti-rabbit IgG antibody (Abcam, ab150077) was added at a 1:200 dilution and again incubated 30 minutes at 4°C protected from light. Control samples were stained with 2° antibody only, or a rabbit polyclonal isotype control (Biolegend, 910801) and 2° antibody to confirm specificity of staining. Cells were then washed 2x with perm wash solution to prepare samples for FACS analysis. FACS was performed using an Intellicyt cytometer.

Results

To first determine the fundamental differences between model cells and primary cells, we compared fresh rCD4s with bcl-2 transduced model cells. 2×10^6 cells were treated with DMSO alone (baseline) or various LRAs: romidepsin, panobinostat, bryostatins, bortezomib, or disulfiram. After a 24 hour treatment, cells were lysed, RNA isolated, and cDNA prepared. cDNA from these samples was used in a Cell Stress and Toxicity qPCR array, which was used to generate datasets from 3 sets of model cells, associated rCD4s, and drug treatments comparing the differences at baseline and post-LRA treatment. We found that several genes (GADD45G, CDKN1A, BBC3 and VEGFA) were upregulated and some (EDN1, TLR4, and IL1B) downregulated at least 5-fold in model cells at baseline compared with rCD4s (Figure 13). By far the most striking of these baseline differences was CDKN1A, the gene that encodes for the cell cycle regulator p21, which was upregulated 15-400-fold. Interestingly, when rCD4s were treated with LRAs of different classes, we saw a similar effect: all treatments upregulated CDKN1A gene expression more than 15-fold.

To further our knowledge of the differences between rCD4s and model cells, we performed a similar comparison using an array measuring activation of various transcription factors. In these experiments, 5×10^6 cells were treated for 72 hours with

DMSO alone or various LRAs. We then performed a nuclear extraction, and incubated the nuclear extract with DNA sequences that activated transcription factors will specifically bind. The assay is qualitative and readouts of an array of factors are normalized for intra-sample comparison. Although each batch of model cells tested revealed different pathways of interest, some factors fit in well with our previous gene expression array data (Figure 14). The transcription factor screen showed increased NFAT, NFkB, Sp1, WT1, and p53 expression at basal levels in one batch of model cells. Factors overexpressed in both model cell batches included HSF1, NRF1, FOXO1, and MEF1.

We then performed experiments using specific pathway inhibitors in combination with HDACi to identify a potential role for our hits: p21, FOXO1, HSF1, and p53. These experiments were done in the J-lat 10.6 cell line, which has an HIV-1 LTR driving GFP expression (79). J-Lats have low expression of GFP at baseline that increases with LTR activity. We found that the specific p21 inhibitor, UC2288, displayed drastic inhibition on romidepsin induced GFP expression in J-Lats (Figure 15A). SKPin C1, an E3 ligase inhibitor that upregulates p27 expression and may thereby downregulate p21, also modestly downregulated LRA mediated GFP expression (Figure 15B). FOXO1 inhibition did not have a drastic effect on GFP expression (Figure 15B). Co-treatment with KRIB11, a heat shock pathway inhibitor, downregulated GFP expression in response to LRA treatment. However, this inhibition was most substantial on bryostatatin-induced GFP expression (Figure 15C), and we chose to instead explore mechanisms of action of HDACi since these are not as well-characterized.

The inhibitor data in J-Lats indicated that p21 was the most promising of hits. We therefore continued this study to specifically characterize a role of p21 in HDACi activity on HIV-1 latency reversal. As a proof-of-concept we first measured p21 activity in rCD4s at baseline and with 48-hour treatment with romidepsin or panobinostat, both potent

HDACi. We found that treatment with either HDACi or with the potent T cell activator PMA/ionomycin substantially increased p21 expression (Figure 16B). In J-Lats, UC2288 caused substantial inhibition of GFP expression stimulated by various LRAs. In cells derived from HIV-1 infected patients, this effect was less substantial, likely due to the low activity of single LRAs on cells treated *ex vivo* (Figure 16A). However, p21 downregulation caused inhibition of HIV-1 expression by the potent combination of bryostatin and romidepsin, suggesting a role for p21. We measured the expected effect of p21 protein downregulation by UC2288 co-treatment (Figure 16B).

We next wanted to see if p21 overexpression would have an effect on the HIV-1 latency reversing potential of HDACi or the PKC agonist bryostatin. To modulate p21 expression, we used the ubiquitin E3 ligase inhibitor SKPin C1, which has been shown to upregulate p27 and so may downregulate p21 protein levels (80). In J-Lats, SKPin C1 appeared to modestly decrease GFP expression. Conversely, in patient cells, we found that SKPin C1 showed substantial synergy with LRAs of all classes to promote HIV-1 expression (Figure 16A). This drug did not have the expected effect of increasing p21 expression (Figure 16B).

Discussion

We sought to determine the fundamental differences between model cells and primary cells, in order to better understand the discrepancy in latent HIV-1 reactivation between these systems and attempt to harness the differences in pathway expression to build a better LRA. Comparing these two cell types gave us a direct comparison of the pathways changed during the process of producing model cells while removing donor variability by receiving primary cell samples from the same, original donor of model cells. We added another layer to this comparison by treating both cell types with various LRAs, as well as comparing differences at baseline. We found that CDKN1A, the p21 gene,

was overexpressed in model cells at baseline and also increased in response to LRA stimulation. We also measured increased WT1 and p53 transcription factor activity in model cells. Both these factors positively regulate p21 (76). Overall, these data sets suggested a followup on activity of p21 in HIV-1 latency reversal. Since HDACi are well characterized to upregulate p21 gene expression, and do not have a supported mechanism of action on HIV-1 activity, we chose to specifically study the role of p21 in HDACi latency reversing potential.

In support of our hypothesis, the p21 inhibitor UC2288 did downregulate the latency reversing potential of HDACi in J-lats and the combination of the HDACi romidepsin with bryostatin in patient cells. All single LRAs only had modest effect on HIV-1 expression in *ex vivo* treated rCD4s, whereas a combination of the PKC agonist bryotatin with romidepsin caused more robust HIV-1 expression, consistent with previous reports (47, 63). It is likely because of the scale of this effect that we were unable to measure downregulation by UC2288 on single LRAs. Our results displaying substantial synergy of the E3 ligase inhibitor SKPinC1 with LRA treatment were unexpected, and need to be followed up on. The first step would be to test protein targets of this E3 ligase including p21 for LRA activity. We also need to confirm that p21 is in fact downregulated by SKPin C1 treatment, as p21 data from J-Lats and patient cells were inconsistent with each other.

To follow up on a role for p21 in HDACi activity, specific knockdown or overexpression experiments can be performed. Transfection with RNAi or CRISPR constructs can be used to downregulate p21 expression without off-target effects that may be present during treatment with drugs such as UC2288 or SKPin C1. If p21 has a specific role in HDACi activity, downregulation of this protein (confirmed by staining) should abrogate their effect on HIV-1 expression. p21 can also be upregulated by RNAi or CRISPR knockdown of upstream inhibitors, however this strategy is less targeted and

may affect other pathways. We have not yet used these approaches, as transfection is difficult in unactivated primary cells and can cause high levels of cellular toxicity.

Our qPCR and transcription factor screens display good validity, in that many transcription factors and pathways implicated in our screen have been previously suggested to play a role in HIV-1 expression or latency reversal. This includes the factors Sp1, NFAT, NFkB, and Hsf1 (53, 81). Other “hits” from our original screen can be followed up on in patient rCD4s, as these may respond differently than cell lines such as J-Lats. Often cell lines have many dysregulated genes, including proto-oncogenes or those involved in cell cycle arrest like p21, so any genes in these categories may be particularly important to test in primary cells. For these reasons, it might be most useful in the future to directly screen inhibitors in primary rCD4s.

Table 1: Definitions and examples of cure.

Cure type	Definition	Examples
Sterilizing cure	Complete eradication of replication-competent HIV-1	Berlin patient – following bone marrow transplant from a CCR5 delta32/delta32 donor, cART treatment was stopped and the patient has not experienced viral rebound
ART-free remission	Reduction of HIV-1 reservoir sufficient to greatly increase time to viral rebound when treatment is stopped	<p>Boston patients – 2 individuals who received bone marrow transplants and achieved complete chimerism with no detectable HIV, but rebounded 3 and 8 months after cART interruption</p> <p>Mississippi baby – perinatally infected infant with high viremia at birth, began cART very early with later discontinuation of treatment. Viral rebound 26 months after cART interruption</p>
Functional cure	Immune control over HIV-1 infection; does not necessarily require reduction or elimination of HIV-1 reservoirs	<p>Elite controllers – individuals who maintain undetectable viral load without cART due to unique immune control over HIV</p> <p>VISCONTI cohort – cART initiation during acute infection may have induced immune control in some patients, who maintain low-level viremia after cART interruption</p>

Table 2: Characteristics of HIV-1 infected participants.

