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Everolimus, an mTORC1/2 inhibitor, in ART-suppressed individuals who received solid organ transplantation: A prospective study

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Abstract

Pharmacologic inhibition of the mammalian target of rapamycin (mTOR) in the setting of renal transplantation has previously been associated with lower HIV-1 DNA burden, and *in vitro* studies suggest that mTOR inhibition may lead to HIV transcriptional silencing. As prospective clinical trials are lacking, we conducted an open label, single-arm study to determine the impact of the broad mTOR inhibitor, everolimus, on residual HIV burden, transcriptional gene expression profiles, and immune responses in HIV-infected adult solid organ transplant (SOT) recipients on antiretroviral therapy (ART). Whereas everolimus therapy did not have an overall effect on cell-associated HIV-1 DNA and RNA levels in the entire cohort, participants who maintained everolimus time-averaged trough levels >5 ng/mL during the first two months of therapy had

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significantly lower RNA levels up to 6 months after the cessation of study drug. Time-averaged everolimus trough levels significantly correlated with greater inhibition of mTOR gene pathway transcriptional activity. Everolimus treatment also led to decreased PD-1 expression on certain T cell subsets. These data support the rationale for further study of the effects of mTOR inhibition on HIV transcriptional silencing in non-SOT populations, either alone or in combination with other strategies.

Keywords

HIV; mTOR inhibition; everolimus; PD1; HIV reservoir

1 INTRODUCTION

The role of immune-based therapeutics is a key HIV curative research priority.[1] The mammalian target of rapamycin (mTOR) is a key regulatory kinase that controls cell-cycle progression, and mTOR inhibition leads to significant immune modulation that is used to prevent or treat graft-versus-host disease and organ transplant rejection.[2–7] Despite its immunosuppressant qualities, mTOR inhibition leads to several potential beneficial immune regulatory functions that have activity against various viral pathogens, including HCV, CMV, HHV8, HPV, and HIV.[8–17] In addition, we previously reported that exposure to the mTOR inhibitor, sirolimus, in HIV-infected, antiretroviral therapy (ART)-suppressed renal transplant recipients was associated with lower post-transplant quantities of CD4+ T cell-associated HIV-1 DNA[11].

Sirolimus regulates several immune functions that likely play an important role in observed *in vitro* anti-HIV-1 replication effects, including reducing expression of CCR5, a chemokine coreceptor used by HIV for cell entry,[14,15,18,19] enhancing adaptive immunity,[20] and limiting CD4+ T-cell homeostatic proliferation.[21] The inhibitory effect of sirolimus on cell-cycle progression also appears to be limited to T-cells that are activated by cytokines and not by antigen-TCR engagement.[22,23] HIV persists for a large part in CD4+ T cells that have the ability to proliferate, and as a result, persist indefinitely despite suppressive ART. As a result, mTOR inhibition has the potential to reduce HIV-1 burden by preventing the proliferation of infected cells while preserving viral-specific adaptive immune function.[22–25] Recent *in vitro* studies suggest that blocking mTOR may promote HIV latency through viral transcriptional silencing,[26] an effect that could be taken advantage of in achieving long-term ART-free HIV remission by keeping cells in a more permanent, transcriptionally quiescent state. However, there is a paucity of prospective mTOR inhibition trials in HIV-infected individuals.

Based on these prior observations and our retrospective data suggesting that mTOR inhibition may reduce cellular HIV DNA burden, we conducted an open label, single-arm, exploratory study of everolimus, a dual mTORC1/2 inhibitor in 10 HIV-infected, ART-suppressed liver or kidney transplant recipients to: (1) determine the effect of everolimus on HIV DNA and RNA in CD4+ T cells in HIV infected patients on stable antiretroviral regimens in the context of mTOR signaling and related transcriptomic signatures, (2)

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determine the effect of everolimus on residual, low-level plasma HIV-1 RNA, and, (3) to determine the safety and tolerability of everolimus at standard immunosuppressive doses in HIV-infected transplant recipients who are on stable ART and concomitant immunosuppressive regimens. Overall, we hypothesized that mTOR inhibition with everolimus will reduce CD4+ T-cell associated HIV-1 DNA and RNA and low-level viremia despite otherwise suppressive ART. Everolimus is being using with increasing frequency in immunosuppressive regimens in transplant recipients as obtaining therapeutic drug levels is facilitated by more favorable pharmacokinetics as compared to the TORC1-inhibitor, sirolimus.

2 METHODS

2.1 Study population, inclusion and exclusion criteria

Ten HIV-infected adult solid organ transplant (SOT) recipients on stable ART and non-mTOR based immune suppressive regimens for allograft rejection prevention were recruited in this open label, single-arm everolimus trial. Inclusion criteria included HIV −1 infected adults aged 18 years or over on combination ART who underwent SOT (kidney or liver) with HIV-1 plasma RNA <50 copies/ml for at least 2 years and the most recent viral load within 3 months of screening. A single, isolated episode of detectable HIV RNA 500 copies/ml did not exclude participation. Participants were required to have two CD4+ T cell counts greater than 350 cell/μl in the six months prior to screening. Key exclusion criteria included patients who were intending to modify antiretroviral therapy in the next 6 months, any serious illness requiring hospitalization or parenteral antibiotics within the preceding 3 months, hemoglobin <11.5 g/dL, thyroid stimulating hormone consistent with hypothyroidism, significant renal disease (eGFR < 60 ml/min) or acute nephritis, clinically active hepatitis as evidenced by jaundice or Grade 2 liver function test abnormalities, hepatic cirrhosis, decompensated chronic liver disease, and no prior mTOR inhibitor use. Given concerns regarding safety of changing immunosuppressive therapy in renal transplant recipients with HIV who have a higher incidence of rejection[27], enrollment in this group was targeted to those with stable renal function and no evidence of rejection years following transplantation.

2.2 Data collection

Data were collected from medical records and biologic measurements were performed at screening, baseline (month 0), month 2 (treatment), month 6 (end of treatment), and month 12 (6 months post-treatment). Safety labs for kidney and liver transplant and blood everolimus levels were performed as standard-of-care. Primary analysis endpoints were paired change in measures of HIV persistence from baseline to month 6 of everolimus therapy.

2.3 Drug dosing and concomitant immunosuppressive therapy

Everolimus replaced a calcineurin inhibitor if the participant had undetectable calcineurin inhibitor levels for a target trough level of 5–7 ng/mL. For participants with higher calcineurin inhibitor blood levels, dosing was reduced by 50% along with addition of everolimus for the same trough goal. The modest everolimus trough goal was used given that

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combination of higher doses of everolimus with other anti-proliferative agents may lead to leukopenia or lymphopenia, but the goal was within previously evaluated therapeutic ranges for organ rejection[28].

2.4 Ethics statement

The University of California San Francisco Committee on Human Research approved the study and written informed consent was obtained from all participants. Patient-level information was stored in encrypted electronic databases or under lock and key.

2.5 HIV DNA and RNA and measurements

PBMC were isolated from whole blood and CD4 T cells were enriched by negative selection. Bulk CD4+ T cell or PBMC-associated HIV DNA and unspliced RNA were quantified using real-time PCR methods as described.[29–31] Values were normalized to genomic DNA quantification of a human housekeeping gene (CCR5; genomic DNA quantitation is not impacted by changes in gene transcriptional changes).[31] Plasma HIV RNA was quantified in a single-copy assay (SCA) using repetitive sampling in the Panther system (Hologic).[32,33] Up to 18 replicates were tested for each sample in order to determine plasma RNA levels as low as 0.18 copies/mL.

