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Rapamycin-mediated mTOR inhibition uncouples HIV-1 latency reversal from cytokine-associated toxicity

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Current strategies for HIV-1 eradication require the reactivation of latent HIV-1 in resting CD4+ T cells (rCD4s). Global T cell activation is a well-characterized means of inducing HIV-1 transcription, but is considered too toxic for clinical applications. Here, we have explored a strategy that involves a combination of immune activation and the immunosuppressive mTOR inhibitor rapamycin. In purified rCD4s from HIV-1–infected individuals on antiretroviral therapy, rapamycin treatment downregulated markers of toxicity, including proinflammatory cytokine release and cellular proliferation that were induced after potent T cell activation using αCD3/αCD28 antibodies. Using an ex vivo assay for HIV-1 mRNA, we demonstrated that despite this immunomodulatory effect, rapamycin did not affect HIV-1 gene expression induced by T cell activation in these rCD4s. In contrast, treating activated rCD4s with the immunosuppressant cyclosporin, a calcineurin inhibitor, robustly inhibited HIV-1 reactivation. Importantly, rapamycin treatment did not impair cytotoxic T lymphocyte (CTL) recognition and killing of infected cells. These findings raise the possibility of using rapamycin in conjunction with T cell–activating agents in HIV-1 cure strategies.

Introduction

HIV-1 persists in a latent reservoir in resting memory CD4+ T cells despite combination antiretroviral therapy (cART) (1–3). 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 (4) involves reactivation of proviral expression in latently infected cells to allow their elimination by immune mechanisms including CD8+ cytotoxic T lymphocytes (CTLs) (5). Agents eliciting global T cell activation were used in the original detection of the reservoir (1, 2) and effectively reverse latency. However, in clinical settings they induce severe adverse reactions by causing systemic release of proinflammatory cytokines (6, 7). 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 (6), this approach has been abandoned in favor of latency-reversing agents (LRAs) that induce HIV-1 transcription without cellular activation. No single LRA reactivates HIV-1 gene expression to levels induced by T cell activation (8). The most effective single agents are PKC agonists, which affect a subset of the pathways stimulated by T cell activation (9). Thus, effective latency reversal may require some immune activation. Here, we explore the idea that immunomodulatory compounds in conjunction with T cell activation may limit toxicity while allowing maximal reactivation of latent HIV-1.

Conflict of interest: The authors have declared that no conflict of interest exists.
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Adverse reactions elicited by the activation of large numbers of T cells are typically attributed to "cytokine storm" (6). A classic example is toxic shock syndrome, caused by a staphylococcal superantigen that activates all cells expressing a Vβ2 T cell receptor (TCR) (about 10% of total T cells) (10). 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 (11).

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 because of similarities between the HIV-1 long-terminal repeat (LTR) and the promoters of key genes upregulated by T cell activation, including *IL2* (12). The widely used immunosuppressant cyclosporin inhibits the calcium-dependent phosphatase calcineurin, blocking nuclear translocation of the transcription factor NFAT, which acts on the *IL2* promoter and the HIV-1 LTR (13). Cyclosporin inhibits HIV-1 expression in a cell line model of latency (14).

To avoid inhibition of transcription factors that act on 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 (15). 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 (ref. 15 and