patient number	age	gender	race	time on suppressive ART (months)	cART regimen ^A
1	52	M	B	36	3TC, RAL, DRV/r
2	67	M	B	32	EFV, 3TC, ABC
3	49	M	W	25	FTC, TDF, EFG/c
4	55	M	B	26	FTC, RAL, DRV/r
5	52	F	B	102	EFV, FTC, TDF
6	56	M	B	24	FTC, TDF, DTG
7	55	F	B	19	FTC, TDF, EVG/c
8	59	M	B	21	FTC, TDF, RAL
9	53	F	B	11	FTC, TDF, ATV/r
10	49	M	unknown ^B	23	3TC, RAL, DRV/r
11	62	M	B	100	FTC, DTG, DRV/r
12	34	F	B	133	FTC, TDF, EFV
13	50	M	B	40	FTC, TDF, ATV/r
14	48	F	B	49	FTC, TDF, EVG/r
15	48	F	B	90	3TC, ABC, DTG
16	56	M	B	52	EFV, FTC, TDF
18	64	M	B	12	3TC, RPV, RAL
19	53	M	B	20	FTC, TDF, RAL
20	45	F	B	39	FTC, TDF, RAL
21	43	M	B	78	EFV, FTC, TDF
22	65	F	B	123	FTC, TDF, RPV
23	69	M	B	10	3TC, ABC, DTG
24	50	M	W	155	3TC, ABC, RAL
25	62	M	B	70	3TC, ABC, TDF
26	53	M	B	18	EFV, FTC, TDF
27	37	M	B	98	FTC, TDF, RPV
28	49	M	B	93	FTC, TDF, RPV
29	44	M	W	57	3TC, ABC, ATV/r
30	44	M	B	113	3TC, ABC, DTG

^AAbbreviations: abacavir (ABC), emtricitabine (FTC), lamivudine (3TC), tenofovir disoproxil fumarate (TDF), efavirenz (EFV), ritonavir-boosted atazanavir (ATV/r), ritonavir-boosted darunavir (DRV/r), cobicistat-boosted elvitegravir (EVG/c), raltegravir (RAL), dolutegravir (DTG), rilpivirine (RPV)

^Bnot reported

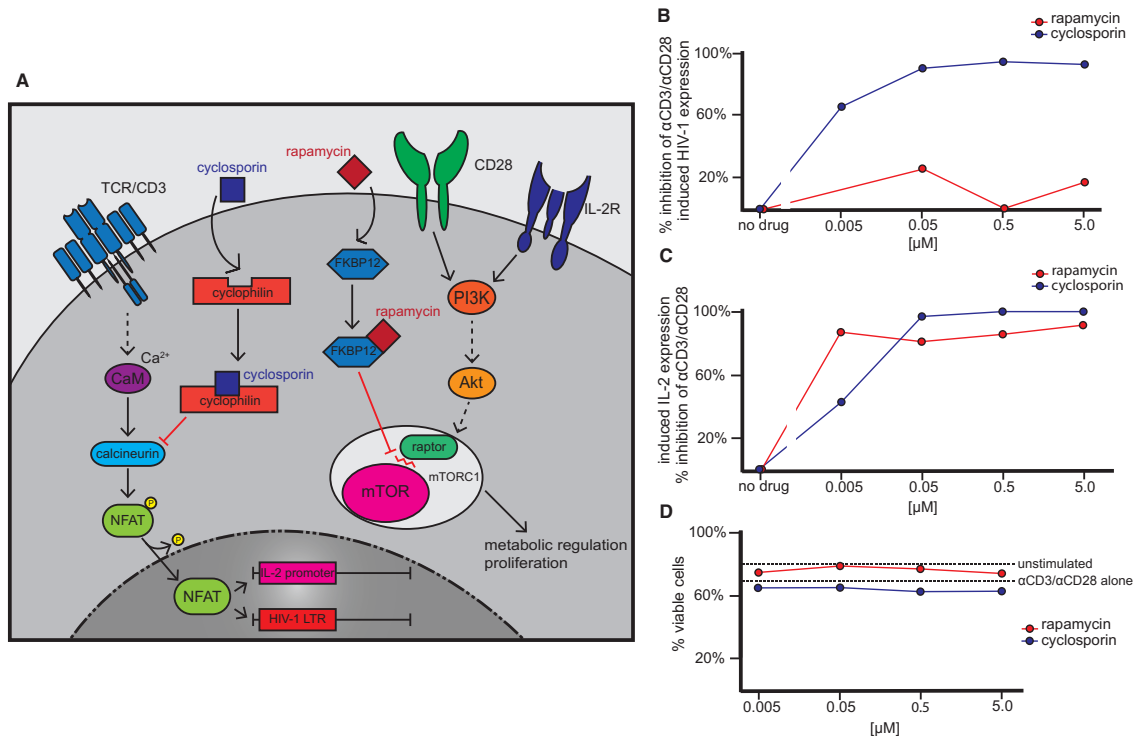


Figure 1: Immunosuppressant mechanisms and dose-response inhibition of HIV-1 or cytokine expression by rapamycin or cyclosporin treatment

A: Accepted mechanisms of action and downstream effects of rapamycin and cyclosporin.

B: RT-qPCR measurements of intracellular HIV-1 mRNA from infected individual derived rCD4s, shown as percent inhibition of $\alpha\text{CD3}/\alpha\text{CD28}$ induced HIV-1 expression by rapamycin or cyclosporin co-treatment.

C: Dose-dependent inhibition of $\alpha\text{CD3}/\alpha\text{CD28}$ induced supernatant IL-2 by increasing concentrations of rapamycin or cyclosporin.

D: Effects of rapamycin or cyclosporin viability of rCD4s stimulated with $\alpha\text{CD3}/\alpha\text{CD28}$, compared with $\alpha\text{CD3}/\alpha\text{CD28}$ treatment alone or unstimulated cells.

Data points are the average of duplicate experiment conditions.

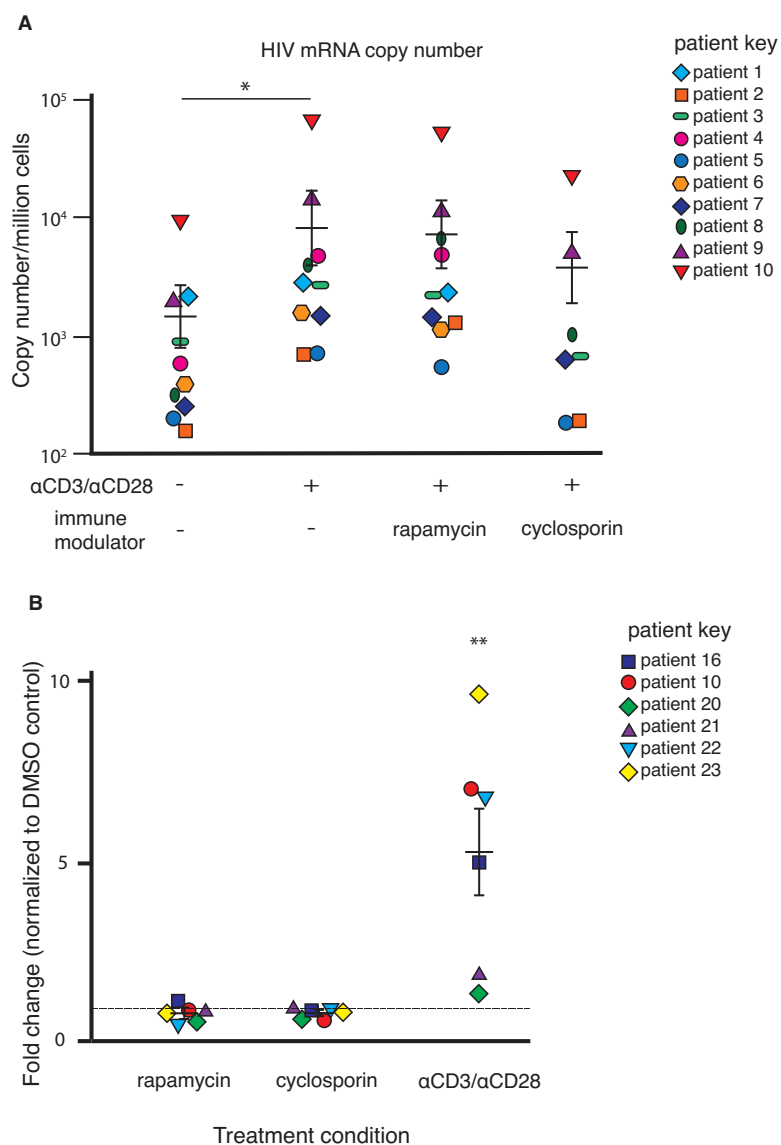


Figure 2: RT-qPCR results for various immunosuppressant treatments.

A: RT-qPCR results for intracellular HIV-1 mRNA in rCD4s purified from infected individuals on cART. Copy number represents copies of HIV-1 mRNA detected per million cells. Symbols represent results from different donors (n=10).

B: Intracellular HIV-1 mRNA levels from cells treated with immunosuppressants alone. Results are shown as fold change over the no stimulation control. Dotted line represents no change from baseline (DMSO alone). Each symbol represents results from a different donor (n=6).