2.6 Immune phenotyping and quantification of HIV and CMV-specific immune responses

A flow cytometric panel was used on PBMC which included markers of CD4+ and CD8+ T cell activation (CD69, CD38, HLA-DR), naive and memory cell subsets (CCR7, CD45RA), CCR5, and immune checkpoint (PD-1). Fresh cells were used to prevent down-regulation of CCR5. Flow cytometry antibody clones and fluorophores are listed in Supplemental Table 2. For HIV and CMV-specific intracellular response assays, PBMCs were stimulated with HIV Clade B Gag pooled peptides (NIH AIDS Reagent Repository) or CMV pp65 peptide for six hours. Intracellular cytokines (IFNγ, TNFα and IL2) and surface markers of perforin and CD107a were measured following stimulation. Detailed flow cytometric and intracellular response methods are shown in the Supplementary Methods.

2.7 Antibody responses and plasma cytokine measurements

Longitudinal quantification of HIV-specific antibody avidity by limiting-antigen enzyme immunoassay and antibody levels using the less sensitive Vitros Anti–HIV-1_2 assay (Ortho Clinical Diagnostics) were performed as described when sufficient plasma was available.[34–36] Multiplexed assays to simultaneously quantify plasma cytokines were performed on plasma samples from baseline (month 0) and month 6 using Luminex-based bead-based assay system.

2.8 RNA-Seq and bioinformatic analysis

RNA was purified from 1 million PBMCs followed by library generation, paired end sequencing and bio-informatic analysis including differential gene expressing testing, gene set variation analyses, and linear regression modeling as previously described[37–43] and detailed in the Supplementary Methods.

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2.9 Statistical analyses

Differences between study month 0 and month 6 were measured using paired, non-parametric Wilcoxon tests and further repeat measures analyses for all time points were performed using Friedman tests with Dunn correction for multiple comparisons. Associations between time-averaged everolimus trough levels or immune phenotypes and measures of HIV persistence were performed using Spearman rank correlation analyses. Analyses were performed using Prism v. 7 (GraphPad) and SPSS v.25 (IBM).

3 RESULTS

3.2 Everolimus safety and tolerability in SOT recipients with HIV

Ten participants were enrolled in the study, all of whom were on suppressive ART. Eight participants received liver and two received kidney transplantations. At the time of enrollment, >120 kidney transplant and >70 liver transplant recipients had been performed at UCSF in people with HIV. The lower number of enrolled individuals reflected inclusion of participants at lowest risk of renal graft rejection (e.*g.* stable renal function without evidence of rejection for several years), and the high percentage of potential liver transplant participants with active HCV infection as enrollment was initiated prior to widely available direct acting anti-HCV therapies. The 10 participants were those who met the stringent inclusion/exclusion requirements and were willing to participate following discussion of potential risks and benefits. All were enrolled >4 years following organ transplantation (range 1,244–4,853 days; mean 2,673 days; Table 1).

Participant demographics, organ transplant information, ART regimen and concomitant immunosuppression during everolimus therapy are shown in Table 1. One individual stopped everolimus therapy due to diarrhea, and one continued beyond the 6-month planned duration for clinical need. All but two individuals who were receiving calcineurin inhibitor-based immunosuppressive therapy continued either cyclosporin or tacrolimus following addition of everolimus. Participants continued long-term mycophenolate sodium or the mycophenolic acid ester prodrug, mycophenolate mofitil (MMF). CD4+ T cell counts remained stable throughout the study (Figure 1).

3.2 Impact of everolimus on measures of HIV persistence

We performed in-depth analysis of cell-associated HIV-1 DNA, unspliced (us)RNA, and low-level residual viremia. There were no significant changes in CD4+ T cell or PBMC-associated HIV-1 DNA or RNA between baseline and the last day of everolimus (study month 6) in the overall cohort (Fig 2A–B). Similarly, there were no significant differences in low-level plasma HIV RNA during this time (Fig 2E). However, participants that achieved a time-averaged everolimus trough level of at least 5 ng/mL during the first two months of study drug experienced a trend towards decreased CD4+ T cell-associated HIV RNA at study month 2 and a significant, sustained reduction in cellular RNA levels at month 12, six months following everolimus cessation (P=0.04 in adjusted non-parametric analyses; Figure 2D). This trough cutoff value was the desired lower trough limit in the study protocol and within range of previously reported efficacy concentrations in transplant recipients.[28]

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Further corroborating the ability for everolimus to block HIV transcription *in vivo*, we observed a significant negative correlation between the fold change of CD4+ T cell-associated HIV-1 RNA between baseline and M2 and time-averaged everolimus trough levels through 2 months of therapy (r= −0.84, P=0.004; Fig 2F) and fold change in HIV-1 RNA between baseline and M12 (last study time point, 6 months following cessation of therapy) compared to time-averaged drug troughs over all 6 months of therapy (M0 to M6; r= −0.7, P=0.043; Fig 2F–G).

3.3 Everolimus therapy reduced PD-1 expression

No changes in the distribution of naive, central memory, effector memory, or effector memory CD45RA+ cells were observed (Fig 3 A, B). However, increases in the percentage of total, central memory, and effector memory CD4+ and CD8+ T cells expressing the early activation marker CD69 were observed between study month 0 and month 6 (primary immunologic analysis time point; Fig 3 B, C), an effect which persisted 6 months following cessation of everolimus. No changes in the frequency of CD8+/HLA-DR+ T cells were observed. Although not significant, a visual decrease in surface expression of CCR5 on various CD4+ and CD8+ T cell subsets was observed at study months 2 and 6 months (Fig 3 D, E). A significant decrease in the frequency of PD-1 expressing CD4+ T_{EMRA} cells was observed at month 6 (P<0.01). The frequency of PD-1 expressing CD4+ and CD8+ central and effector memory cells also appeared to have decreased during everolimus treatment but was not statistically significant.

3.4 HIV DNA and RNA levels were negatively associated with CCR5 expression and positively associated with PD-1 expression

Statistically significant negative correlations were observed between HIV DNA and RNA copies/10^6 CD4+ T cells and the percentage of CD4+ T cells expressing CCR5 and between cell-associated HIV RNA levels and the percentage of CD8+ T cells expressing CCR5 (Fig 4 A–D). Significant positive correlations between cell-associated HIV RNA levels and the percentage of both CD4+ and CD8+ T cells expressing the immune checkpoint marker, PD-1 were observed (Fig 4 E, F).

3.5 HIV and CMV-specific CD8+ T cell responses

Intracellular cytokine staining was performed in order to determine changes to HIV and CMV-specific CD8+ T cell responses following initiation of everolimus. Overall, IFNγ responses to both HIV Gag and CMV pp65 peptides were low and we observed no significant differences between baseline and month 6 or in repeat measures analyses. No significant differences were observed in CD107a expression, a marker of cytotoxic degranulation, following peptide stimulations (Figure 5).