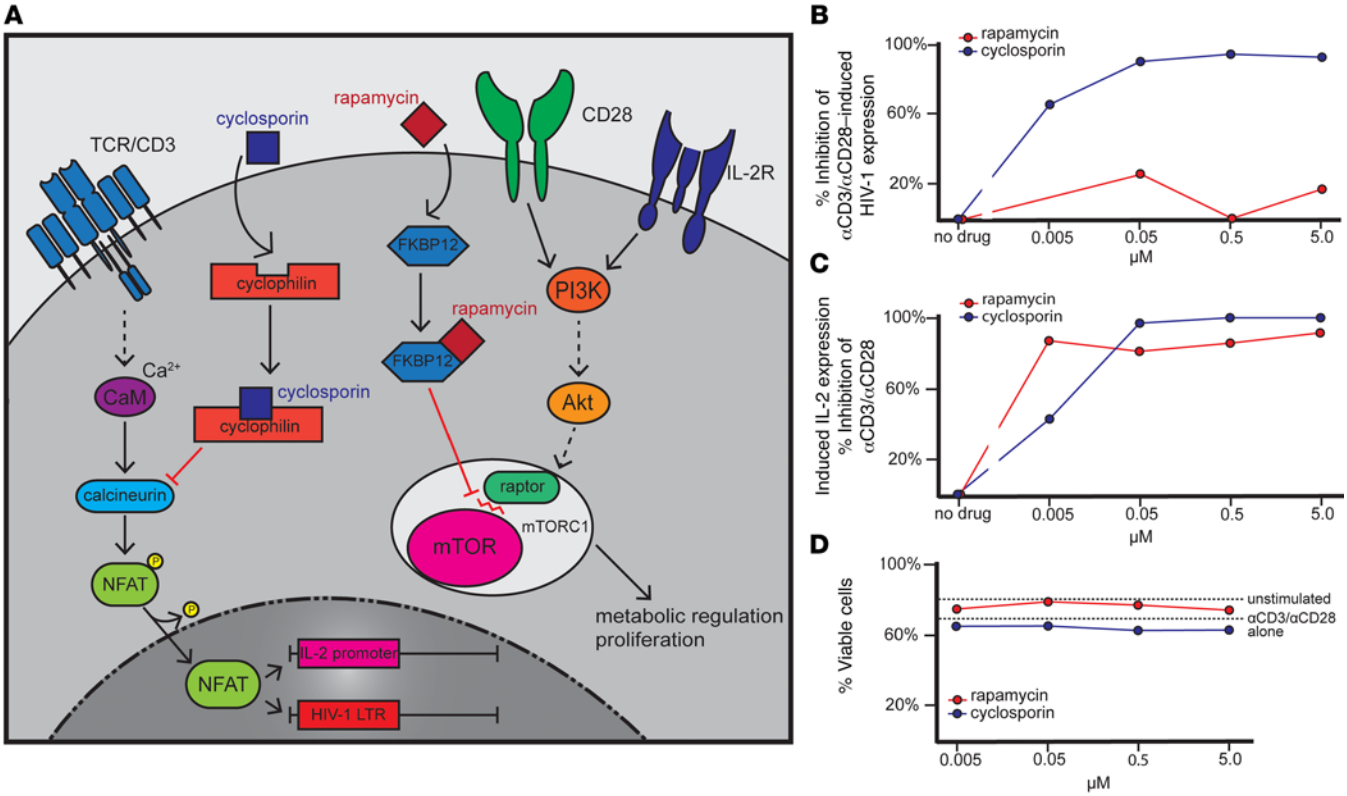

Figure 1. Immunosuppressant mechanisms and dose-response inhibition of HIV-1 or cytokine expression by rapamycin or cyclosporin treatment. (**A**) Accepted mechanisms of action and downstream effects of rapamycin and cyclosporin. (**B**) qRT-PCR measurements of intracellular HIV-1 mRNA from rCD4s derived from infected individuals, shown as percentage inhibition of αCD3/αCD28–induced HIV-1 expression by rapamycin or cyclosporin cotreatment. (**C**) Dose-dependent inhibition of αCD3/αCD28–induced supernatant IL-2 by increasing concentrations of rapamycin or cyclosporin. (**D**) Effects of rapamycin or cyclosporin viability of rCD4s stimulated with αCD3/αCD28, compared with αCD3/αCD28 treatment alone or unstimulated cells. Data points are the average of duplicate experiment conditions.