Data points are the average of duplicate experiment conditions. Error bars represent mean +/- SEM. (*=p < 0.05, **=p < 0.01)

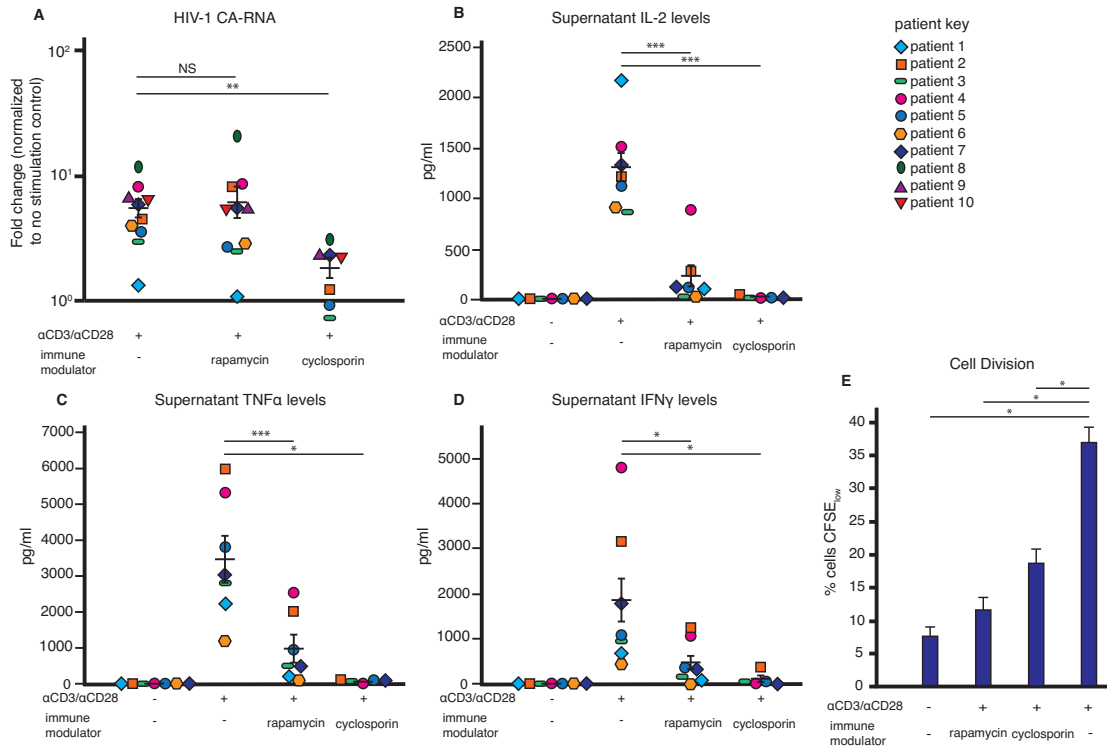


Figure 3: Effects of immunosuppressants on α CD3/ α CD28 mediated HIV-1 expression and pro-inflammatory cytokine release from infected rCD4s.

A: Relative amounts of induced HIV-1 mRNA shown as fold change relative to no stimulation (DMSO alone). All samples represent RT-qPCR measurements of intracellular RNA from infected individual derived rCD4s (n=10).

B: Effect of immunosuppressants on α CD3/ α CD28 induced rCD4 production of proinflammatory cytokine IL-2. Points represent supernatant samples from experiments described in Figure 2A (n=7).

C: Effect of immunosuppressants on α CD3/ α CD28 induced IFN γ release. (n=7).

D: Effect of immunosuppressants on α CD3/ α CD28 induced TNF α release. (n=7).

E: Effect of immunosuppressant co-treatment on T-cell proliferation. Healthy donor PBMC (n=3) were stained with CFSE before stimulation.

(*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$)

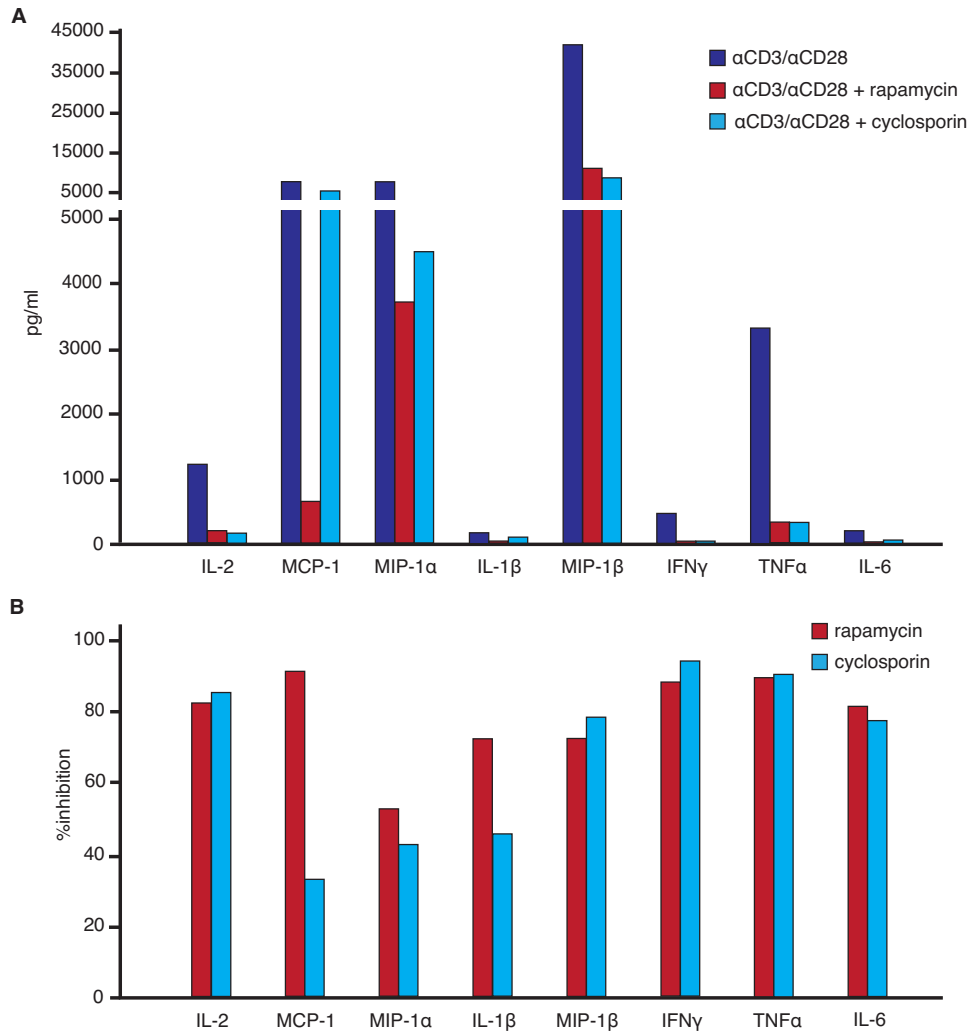


Figure 4: aCD3/aCD28 induced cytokine release by PBMCs is downregulated by immunosuppressant treatment.

A: Quantification of cytokines induced by aCD3/aCD28 alone or cotreatment with rapamycin or cyclosporin. Induced cytokines: IL-2, MCP-1, MIP-1 α , IL-1 β , MIP-1 β , IFN γ , TNF α , IL-6. Bars represent the mean of 3 separate experiments.

B: Inhibition of aCD3/aCD28 induced cytokines by cotreatment with rapamycin or cyclosporin (n=3).

Figure 5: Immunosuppressant effects on cell surface markers and cell viability; representative flow cytometry plots.

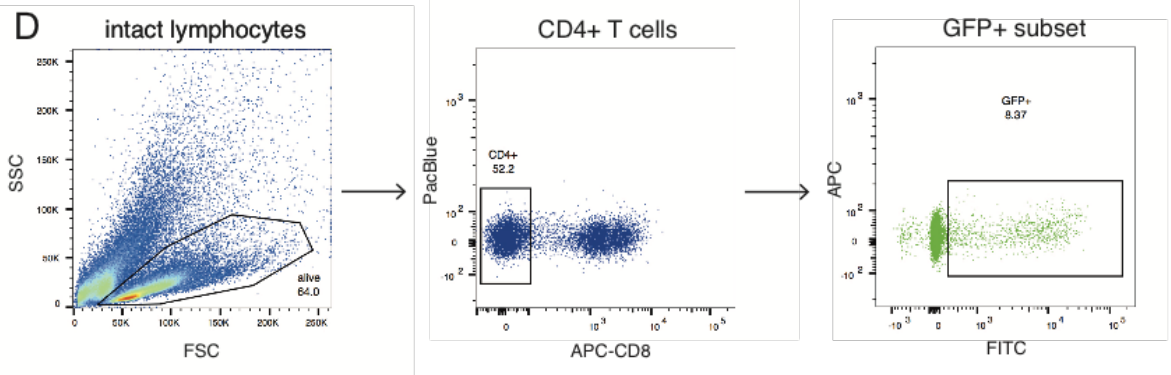
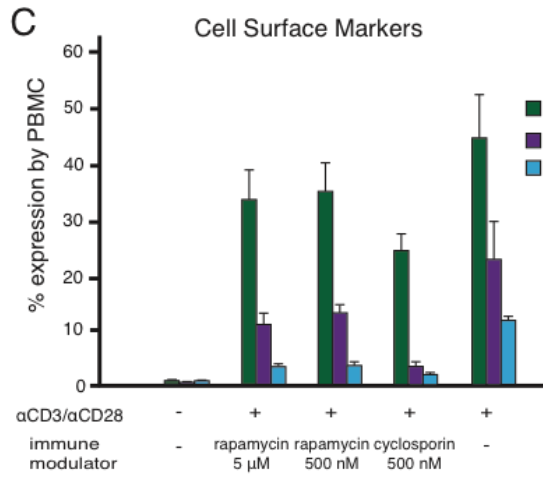
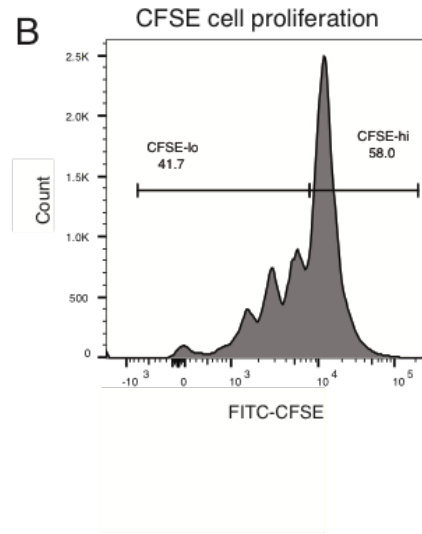
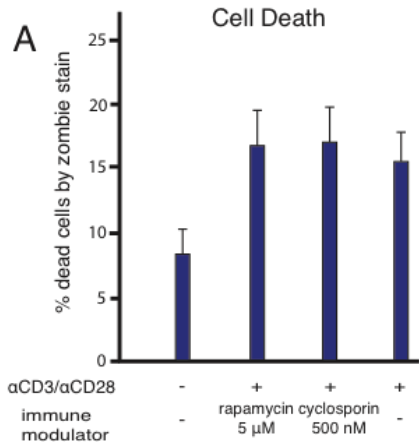
A: Activation and exhaustion cell surface markers. PBMC isolated from patients (n=3) were treated for 24 hours for the indicated conditions before cell surface marker measurement with flow cytometry.