3.6 Downregulation of mTOR signaling pathway gene expression in participants with decreased CD4+ T-cell-associated RNA

Given the significant association between everolimus levels and decreases in cell-associated HIV-1 RNA we explored the relationships between CD4+ T cell RNA and DNA levels following everolimus therapy with changes in Hallmark mTOR and other signaling pathway

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gene expression by RNAseq transcriptome profiling of total PMBCs and linear regression modeling. The differential expression of top genes at baseline, month 1 and 6 of everolimus treatment, and 6 months following cessation of mTOR inhibition (month 12) are shown in Supplementary Figure 1.

Overall, gene expression appears to be broadly downregulated by month 2 and 6 of everolimus therapy compared with baseline, with some return to baseline levels 6 months following cessation of therapy (study month 12). Increased enrichment of the IL-6 JAK-STAT3 and inflammatory signaling pathways by gene set variation analysis at baseline was significantly associated with negative changes in CD4+ T cell-associated HIV-1 DNA levels from baseline to the last study time point (month 12) (Supplementary Figure 2). Down regulation of genes from the Hallmark MTORC1 Signaling pathway from baseline to treatment month 2 correlated with greater decreases in CD4+ T cell-associated HIV RNA during the same interval and over the entire study period (BL to M2 and BL to M12, respectively; Figure 6).

3.7 Everolimus drug levels were associated with decreased mTOR signaling and CCR5 gene expression.

Figure 7 shows results from regression analysis of the top differentially enriched Hallmark pathways by everolimus drug trough values at treatment month 2. Everolimus levels were more variable during the initial months of therapy prior to stabilization by month 6. Regression analysis of the top 50 differentially expressed pathways at treatment month 6 by everolimus time-averaged trough levels from baseline to both month 2 and month 6 are shown in Supplementary Figure 3. Overall, increased trough levels were correlated with decreased mTORC1 pathway expression, among others as detailed in Figure 7 at month 2 and 12. The effect on everolimus drug levels on mTORC1 inhibition persisted up to 6 months following cessation of drug. Regression analyses of DEGs also revealed significant inverse correlations between time-averaged baseline trough levels to treatment month 2 and to month 6 with CCR5 expression at final treatment month 6 as shown in Figure 7.

3.8 Plasma cytokines

Plasma cytokine levels at baseline and month 6 are shown in Supplementary Table 2. In unadjusted analyses, interferon-inducible T-cell alpha chemoattractant (CXCL11) levels significantly decreased, but no significant changes in other cytokines and no significant changes in analytes were observed in adjusted analyses.

3.9 HIV specific antibody responses

HIV-specific antibody levels and limiting antigen antibody avidity are shown in Supplementary Figure 4. Overall, no changes to antibody avidity or level (using the detuned less-sensitive (LS)-Vitros assay) were observed. However, a positive correlation was identified between CD4+ T cell-associated HIV DNA levels and antibody levels ($r=0.42$, $P=0.034$).

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4 DISCUSSION

To our knowledge, this is the first prospective trial to examine the impact of mTOR (TORC1/2) inhibition on measures of HIV persistence and immune responses in the setting of SOT. Overall, 6 months of everolimus in addition to concomitant immunosuppression in SOT recipients on suppressive ART led to decreased CD4+ T cell-associated HIV-1 RNA levels in participants that maintained time-averaged everolimus troughs >5 ng/mL within the first two months of therapy, a time when there is often dose adjustments prior to reaching more stable drug levels. Supporting the above observation that everolimus may dampen HIV transcriptional activity, we observed a significant correlation between increased time-averaged everolimus trough levels and decreased cellular HIV RNA levels, even 6 months following cessation of mTOR inhibitor therapy. Interestingly, the data suggest that lower doses of everolimus may paradoxically facilitate viral transcription, and there may be a complex relationship between dosing and biological effect. However, participants who did not achieve drug trough levels >5 ng/mL did not experience significant changes in cell-associated RNA throughout the study time points.

Transcriptome profiling experiments suggested that individual responses to mTOR inhibition therapy may be important in interpreting and predicting drug effects on measures of HIV persistence and transcriptional activity, and will likely be crucial components in future HIV eradication trials. More specifically, individuals that experienced decreases in mTORC1 pathway gene expression (*i.e.* greater mTOR inhibition) had greater decreases in CD4+ T cell-associated HIV RNA during everolimus therapy. In turn, higher time-averaged everolimus trough levels were significantly correlated with less mTORC1 signaling (*i.e.* greater mTOR inhibition) as would be predicted by the above correlation between drug levels and HIV RNA. These relationships between mTOR signaling and RNA levels are consistent with a recent *in vitro* investigation which suggested that mTOR inhibition promotes HIV transcriptional silencing *in vitro* via the viral transactivator, Tat, and Tat-independent mechanisms.[26] Together, our results suggest that pathway-specific information will likely be important components in future HIV pathogenesis and eradication studies. While care should be taken in over-interpreting these data given the limited sample size and lack of control group or randomization, they provide potential hypotheses for mechanistic interactions between mTOR inhibitor use and HIV persistence and provide rationale to conduct larger prospective trials in otherwise healthy individuals living with HIV.

The reason why everolimus therapy leads to changes in HIV-1 RNA levels in participants achieving a trough level of 5 ng/mL or higher 6 months after stopping treatment is not entirely clear, but there appear to be lasting changes to differential expressed genes at this time point even when excluding the individual that remained on everolimus through study month 12. This suggests that there is a certain degree of immune remodeling, potentially epigenetic in nature, leading to longer-term changes in gene transcriptional activity as a result of a defined period of pharmacologic mTOR inhibition.

The cell-associated HIV DNA results from this prospective clinical trial are in contrast with data we previously reported from a retrospective observational study in renal transplant recipients who were exposed to the TORC1-specific mTOR inhibitor, sirolimus and

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had lower cell-associated HIV DNA levels than those who did not receive sirolimus.[11] Everolimus has been shown to also inhibit signaling through the TORC2 complex, which regulates protein kinase C (PKC) phosphorylation and NF-κB signaling, and this additional activity may be responsible, in part, for differences in outcomes.[44,45] The clinical impact of increased frequency of T cells expressing CD69 is unclear. Although CD69 is an early marker of T cell activation, it also regulates lymphocyte egress from thymus and lymph nodes[46,47] and may play a more complicated role in immune responses, such as differentiation and maintenance of regulatory T cells.[48,49]

All study participants were taking concomitant mycophenolate therapy for years prior to and during the study interval. Mycophenolate has anti-proliferative effects on CD4+ T cells,[50] and a recent modeling study estimated that nearly all infected cells in ART-suppressed patients arise from proliferation rather than replication months after ART initiation.[51] While pre-mycophenolate sampling was not performed in our trial, all individuals had readily detectable cell-associated HIV-1 RNA and DNA after long-term mycophenolate therapy within range of what would be expected in chronically ART-suppressed individuals.[52,53]

Immune checkpoint inhibition by blocking PD-1 or other molecules is currently a major focus of HIV-1 curative strategies.[54–56] In this study, a significant reduction in the frequency of PD-1-expressing CD4+ terminally differentiated memory cells from baseline to month 6 of everolimus therapy were observed. Non-significant reductions were also noted on various CD4+ and CD8+ T cell subsets. However, the reduction in PD-1 expression was modest compared with what has been observed in studies involving direct anti-PD-1 antagonism with monoclonal antibodies, and early clinical reports of anti-PD-1 therapy have demonstrated variable effects on measures of HIV persistence.[54–56] We did identify positive correlations between PD-1 expression and cell-associated HIV-RNA levels, but the exact impact of down-regulation of PD-1 by mTOR inhibition on HIV persistence is unclear, and larger studies will be required.