Figure 1A). Importantly, rapamycin does not affect mTOR complex 2 (mTORC2), which positively regulates PKC activation and downstream pathways in T cell activation (15, 16). It has been used in HIV-1–infected patients undergoing solid-organ and bone marrow transplantation (17). Rapamycin also protects mice from death after superantigen challenge (18). 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, may interfere with CTL killing (19). Cyclosporin may also inhibit CTL function owing to a dependence of CTL degranulation on calcium influx and downstream signaling (20). In contrast, rapamycin has a positive effect on the memory $CD8^+$ T cell response to viral infection (21). We tested whether rapamycin interferes with CTL killing of infected cells in the context of HIV-1 shock-and-kill.

Results and Discussion

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 (8). 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 quantitative reverse transcriptase PCR (qRT-PCR) using primers that detect all correctly polyadenylated HIV-1 transcripts (8). At concentrations as low as 50 nM, cyclosporin inhibited HIV-1 induction by αCD3/αCD28 (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 μM, despite inhibiting IL-2 production at lower concentrations (Figure 1, B and C). Neither drug affected cell viability in the concentration range tested (Figure 1D).

Using dose-response data from the above experiments and published studies (14, 18), 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), αCD3/αCD28 alone, or αCD3/αCD28 plus rapamycin or cyclosporin. HIV-1 mRNA levels increased significantly after treatment with αCD3/