B: Effect of immunosuppressant cotreatment on cell viability. PBMC isolated from healthy donors (n=4) were treated according 24 hours with the indicated drugs, and viability assessed using Zombie Aqua.

C: Representative example of flow cytometry gating for cellular proliferation analysis. Healthy donor derived PBMC (n=3) were stained with CFSE and treated for 24 hours as shown.

D: Representative example of flow cytometry gating for CTL killing assay. Cells were cocultured for 3 days then stained with CD8-APC to gate CD4⁺ T cells (APC-). % GFP⁺ was calculated as a subset of total CD4⁺ T cells. Fluorescent markers were gated against fluorescent channels to exclude autofluorescent cells.

Data points are the average of duplicate experiment conditions. Error bars represent mean +/- SEM.



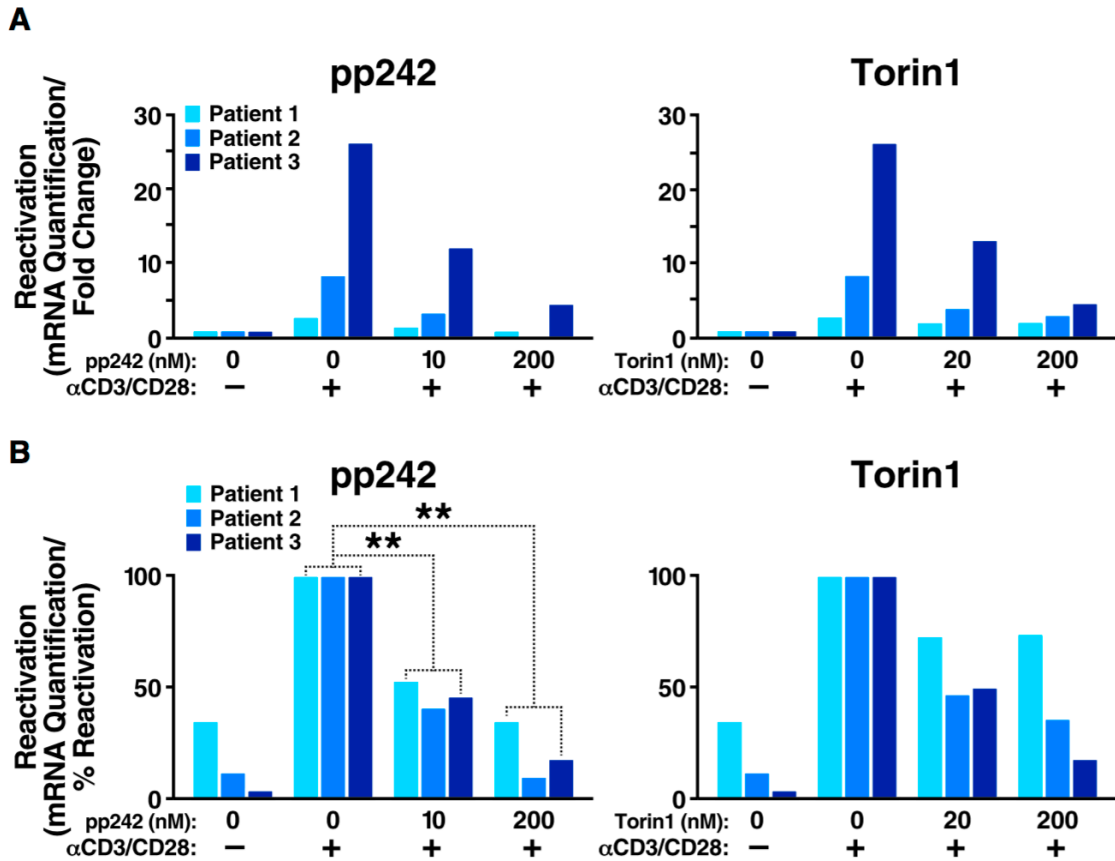


Figure 6: pan-mTOR inhibitors downregulate αCD3/αCD28 mediated HIV-1 expression
A: Dose-response inhibition of HIV-1 expression shown as fold change to highlight inter-patient variation. Data points represent duplicate experiment conditions.
B: Inhibition of αCD3/αCD28 mediated HIV-1 expression by pp242 or Torin1, displayed as % max normalized to αCD3/αCD28 alone. (*=p < 0.05, **=p < 0.01)

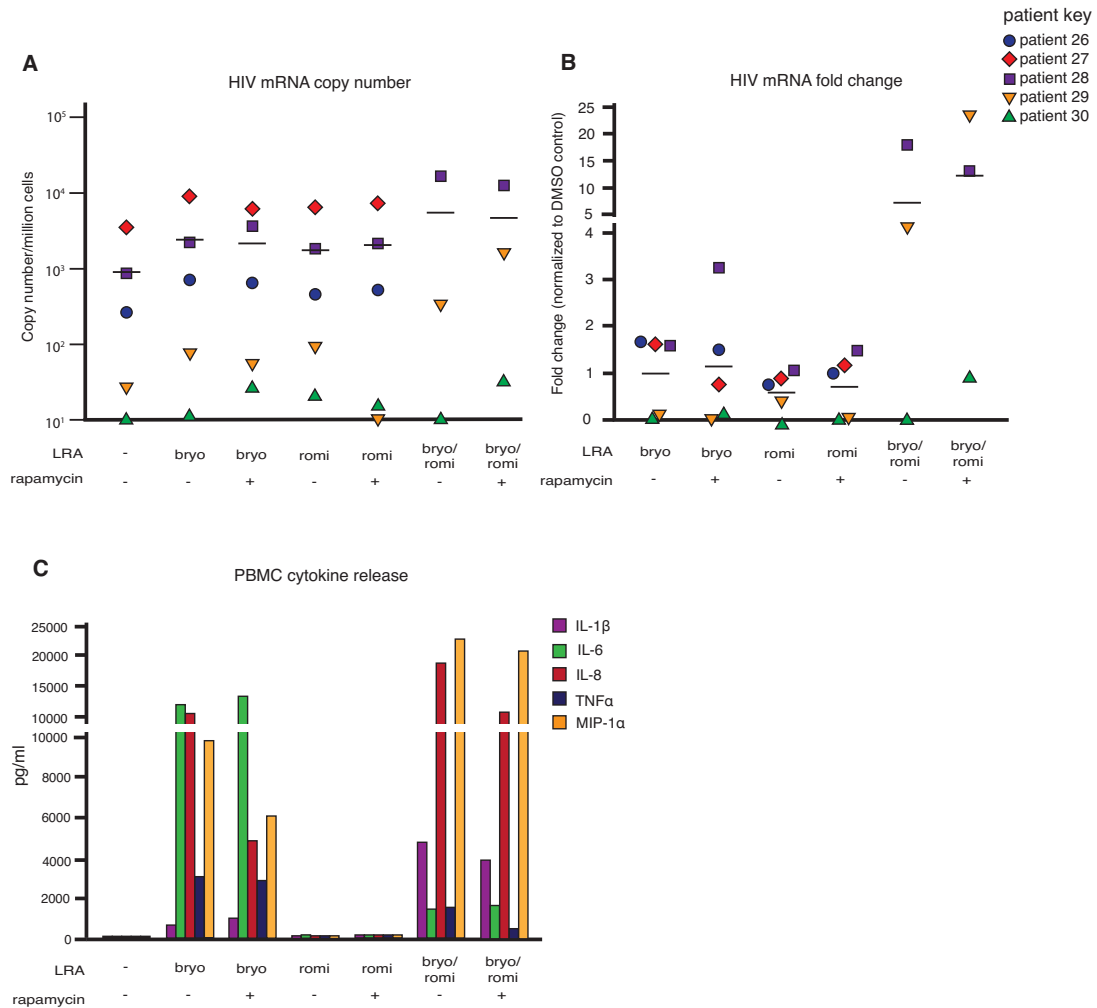


Figure 7: The effect of rapamycin on LRA induction of HIV-1 mRNA and PBMC cytokine release.

A: RT-qPCR measurements of intracellular HIV-1 mRNA in rCD4s purified from infected individuals on cART, shown as copies of HIV-1 mRNA detected per million cells. Symbols represent results from different donors (n=5). Treatments include bryostatin-1 (bryo), romidepsin (romi), a combination of bryostatin-1 and romidepsin (bryo/romi), all with or without rapamycin. Data points are the average of duplicate experiment conditions.

B: Relative amounts of induced HIV-1 mRNA from A shown as fold change relative to no stimulation (DMSO alone). Data points are the average of duplicate experiment conditions.

C: Effect of immunosuppressants on LRA induced production of cytokines by healthy donor PBMC. Cytokines shown have been previously suggested to be associated with bryostatin administration to patients. Bars represent the mean of 3 separate experiments.