Major histocompatibility complex (MHC) class I or class II restricted antigen presentation by dendritic cells is not inhibited by mTOR inhibition, at least *in vitro*, which may preserve the capacity of the immune system to generate novel T-cell responses.[25,57] Interestingly, no significant changes in T cell responses to either HIV or CMV-specific peptide stimulations were observed in this study. Calcineurin inhibitors, on which a majority of participants remained after addition of everolimus, inhibit MHC-restricted antigen presentation pathways,[25] and may have negated or muted any beneficial effects of mTOR inhibition on viral-specific immunity or generation of novel memory responses.

This study had several limitations, such as concomitant use of other immunomodulatory medications that may have masked or altered the effects of mTOR inhibition on HIV persistence. The trial did not include a control group, sample size was also limited, and the study was powered only to detect large changes in measures of HIV DNA and RNA and T cell phenotype and function. Nonetheless, several key biological indicators of mTOR inhibition were observed, including decreased PD-1. Furthermore, despite use of other immunomodulating agents, we observed significant associations between mTOR transcriptional pathways and HIV transcriptional activity. The study is also limited by the

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investigator concerns not to push the TOR dosage to achieve higher levels, as the recipients were already on immunosuppression. Indeed, the impact of everolimus was seen at trough levels >5 ng/ml.

Despite these limitations, it is evident that future trials examining the impact of TOR inhibitors on the HIV reservoir should maximize the likelihood of achieving adequate drug exposure to down regulate the mTOR pathway. This would include the utilization of ART regimens based on the integrase inhibitors and if possible avoiding regimens that include protease inhibitors. For transplant practitioners, it is particularly interesting to note that trough levels <5 ng/ml failed to impact the mTOR pathway, and demonstrate the importance of sufficient drug exposure to see the desired effect of blocking the alloimmune response. Indeed, the two-three fold higher rejection rates following kidney transplantation in HIV positive recipients are in part related to interactions between anti-retroviral and immunosuppressive drugs. These data also support the rationale for further study of the effects of mTOR inhibition on HIV persistence, either alone or in combination with other HIV curative strategies, in otherwise healthy ART-suppressed individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflicts of Interest.

SGD has received grant support from Gilead, Merck and ViiV. He has consulted from AbbVie, Janssen and Shionogi. He is a member of the scientific advisory boards for BryoLogyx and Enochian Biosciences. TJH receives grant support from Gilead and Bristol Myers Squibb, and has consulted for Merck.

Data Availability Statement:

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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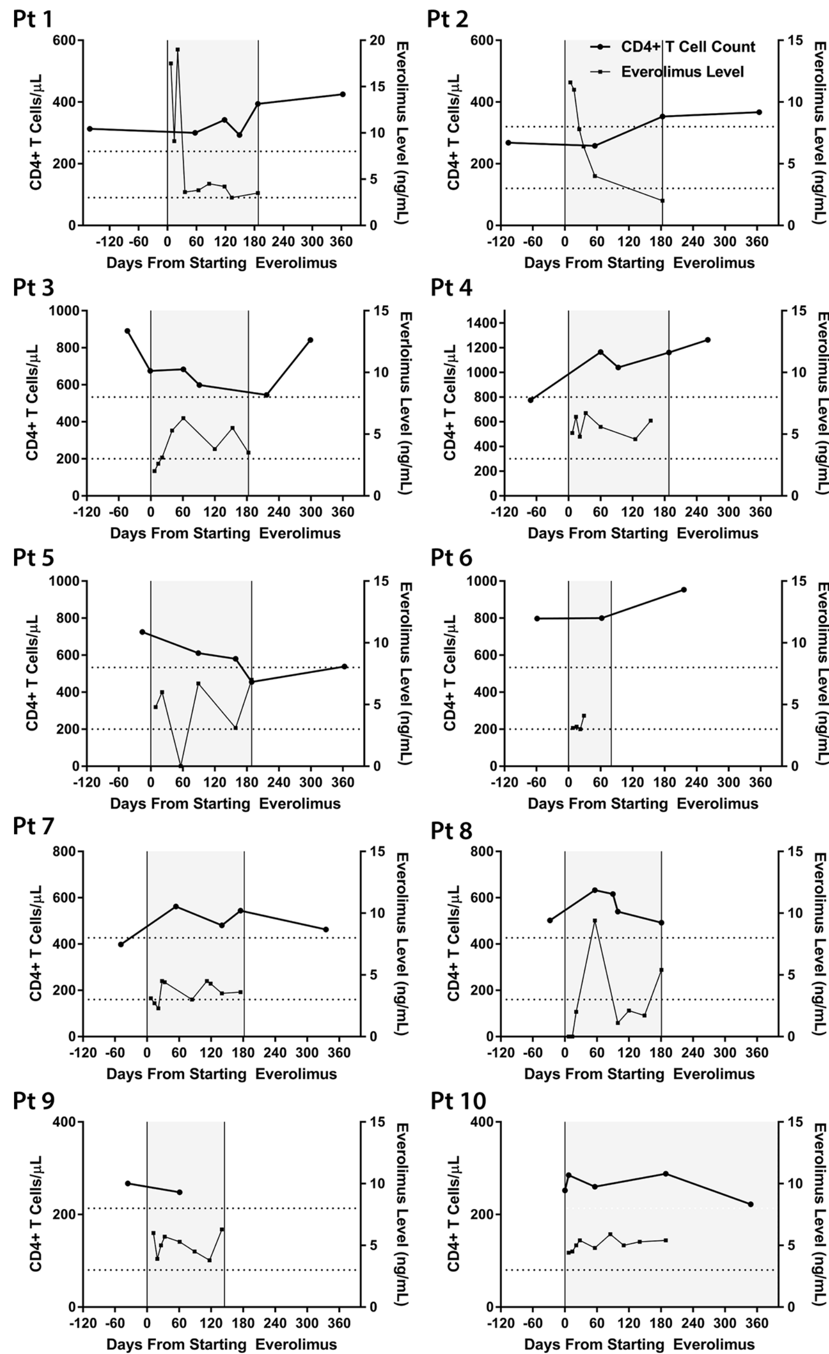

Figure 1. CD4+ T cell counts and everolimus levels in HIV-infected solid-organ transplant recipients on suppressive ART.

Peripheral blood was collected prior to starting everolimus, 2 and 6 months on everolimus, and 6 months following discontinuation of study drug (study month 12). A majority of participant received 6 months of everolimus therapy either substituted for or in addition to calcineurin inhibitor-based immune suppression. Participant 6 discontinued everolimus at study month 2 due to diarrhea. Participant 10 continued on everolimus therapy through the 12 month sample time point. Shaded areas represent periods of everolimus exposure. Dotted lines represent the therapeutic target range for everolimus concentrations.