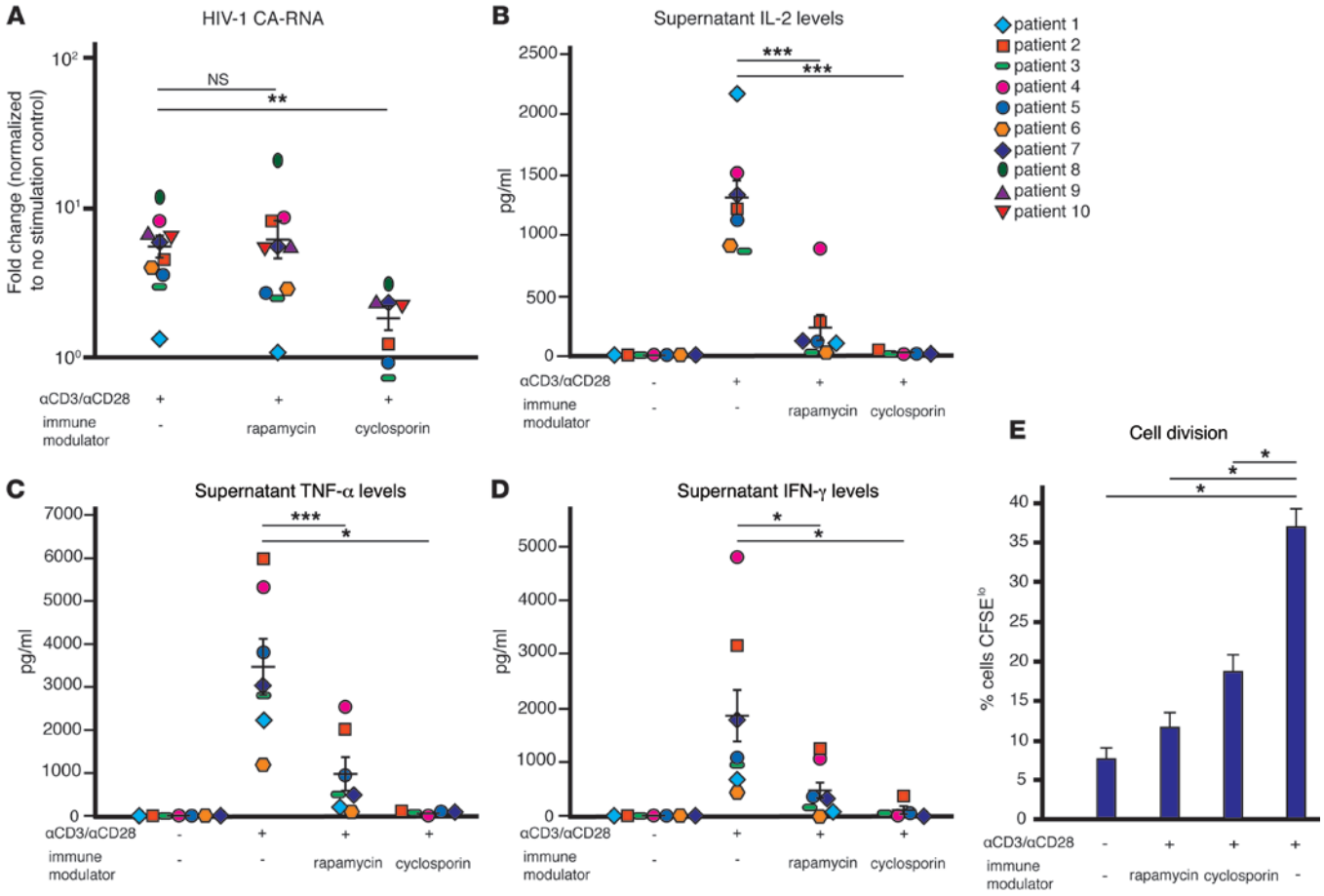

Figure 2. Effects of immunosuppressants on αCD3/αCD28–mediated HIV-1 expression and proinflammatory cytokine release from infected rCD4s. (**A**) Relative amounts of induced HIV-1 mRNA shown as fold change relative to no stimulation (DMSO alone). All samples represent qRT-PCR measurements of cell associated RNA (CA-RNA) from rCD4s derived from infected individuals (n = 10). (**B**) Effect of immunosuppressants on αCD3/αCD28–induced rCD4 production of proinflammatory cytokine IL-2. Points represent supernatant samples from experiments described in **A** (n = 7). (**C**) Effect of immunosuppressants on αCD3/αCD28–induced TNF-α release (n = 7). (**D**) Effect of immunosuppressants on αCD3/αCD28–induced IFN-γ release (n = 7). (**E**) Effect of immunosuppressant cotreatment on T cell proliferation. Healthy donor PBMCs (n = 3) were stained with CFSE before stimulation. 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, **P < 0.01, ***P < 0.001).

αCD28 alone for 24 hours (P = 0.048; Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI89552DS1). To account for substantial interpatient baseline variability, the data were normalized to DMSO control (Figure 2A). We observed no significant difference in HIV-1 RNA induction by αCD3/αCD28 with or without cotreatment with 5 μM rapamycin. However, cyclosporin significantly suppressed αCD3/αCD28–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 αCD3/αCD28 for 24 hours. αCD3/αCD28 significantly increased HIV-1 mRNA transcripts (P = 0.008), while rapamycin or cyclosporin had no effect (Supplemental Figure 1B).

We also examined supernatants from the cultures described in Figure 2A for proinflammatory cytokines. IL-2, TNF-α, and IFN-γ were induced at high levels in rCD4s, whereas other cytokines tested were not substantially produced after αCD3/αCD28 treatment. Cotreatment with αCD3/αCD28 and rapamycin significantly decreased release of IL-2, TNF-α, and IFN-γ compared with αCD3/αCD28 alone in these rCD4s (P = 0.0009, 0.0002, and 0.019, respectively; Figure 2, B–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). Cotreatment also inhibited production of IL-2, MCP-1, MIP-1α, IL-1β, MIP-β, IFN-γ, TNF-α, and IL-6 by unfractionated peripheral blood mononuclear cells (PBMCs) (Supplemental Figure 2A). Rapamycin inhibited MIP-1α production by over 50% and all other measured cytokines by over 80% (Supplemental Figure 2B). This effect was not due to cellular toxicity (Supplemental Figure 3A).