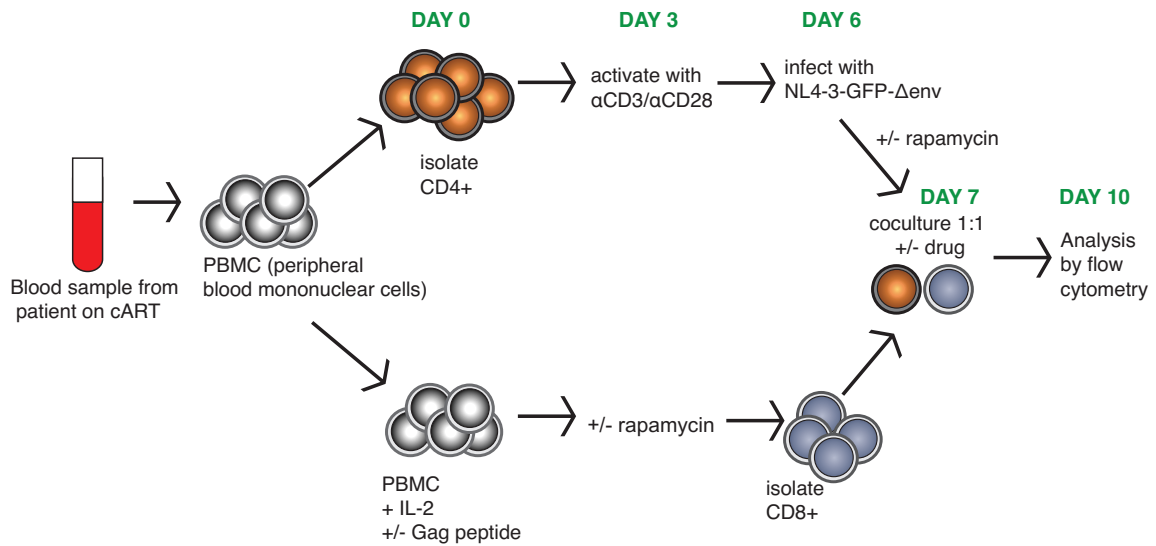


Figure 8: CTL killing assay schematic

Whole blood was collected from HIV-1 infected individuals on suppressive cART and PBMC purified. PBMC samples were split in half; one half was purified for CD4⁺ T cells and infected with NL4-3- Δ env-GFP for flow cytometry readout. The other half of PBMCs was prestimulated with IL-2 alone or plus gag peptide mixture for 6 days, then purified for CD8⁺ T cells. Cells were cocultured at a 1:1 E:T ratio and analyzed via flow cytometry.

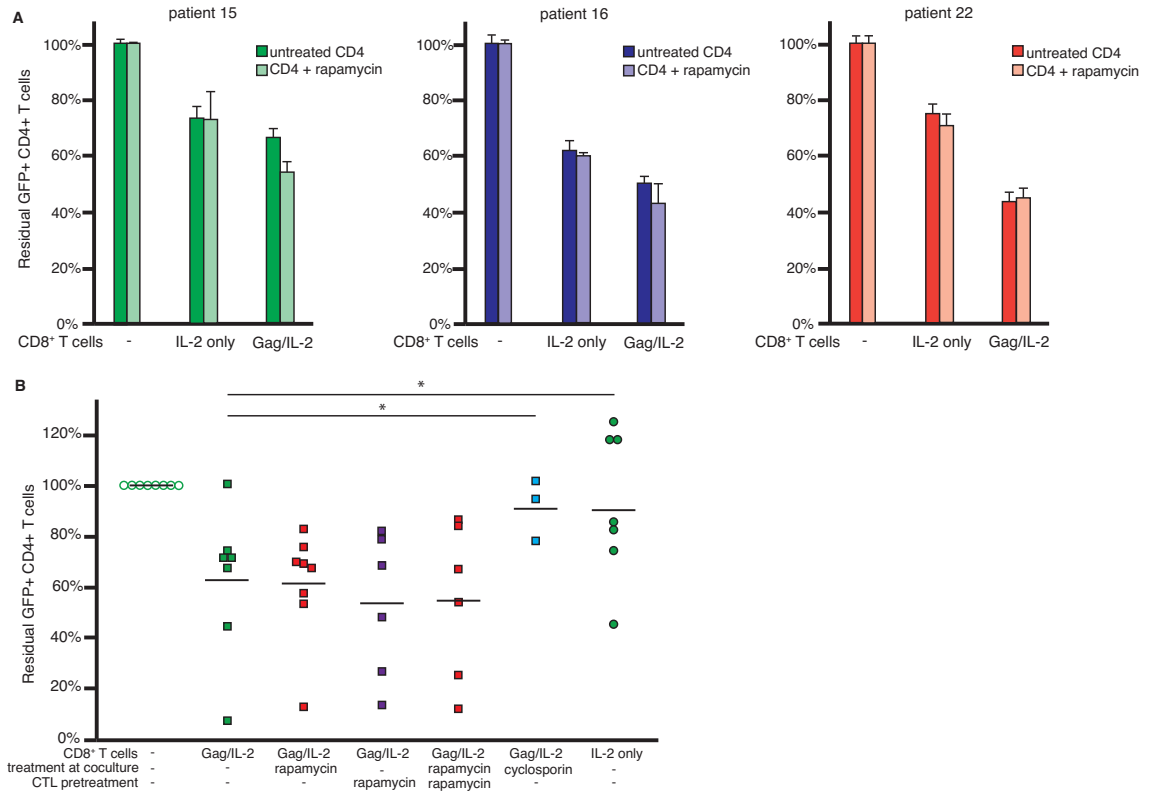


Figure 9: Rapamycin treatment does not reduce CTL killing.

A: Rapamycin does not affect viral peptide production and presentation of epitopes to CTLs (n=3). Activated CD4⁺ T-cells were infected with NL4-3-Δenv-GFP and cultured in media alone, or with rapamycin for 24 hours before coculture. Infected CD4⁺ were cocultured at a 1:1 E:T ratio with CD8⁺ T-cells prestimulated with IL-2 alone or Gag and IL-2. After 72 hours, reduction in infected cells was measured by flow cytometry.

B: CTL mediated killing of HIV-1 infected cells is impaired by the presence of cyclosporin, but not rapamycin (n=8). As above, CD4⁺ T-cells were infected with NL4-3-Δenv-GFP. Infected CD4⁺ were cultured alone, or cocultured at a 1:1 E:T ratio with CD8⁺ T-cells. As indicated, some CD8⁺ were pre-treated with rapamycin for 3 days before coculture, or cocultured in the presence of rapamycin or cyclosporin.

Data points are the average of duplicate experiment conditions. Two-tailed paired student's t-test was used to determine statistical significance. (*=p < 0.05)

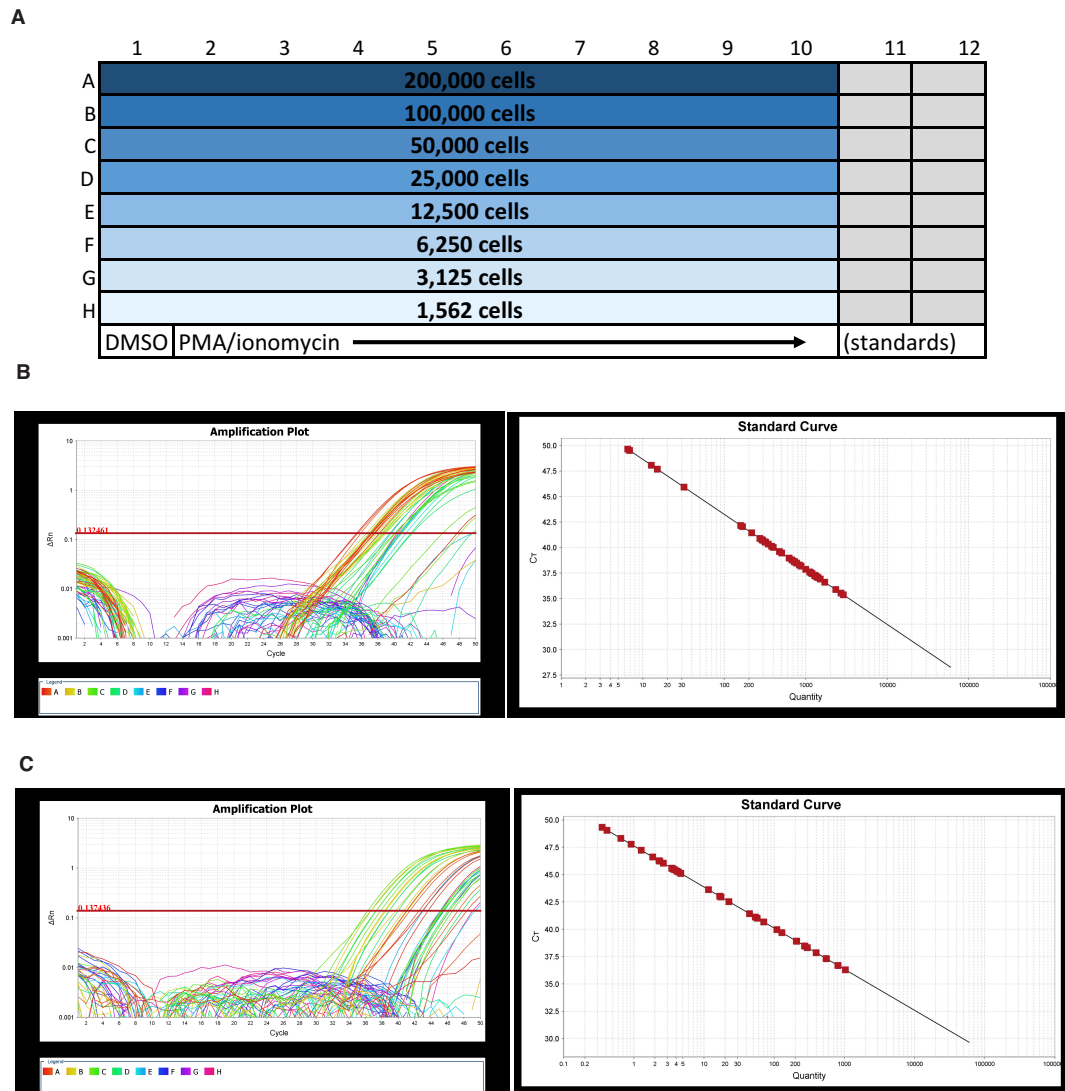


Figure 10: QVIA assay schematic and representative qPCR plots

A: QVIA, the quantitative viral induction assay, requires only 4×10^6 CD4⁺ T cells to set up. Cells are plated starting at 200K in row A, and subsequently diluted 2x down to row H. Columns 11 and 12 are left empty for later qPCR standards.