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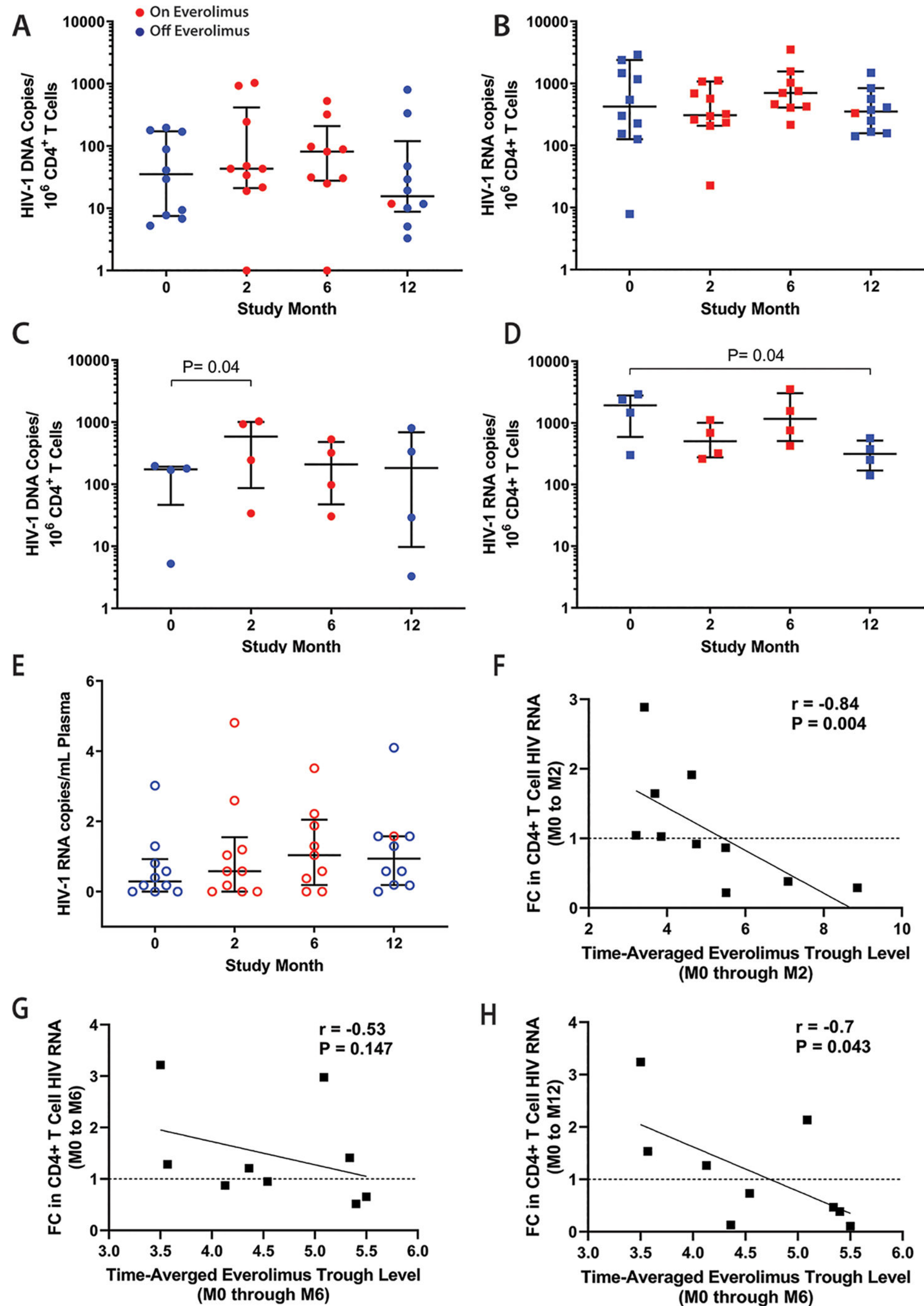

Figure 2. Measures of HIV persistence prior to, during and following everolimus therapy.

(**A**) CD4+ T cell-associated HIV-1 DNA, (**B**) unspliced CD4+ T cell-associated RNA measures for each time point are shown. Changes between baseline (study month 0) all other time points were observed using Freidman’s test with Dunn correction for multiple comparisons (N=9, as participant 6 did not have sampling on month 6). Cell associated DNA and RNA levels for participants who maintained time-averaged drug trough measures of >5 ng/ml are shown in (**C**) and (**D**). Low-level plasma HIV RNA measures by single copy assay for each time point are shown in (**E**). Correlations between fold changes (FC) in CD4+ T

cell-associated HIV-1 RNA and time-averaged everolimus trough levels in ng/mL are shown in (**F-H**). Significant negative correlations between CD4+ T cell-associated HIV-1 RNA FC at M2 and M12 and time-averaged everolimus trough levels between BL (M0) and M2, and BL and M6 (last date of study drug) were observed.

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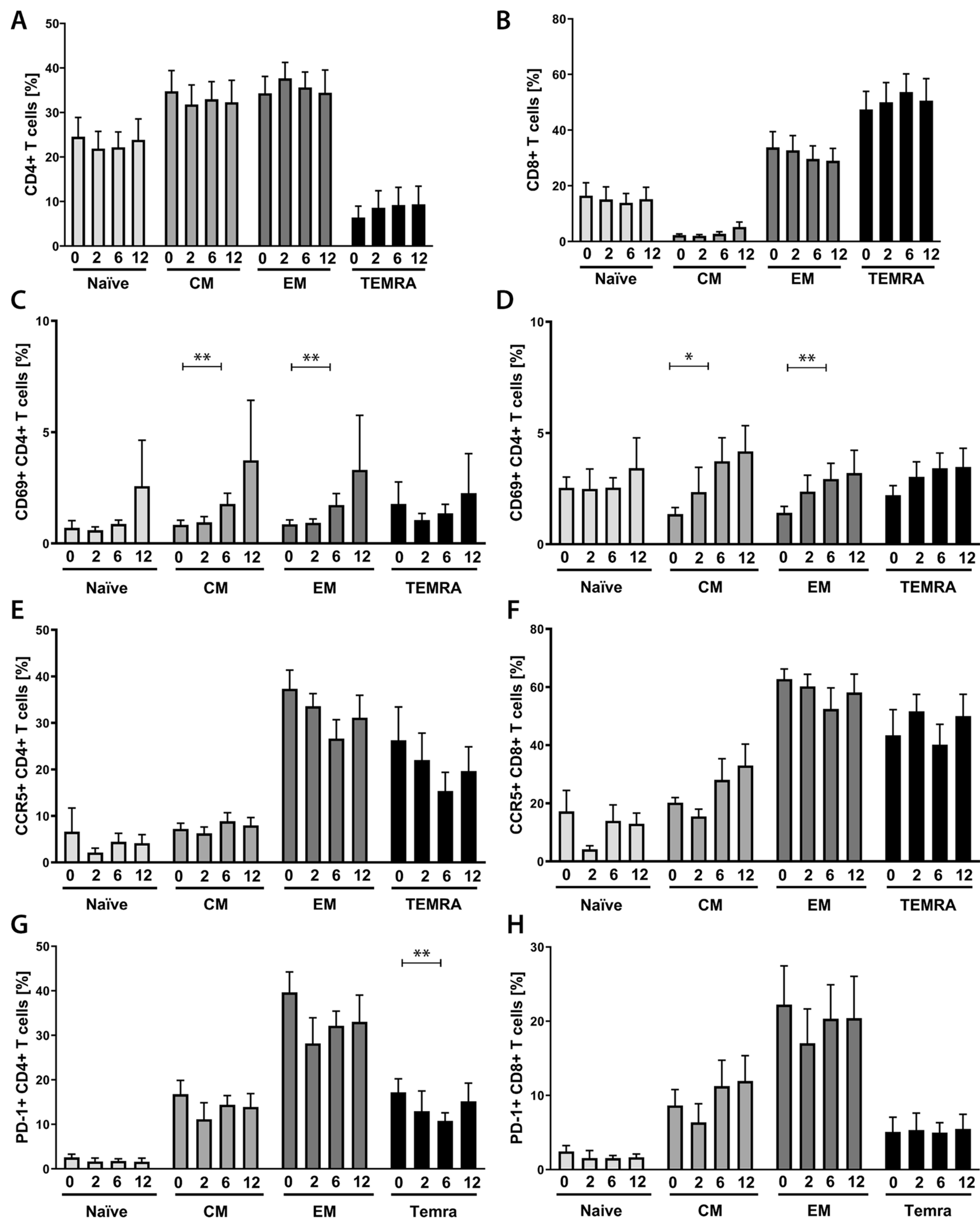