We then measured the effect of each treatment on cell proliferation, another potential indicator of clinical toxicity, using a CFSE assay with healthy donor PBMCs. We treated 5×10^6 cells per condition with DMSO alone, αCD3/αCD28, or αCD3/αCD28 and rapamycin or cyclosporin for 24 hours (Supplemental Figure 3B). αCD3/αCD28 stimulation caused significant proliferation (P = 0.014) that was substantially inhibited by rapamycin and cyclosporin (P = 0.012 and 0.018, respectively) (Figure 2E).

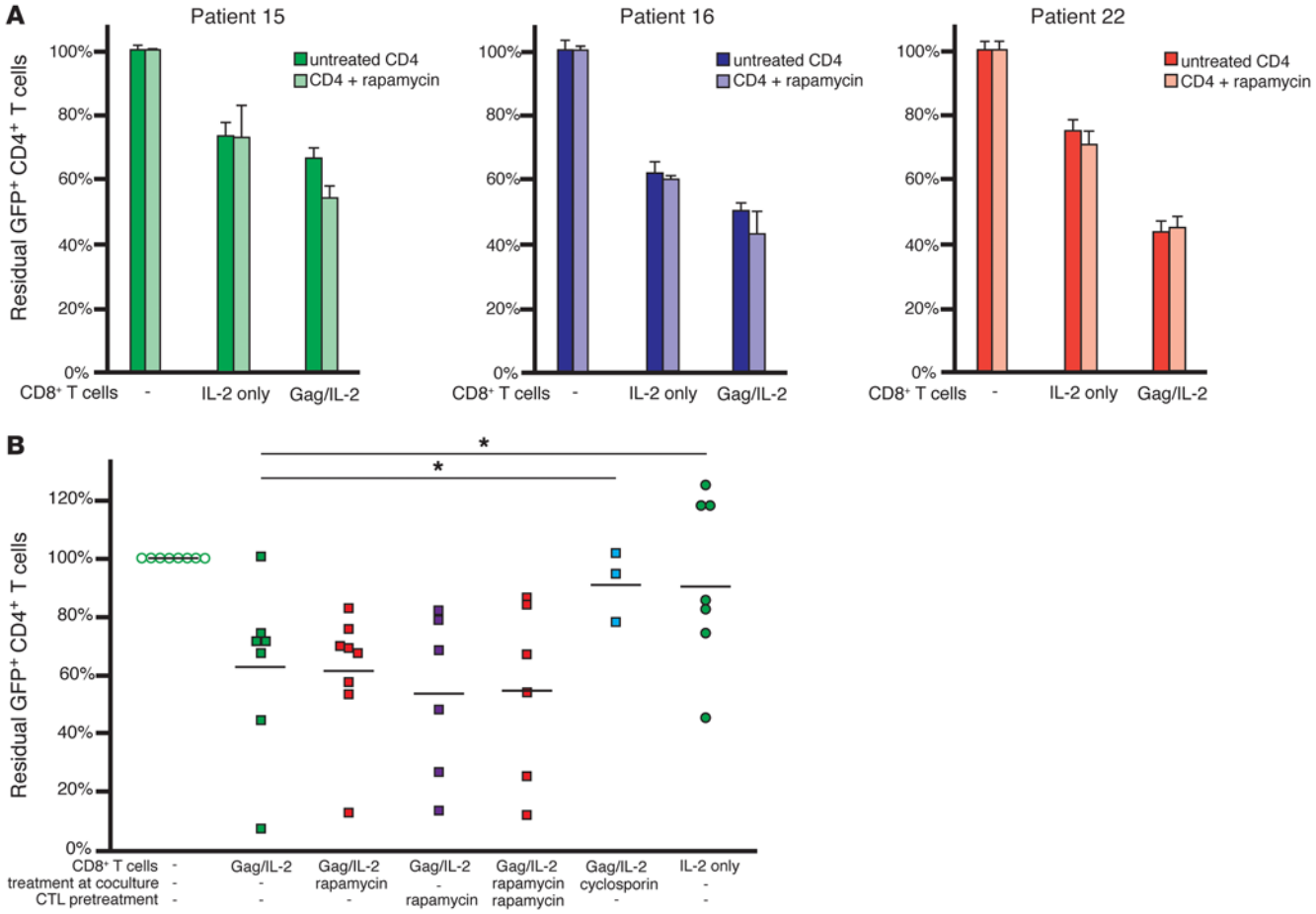

Figure 3. Rapamycin treatment does not reduce CTL killing. (**A**) Rapamycin does not affect viral peptide production and presentation of epitopes to CTLs (n = 3). Activated CD4+ T cells were infected with NL4-3-Δenv-GFP and cultured in media alone, or with rapamycin for 24 hours before coculture. Infected CD4+ T cells 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+ T cells were cultured alone, or cocultured at a 1:1 E/T ratio with CD8+ T cells. As indicated, some CD8+ T cells were pretreated 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).

αCD3/αCD28 treatment strongly induced activation marker expression on PBMCs from infected individuals after 24 hours. Cotreatment 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) (Supplemental Figure 3C), 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 because of inhibition of HIV-1 gene expression.

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 (Supplemental Figure 5, A and B). A modest increase in HIV-1 mRNA was seen with bryostatin-1 or romidepsin alone compared with a more substantial induction by the combination, consistent with previous reports (8, 22). We also tested the release of cytokines suggested to be associated with bryostatin-1 administration in vivo (23). Similar to the robust inhibition of PBMC cytokine release induced by αCD3/αCD28 treatment seen above, IL-8 and MIP-1α were substantially (>50%) reduced by addition of rapamycin, whereas IL-1β, IL-6, and TNF-α were not significantly inhibited (Supplemental Figure 5C). In contrast to global immune activation by stimuli including αCD3/αCD28, 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 Supplemental Figure 4 and ref. 24). Activated CD4+ T cells from infected donors were superinfected 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 prestimulated with a Gag peptide mixture and IL-2 for 6 days.