B: A representative qPCR amplification plot and standard curve representing QVIA data. QVIA readout is characterized by a gap in Ct (cycle threshold) values for at least 2 cycles. Anything below this gap is considered negative: it is likely either a non-induced provirus or a defective provirus that is not fully transcription-competent.

C: A representative qPCR amplification plot and standard curve representing QVIA data. Here the gap is less obvious on the amplification plot, but can be clearly seen on the standard curve.

A

	DMSO	PMA/iono	PMA/iono	PMA/iono	PMA/iono	PMA/iono	PMA/iono	PMA/iono	PMA/iono	PMA/iono
cell count	1	2	3	4	5	6	7	8	9	10
200000	A									
100000	B									
50000	C									
25000	D									
12500	E									
6250	F									
3125	G									
1562.5	H									

B

	DMSO	PMA/iono	PMA/iono	PMA/iono	PMA/iono	PMA/iono	PMA/iono	PMA/iono	PMA/iono	PMA/iono
cell count	1	2	3	4	5	6	7	8	9	10
200000	A									
100000	B									
50000	C									
25000	D									
12500	E									
6250	F									
3125	G									
1562.5	H									

Figure 11: Measuring the intra-assay variability of QVIA.

Two plates were set up from the same patient, same blood draw; separately: to measure the intra-assay variability of QVIA. Red wells are qPCR positive.

A. Inducible proviruses per million (IPPM) = 6.654

B. IPPM = 6.243

patient ID	baseline	week 13	week 26	donor HIV status	transplant type	date of transplantation
10577_R	10.634	25.381	37.902	positive	liver	3/19/16
83234_R	1.473	0.193	0.193	negative	kidney	3/19/16
34246_R	0.858	10.098	2.683	negative	kidney	4/19/16
19427_R	34.377	42.62		negative	liver	5/9/16
34368_R	5.484	11.238		negative	kidney	5/10/16
95475_R	2.318	7.835		negative	liver	6/9/16
27098_R	5.496	23.48		positive	kidney	7/13/16
83302_R	0.869	1.192		negative	kidney	8/3/16
average	7.688625	15.254625				

Table 3: Transplant patient characteristics.

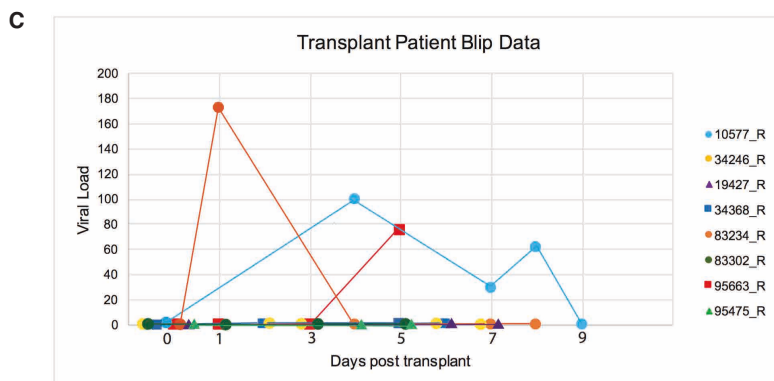
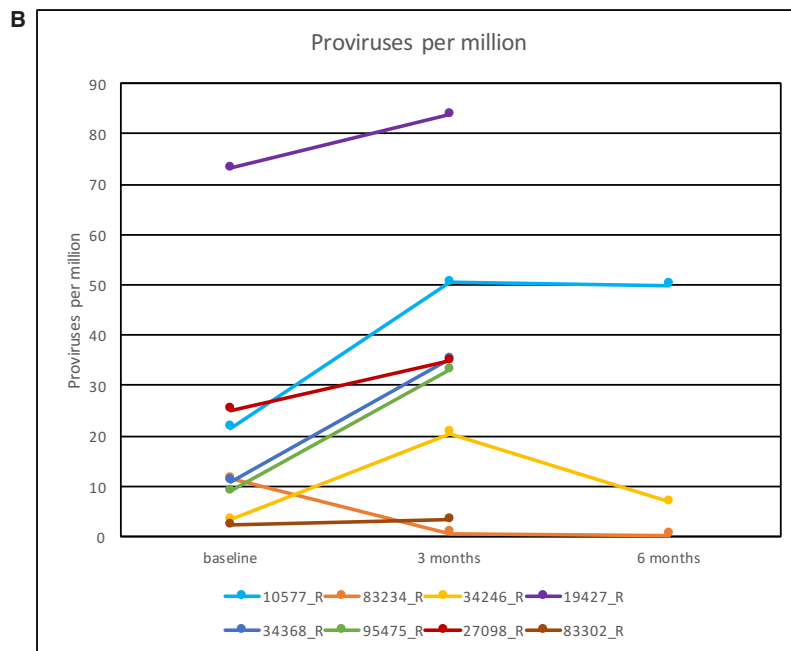
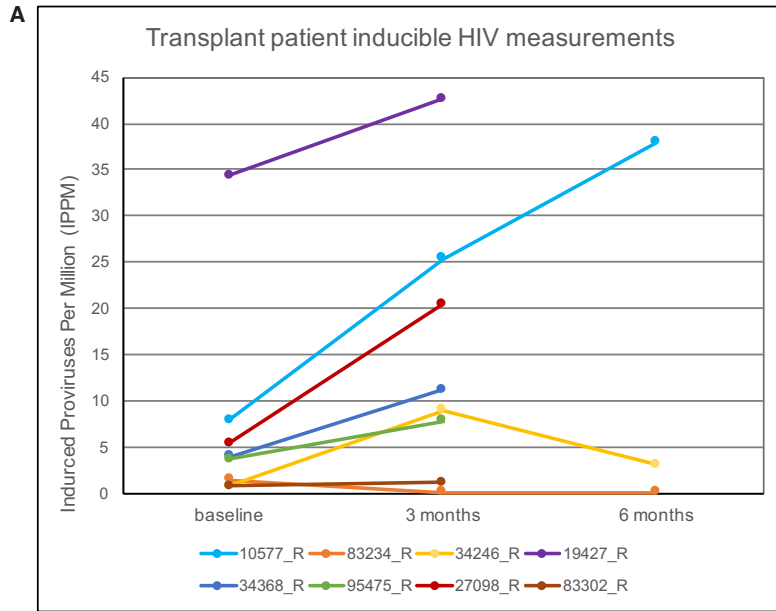
IPPM measurements, donor HIV status, transplant type, and date of transplantation for patients with 13 and 26-week followup data.

Figure 12: Transplant patient reservoir measurements

A: Induced proviruses per million (IPPM) measurements pre-transplant (baseline), and 13 and 26 weeks post-transplant.

B: Total proviruses per million (PPM) in each sample from 11A.

C: Measurements of increases in viral load above the clinical limit of detection (“blips”) within 10 days post-transplant. Not all patients or timepoints are represented due to limited availability of samples.