Figure 3.

Percentages of CD4+ and CD8+ T cell subsets expressing CCR5, PD-1 and CD69 prior to, during and following everolimus therapy. No changes in the distribution of naive, central memory, effector memory, or effector memory CD45RA+ cells were observed (A, B). However, increases in the percentage of total, central memory, and effector memory CD4+ and CD8+ T cells expressing the early activation marker CD69 were observed between study month 0 and month 6, the last time of sirolimus (C, D). Although not significant, a visual change in surface expression of CCR5 on various CD4+ and CD8+ T cell subsets

are shown in E and F. A significant decrease in PD-1 expression from month 0 to month 6 was observed in CD4+ TEMRA cells (G) but not CD8 TEMRA cells (H). Differences between study month 0 and month 6 were measured using paired, non-parametric Wilcoxon signed-rank tests (* $P<0.05$, ** $P<0.01$). These significant differences were also identified in a secondary repeat measures analyses using Freidman's test with Dunn correction for multiple comparisons.

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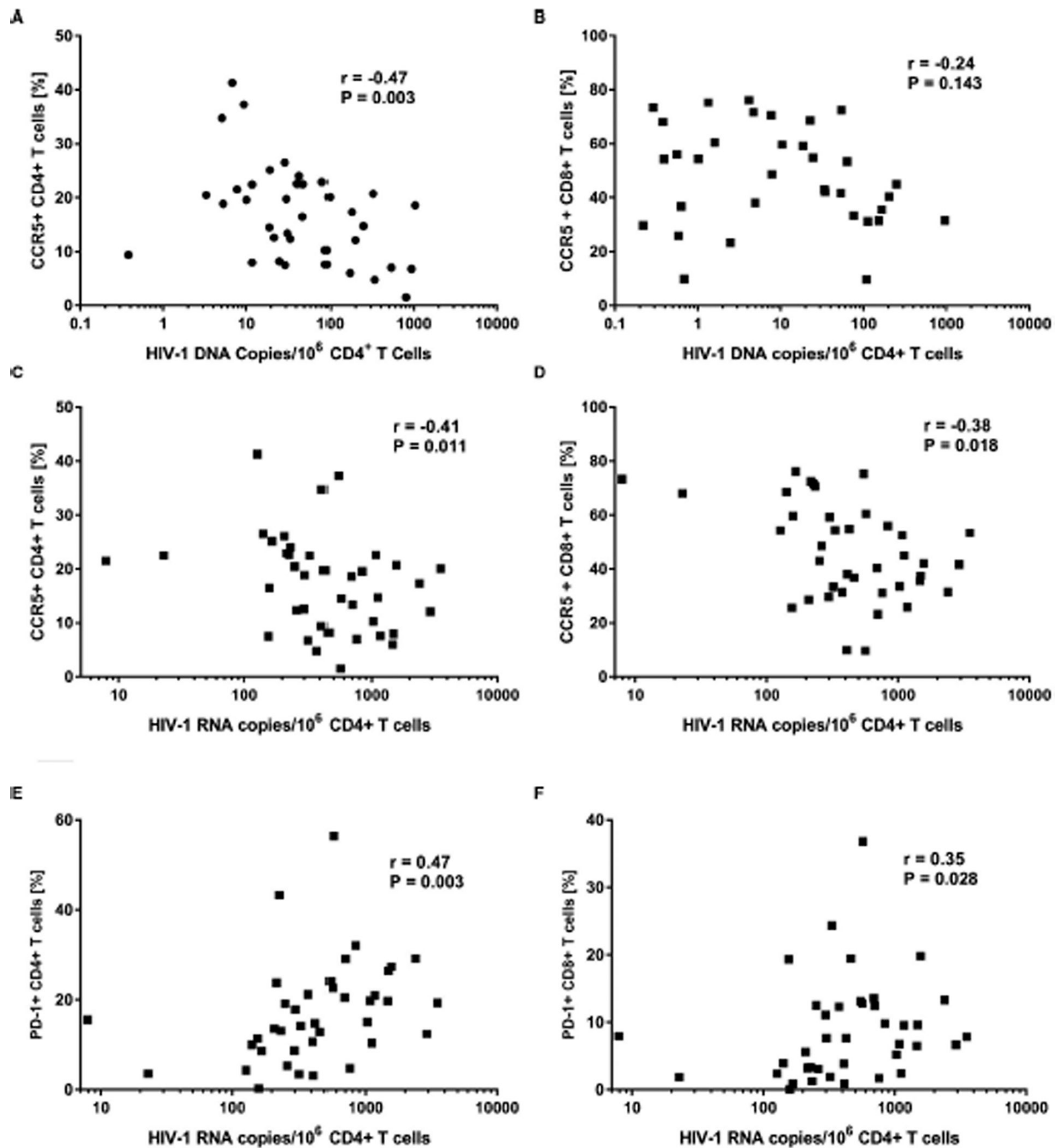

Figure 4.

Associations between CCR5 or PD-1 expression and cell-associated HIV DNA and RNA levels are shown. Modest statistically significant negative correlations were observed between HIV DNA and RNA copies/10^6 CD4+ T cells and the percentage of CD4+ T cells expressing CCR5 (A, C) and between cell-associated HIV RNA levels and the percentage of CD8+ T cells expressing CCR5 (B, D). Significant positive correlations between cell-associated HIV RNA levels and the percentage of both CD4+ and CD8+ T cells expressing

the immune checkpoint marker, PD-1 were observed (E, F). Correlation coefficients and P values were calculated using Spearman rank tests.