For each patient, a decrease in GFP$^+$ CD4$^+$ cells was observed when infected CD4$^+$ T cells were cocultured with prestimulated 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 before coculture did not prevent CTL killing, showing that epitope presentation by infected CD4$^+$ T cells was not affected (Figure 3A and Supplemental Figure 3D). 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 CD4$^+$ T cells, whereas cyclosporin had the expected inhibitory effect ($P = 0.027$) (Figure 3B). In addition, pretreatment of CD8$^+$ T cells with rapamycin for 3 days before coculture had no effect on the killing of infected cells (Figure 3B). These findings are consistent with previous reports of the effect of these immunosuppressants on CTLs (20, 21).

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. Interestingly, Stock et al. (17) reported that HIV-1–infected kidney transplant recipients who received rapamycin (sirolimus) for immunosuppression had lower levels of proviral DNA in PBMCs compared with HIV-1–infected transplant recipients who received cyclosporin or tacrolimus, both calcineurin inhibitors. We propose that the high levels of immune activation induced by transplantation (25) could reactivate expression of latent HIV-1. Our 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. This clinical finding suggests that our ex vivo results may be applicable to HIV-1–infected patients requiring solid-organ transplant.

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, we tested a latency reversal strategy that allows signaling downstream of TCR stimulation to effectively activate HIV-1 gene expression while controlling toxicity by cotreatment 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.

Methods

Statistics. Two-tailed paired Student's *t* test was used to determine statistical significance where indicated. Error bars represent mean ± SEM. We considered *P* less than 0.05 to be statistically significant.

Study approval. The study was approved by the Johns Hopkins Institutional Review Board. Written informed consent was obtained from all participants.

A complete, detailed description of methods can be found in the supplemental material.

Author contributions

ARM and RFS conceived of the study and designed experiments. ARM and RAP conducted experiments and analyzed data. CMD and RFA obtained institutional review board approval and managed study participant recruitment. AC recruited study participants. ARM and RFS wrote the manuscript.

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