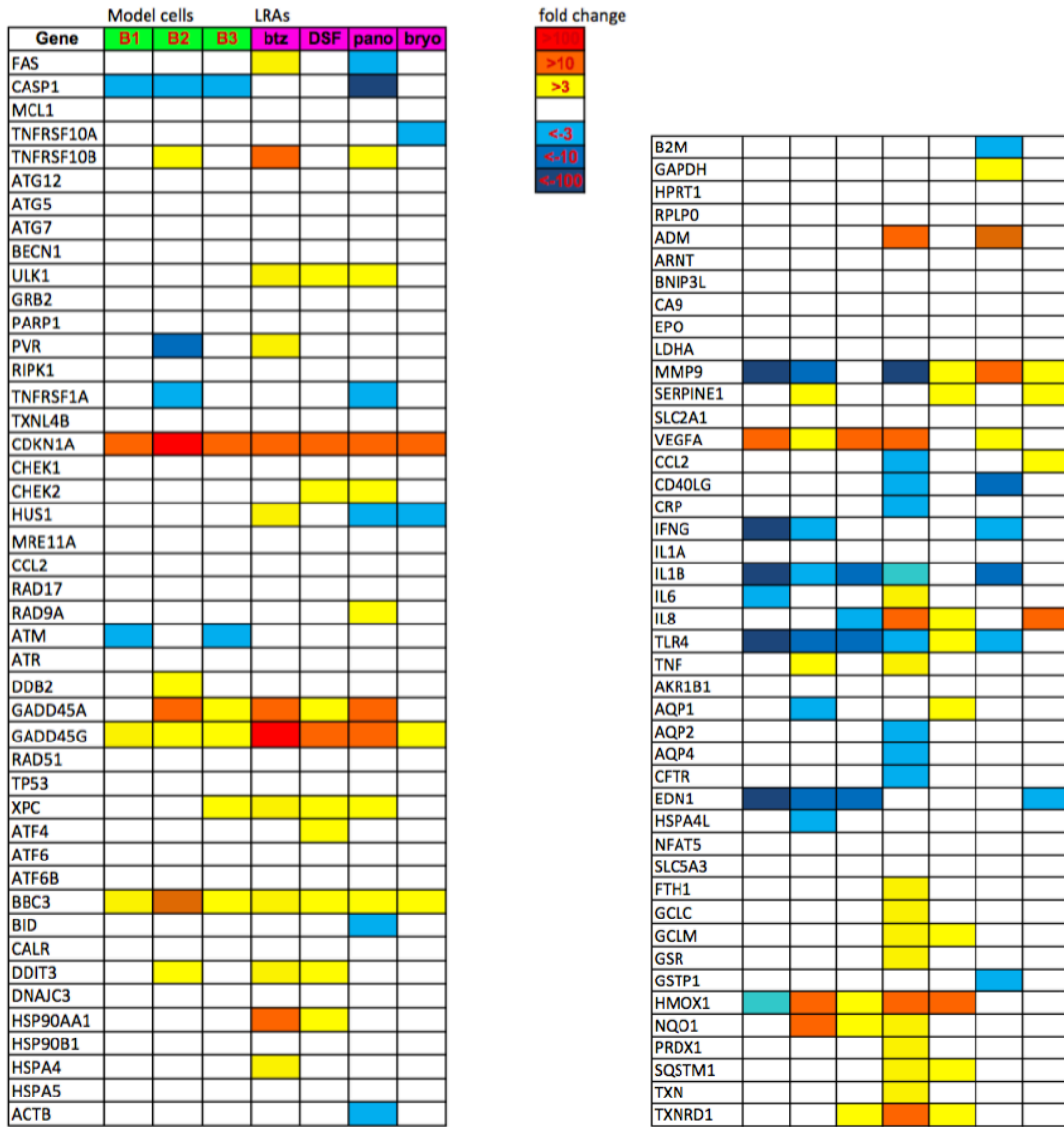
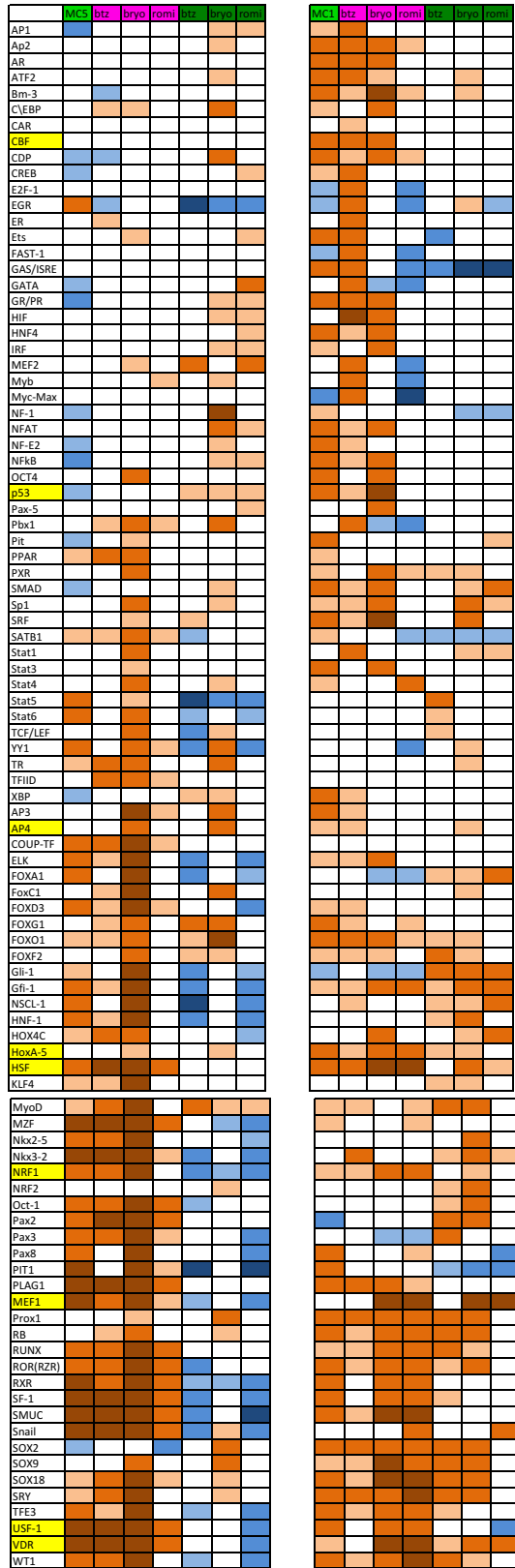


Figure 13: qPCR array heat-map.

Colors represent differences in gene expression in model cells or in response to LRA stimulation in rCD4s. For model cells, changes are shown as model cells/rCD4. For LRA conditions, changes are shown as LRA treated/unstimulated. Model cells are represented in batches as B1, B2, and B3. Abbreviations: bortezomib = btz, disulfiram = DSF, panobinostat = pano, and bryostatin = bryo.

Figure 14: Transcription factor array heat map.

Freshly isolated rCD4s were compared to model cells at baseline and post-LRA treatment. Nuclear extracts were used for transcription factor analysis and comparison. Blue represents downregulation, orange represents upregulation, and colors darken with greater changes. Transcription factors of interest are highlighted. Abbreviations: bortezomib = btz, panobinostat = pano, and bryostatin = bryo.



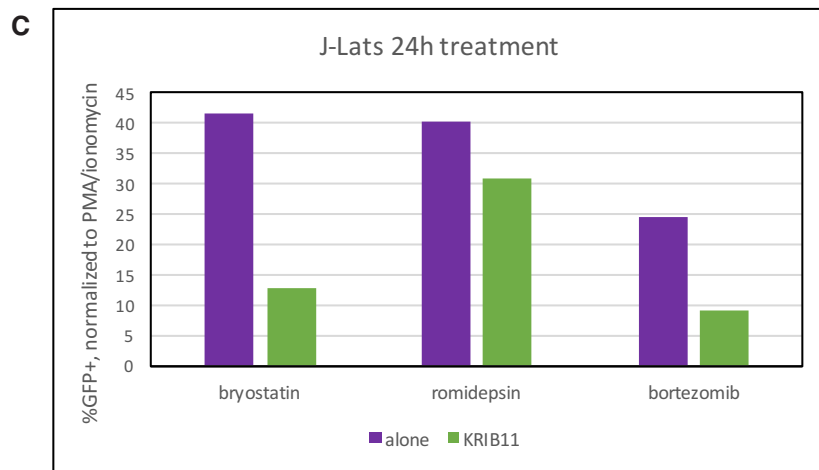
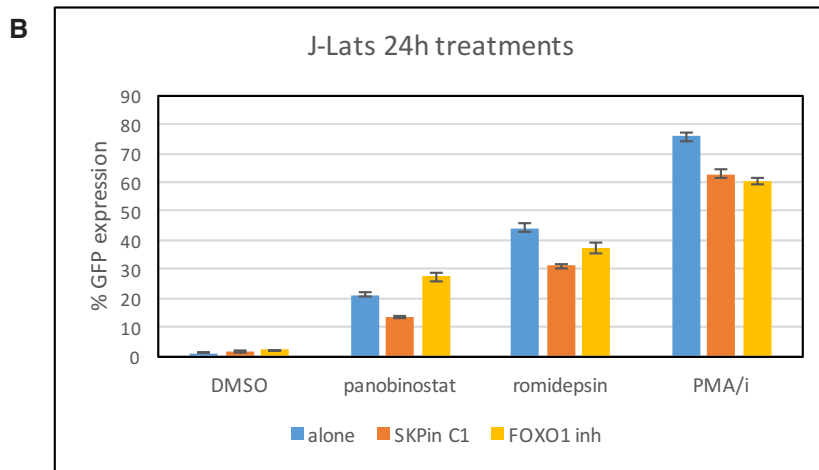
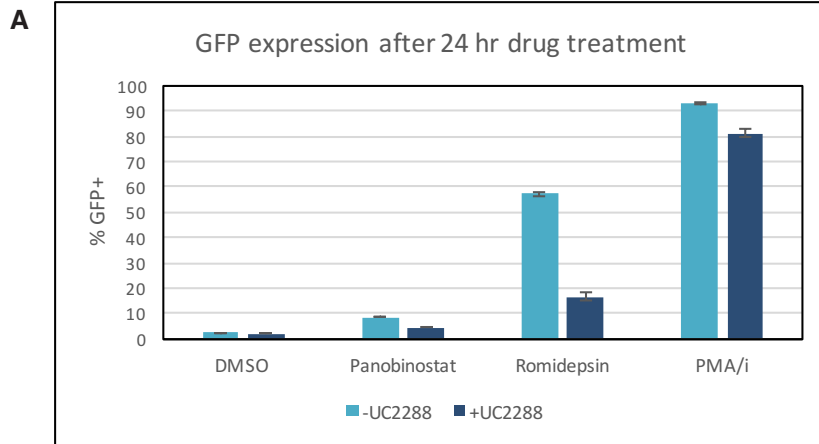
model cells compared to rCD4 at baseline
 rCD4, post LRA treatment
 model cells, post LRA treatment

Figure 15: J-Lat 10.6 cells co-treated with LRAs and modulators of pathways identified as "hits".