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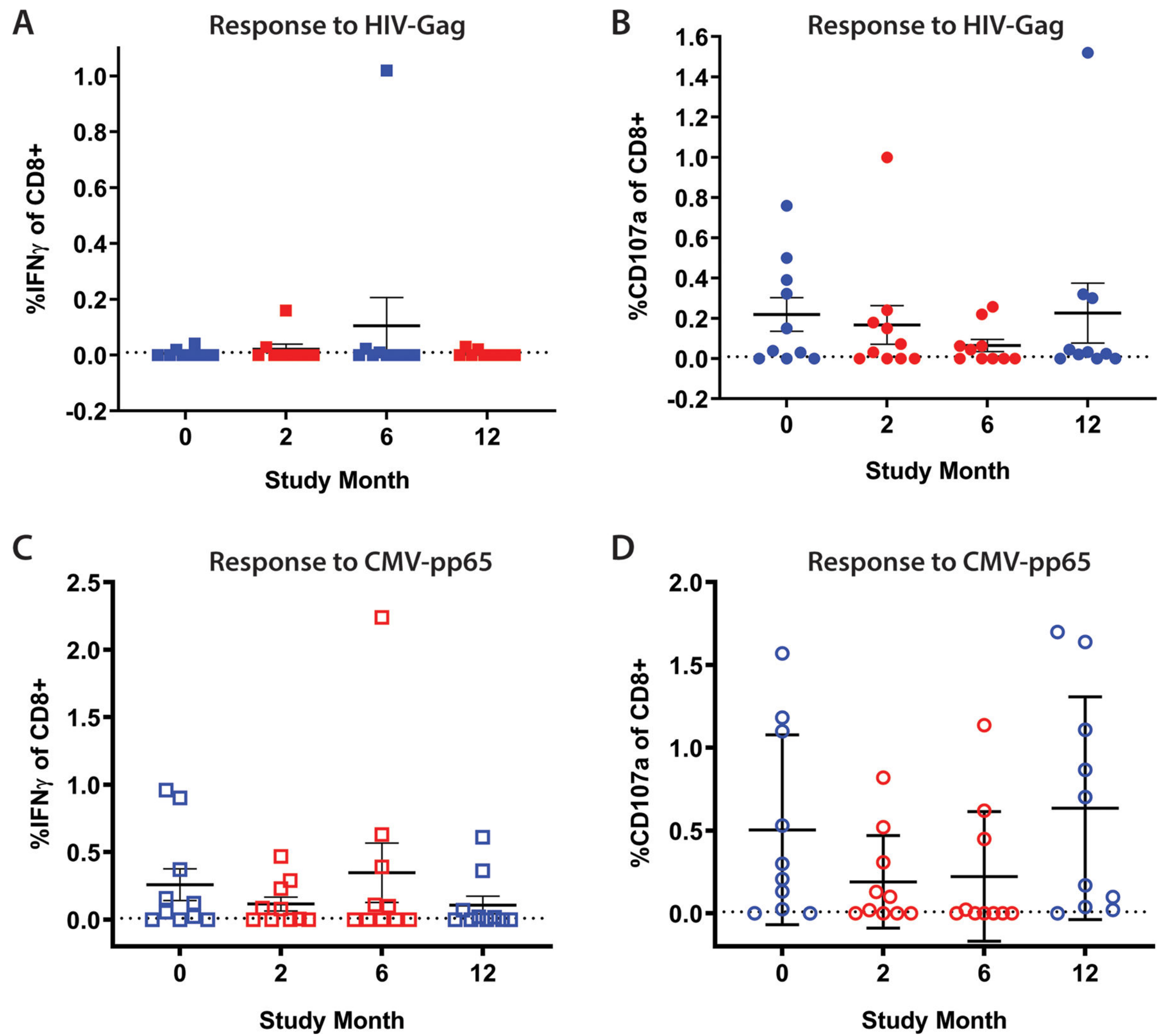

Figure 5.

Responses to HIV and CMV peptides are shown. Overall, no significant differences in the percentage of IFNγ or CD107a expressing CD4+ or CD8+ T cells were observed for either HIV-Gag (A, B) or CMV-pp65 peptides (C, D) between baseline and month 6 or in repeat measures analysis.

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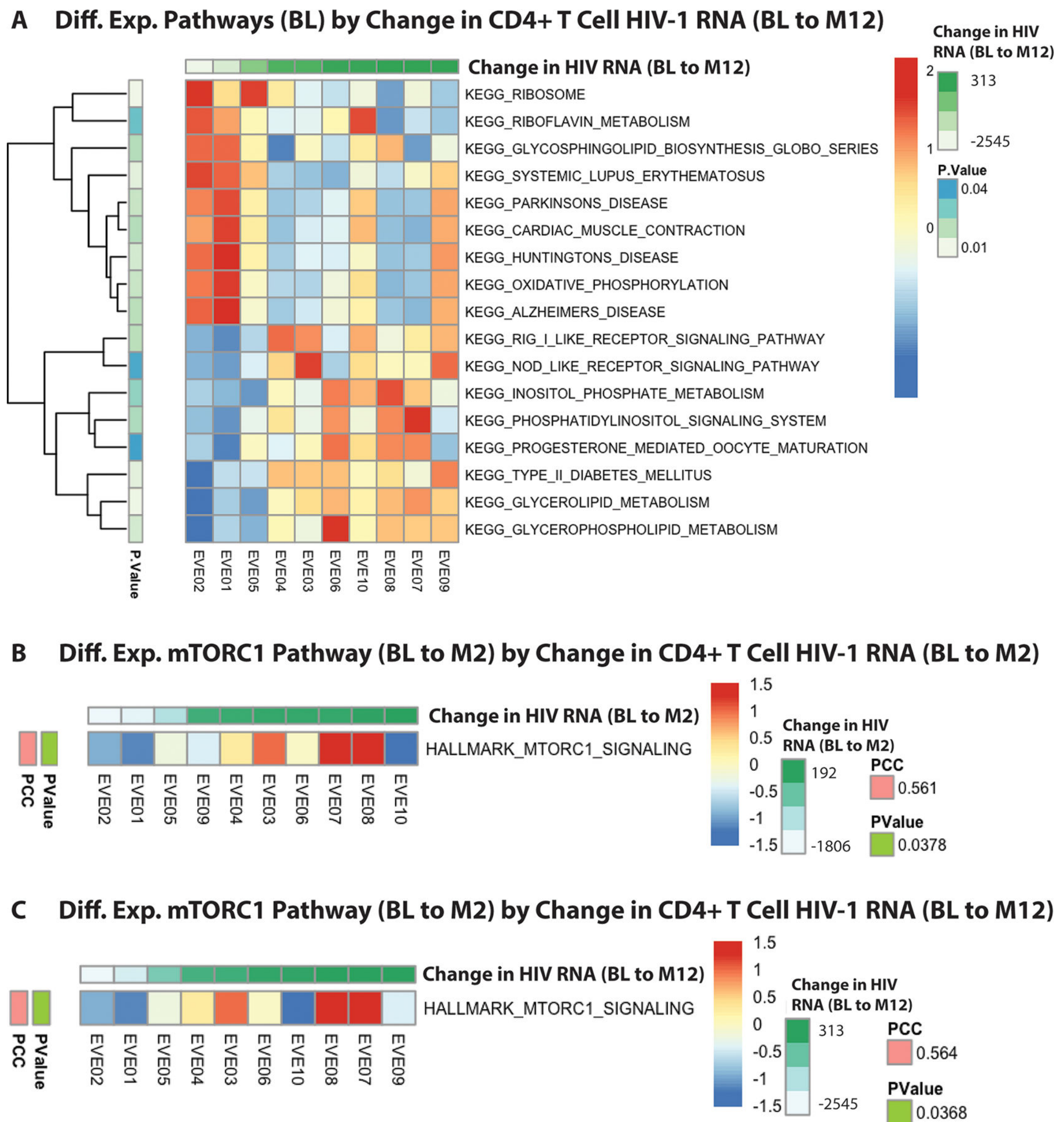

Figure 6.

Differentially enriched pathways at BL (**A**) and from BL to M2 (**B, C**) by change in CD4+ T cell HIV-1 RNA are shown. Response to two months of everolimus therapy in terms of changes in CD4 HIV RNA was significantly associated with both positive and negative changes in gene pathway enrichment (**A**). A greater decrease in mTORC1 signaling was significantly associated with greater decreases in CD4+ T cell associated HIV RNA from baseline to month 2 and month 12 as shown in (**B** and **C**) suggesting sustained impact on mTOR signalling and HIV transcriptional silencing up to 6 months following cessation of everolimus (Pt 10 who remained on therapy through M12).

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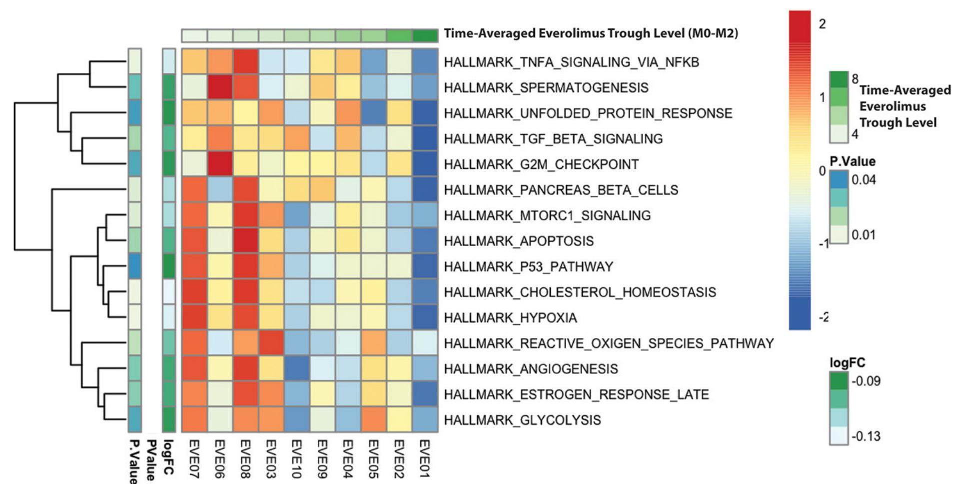

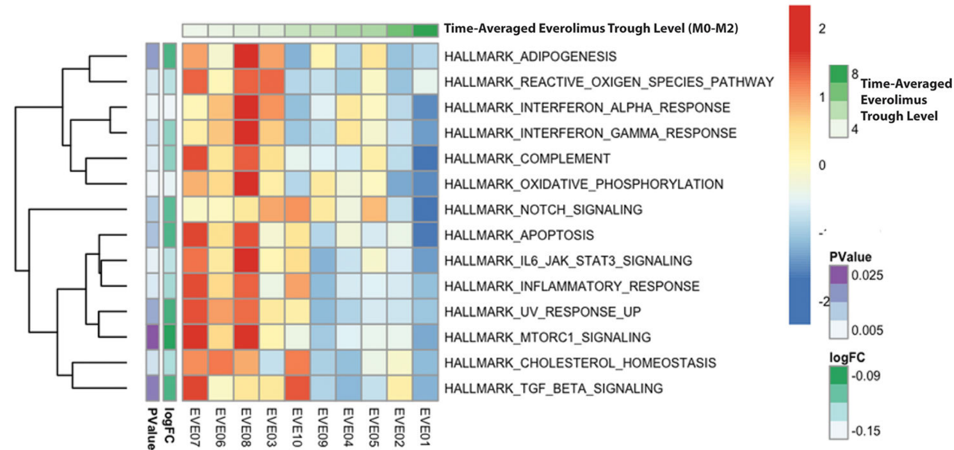

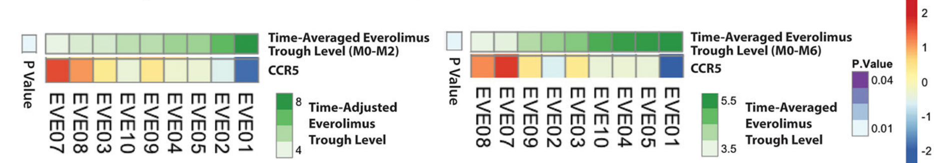

Figure 7.

Differentially enriched pathways from BL to M2 (**A**) and BL to M12 (**B**) by time-averaged everolimus trough level (BL through M2). Increased everolimus trough levels during the first two months of treatment were significantly correlated with decreased mTORC1, TNFα and TGFβ Hallmark pathways among others involved in apoptosis and cell stress response over the first 2 months of mTORC inhibitor therapy. Of note, downregulation of mTORC1 signaling was also seen at M12, 6 months following cessation of everolimus treatment (with the exception of Pt 10 who remained on therapy through M12). Everolimus trough

levels from baseline to through M2 and through M6 inversely correlated with CCR5 gene expression at M6 (**C**).

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Table 1.

Participant demographics, organ transplant type, ART regimen and concomitant immunosuppression during everolimus therapy

Participant	Age	Sex	Type of Organ Transplant	Years Post Transplant Everolimus Initiated	Baseline CD4+ T Cell Count (cells/μL)	Everolimus Therapy (Days)	Immunosuppression Prior to Everolimus	Concomitant Immunosuppression with Everolimus	ART Regimen
1	49	M	Liver	9.2	313	188	Cyclosporin, MMF	MMF	TDF/FTC/RPV/DTG
2	61	M	Liver	3.4	268	183	Tacrolimus, MMF	Tacrolimus, MMF	TDF/FTC/RAL
3	63	M	Kidney	4.3	675	183	Tacrolimus, MMF	MMF	ABC/3TC/EFV
4	57	M	Liver	7.8	775	188	Cyclosporin, Mycophenolate	Cyclosporin, Mycophenolate	TDF/FTC/RAL
5	57	M	Liver	8.5	725	189	Cyclosporin, MMF	Cyclosporin, MMF	TDF/FTC/FPV
6	51	F	Kidney	6.9	798	80[a]	Cyclosporin, Mycophenolate	Cyclosporin, Mycophenolate	ABC/3TC/DTG
7	55	M	Liver	7.1	398	182	Cyclosporin, MMF	Cyclosporin, MMF	TDF/FTC/EFV
8	59	M	Liver	5.0	502	181	Cyclosporin, Mycophenolate	Cyclosporin, Mycophenolate	TDF/FTC/EFV
9	62	M	Liver	7.8	267	145	Cyclosporin, Mycophenolate	Cyclosporin, Mycophenolate	TDF/FTC/RAL
10	61	M	Liver	13.3	252	>348[b]	MMF	MMF[c]	TDF/FTC/r/DRV

MMF = mycophenolate mofetil; TDF =tenofovir; RPV = rilpivirine; DTG = dolutegravir; RAL = raltegravir; ABC = abacavir; 3TC = lamivudine; EFV = efavirenz; r/DRV = ritonovir boosted darunavir; FPV = fosamprenavir

[a] participant stopped everolimus due to diarrhea

[b] participant continued on everolimus therapy throughout the 12 month study duration

[c] MMF stopped 2 months following everolimus initiation