A: Co-treatment of HDACis panobinostat or romidepsin with UC2288, a specific p21 inhibitor.

B: Treatment of J-Lats with LRAs alone or with SKPin C1, a p27 activator, or a specific FOXO1 inhibitor.

C: J-Lats treated with LRAs with or without KRIB11, an inhibitor of the heat shock pathway. Percent GFP+ is normalized such that 0% = DMSO alone, and 100% = PMA/ionomycin treatment.



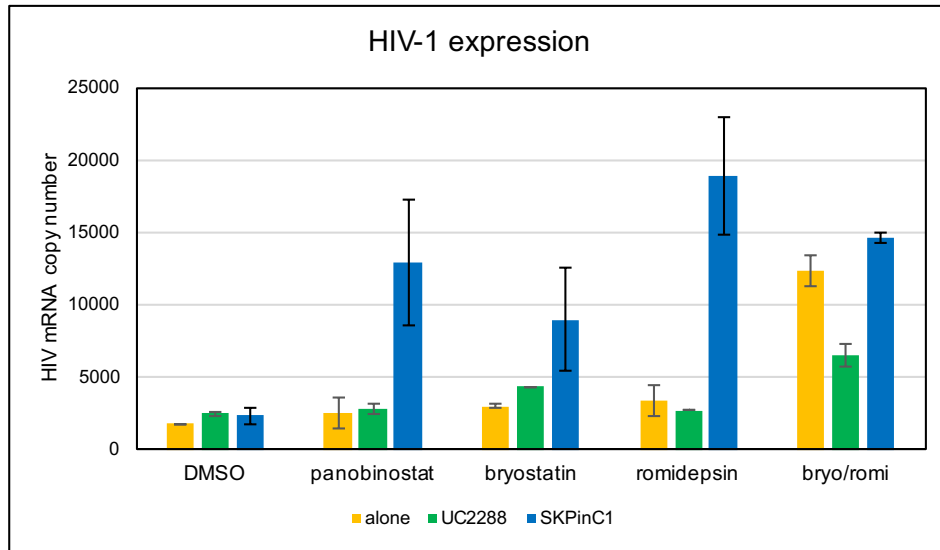
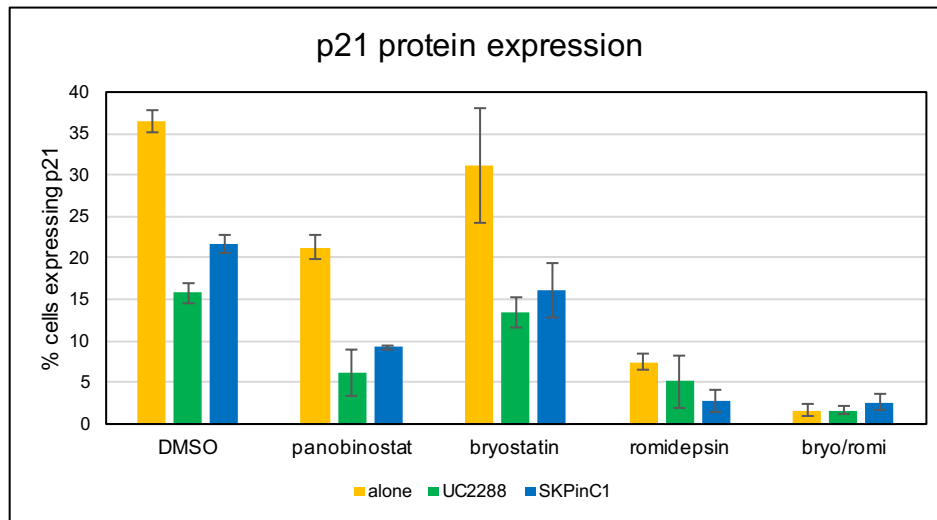
A**B**

Figure 16: HIV-1 mRNA and p21 expression analysis in ex vivo treated rCD4s.

A: SKPinC1 exhibits synergy with single LRA treatment; UC2288 inhibits HIV-1 expression in response to a combination of bryostatin and romidepsin treatment. Treatments were done on rCD4s from HIV-1 suppressed patients; data points represent duplicate experiment conditions.

B: UC2288 and SKPinC1 downregulate p21 expression in rCD4s. Data points represent samples from the same wells as in 15A.

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EDUCATION

The Johns Hopkins University School of Medicine Baltimore, MD
August 2012 – February 2017
PhD, Pharmacology and Molecular Sciences

Emory University Atlanta, GA
August 2009 – May 2012
Bachelor of Science, Biology; Bachelor of Arts, Psychology
GPA 3.55

Cornell University Ithaca, NY
August 2008 – May 2009
GPA: 3.4

RESEARCH EXPERIENCE

Laboratory of Dr. Robert Siliciano, Department of Medicine, Johns Hopkins University School of Medicine
May 2013 – present

- Demonstration of a novel use for rapamycin as an immune modulator during reactivation of latent HIV-1 expression by *ex vivo* treatment of primary cells
- Development of a novel limiting dilution qPCR based assay to measure the size of the latent HIV-1 reservoir in infected patients, including optimization and validation
- Characterization of a primary-cell model for HIV-1 latency; including screening of potential pharmacologic latency reversing agents, modulation of transcription factors to optimize effective HIV-1 induction, and identification of a novel HIV latency reversal mechanism
- Management of multiple inter- and intra-institution research collaborations

Centers for Disease Control and Prevention, Division of Viral Hepatitis, Atlanta, GA
June 2011 – May 2012

- Development of a novel qPCR assay for hepatitis D virus; including sequence analysis, primer design, assay optimization, testing and analysis of clinical samples

Laboratory of Dr. Steven L'Hernault, Biology Department, Emory University
September 2010 – May 2012

- Genetics and development of *C. elegans*, including performing genetic crosses of various strains and development of transgenic organisms

MENTORING

Sarah Jang, undergraduate student, Johns Hopkins University *01/2014-present*

- Role: Primary advisor during undergraduate research

Abena Kwaa, PhD student, Johns Hopkins School of Medicine *08/2015-12/2015*

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Tutor for Molecular Biology and Genomics *10/2013-12/2013*

RESEARCH SKILLS

Molecular Biology & Biochemistry

- DNA and RNA isolation, qRT-PCR, western blotting, gel electrophoresis, gel extraction and DNA sequencing, molecular cloning, luciferase-based assays, CRISPR genome editing

Cell Biology

- Primary-cell and cell-line culture and maintenance, magnetic based cellular isolations, eukaryotic transfection including lipofection and electroporation

Immunology & Virology

- Multicolor flow-cytometry, cell sorting, ELISA, IHC, viral genome editing, viral transductions, virus concentration and purification, multiplex cytokine assays, BSL-2/3 virus handling and safety

Organismal Biology

- *C. elegans* maintenance and genetics

PUBLICATIONS

Martin AR, Pollack RA, Capoferri A, Ambinder RF, Durand CM, Siliciano RF. *Rapamycin-mediated mTOR inhibition uncouples HIV-1 latency reversal from cytokine-associated toxicity*. The Journal of Clinical Investigation, Feb 2017.

Besnard E*, Hakre S*, Kampmann M*, Lim HW, Hosmane NN, **Martin A**, Lin H, Bassik MC, Verschueren E, Chan J, Svensson JP, Gramatica A, Conrad RJ, Ott M, Greene WC, Krogan NJ, Siliciano RF, Weissman JS, Verdin E. *The mTOR complex controls HIV latency*. Cell Host and Microbe, Dec 2016.

Martin AR and Siliciano RF. *Progress toward HIV eradication: case reports, current efforts, and the challenges associated with cure*. Annual Reviews of Medicine, Jan 2016. [Review]

Laird GM*, Bullen CK*, Rosenbloom DI, **Martin AR**, Hill AL, Durand CM, Siliciano JD, Siliciano RF. *Ex vivo analysis identifies effective HIV-1 latency-reversing drug combinations*. The Journal of Clinical Investigation, May 2015.

Kodani M, **Martin A**, Mixson-Hayden T, Drobeniuc J, Gish RR, Kamili S. *One-step real-time PCR assay for detection and quantitation of hepatitis D virus RNA*. Journal of Virological Methods, Nov 2013.

Under review:

Pollack RA, Jones RB, Perteza M, Bruner KM, **Martin AR**, Thomas AS, Capoferri AA, Huang S, Karandish S, Hao H, Halper-Stromberg E, Yong P, Kovacs C, Benko E, Siliciano RF, Ho Y. *Expression of defective HIV-1 proviruses impairs reservoir measurement and elimination*.

(*co-first author)

PRESENTATIONS & AWARDS

Martin AR, Redd AD, Laird GM, Siliciano RF, Brown D, Ostrander D, Quinn T, Segev D, Tobian A, Durand CM. *Inducible HIV-1 latent reservoir increases after HIV+ or HIV- solid organ transplant*. Conference on Retroviruses and Opportunistic Infections (CROI), Seattle, WA, February 2017.

- Young Investigator Scholarship

Martin AR, Pollack RA, Capoferri A, Siliciano RF. *Immune modulation with rapamycin downregulates toxic effects of T cell activation without impairing HIV-1 latency reversal and CTL response*. Keystone Symposia Conference on HIV Persistence: Pathogenesis and Eradication, Olympic Valley, CA, March 2016.

- Keystone Symposia Travel Scholarship

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