Modeling simian immunodeficiency virus (SIV) latency in primary rhesus macaque CD4^{+} T cells

Lea M. Matschke[a] and Matthew R. Reynolds[a,b]#

[a]*Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison WI*

[b]*Wisconsin National Primate Research Center, University of Wisconsin, Madison WI*

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Address correspondence to Matthew Reynolds, matt.reynolds@wisc.edu

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Abstract

Simian immunodeficiency virus (SIV)-infected rhesus macaques are valuable models for HIV cure research, offering insights into tissue reservoirs and enabling the evaluation of reservoir-reduction strategies. However, low frequencies of latently infected cells *in vivo* limit mechanistic studies of viral latency *ex vivo.* While *in vitro* latency models have advanced our understanding of HIV persistence, comparable models for SIV are lacking. To address this gap, we developed an *in vitro* model of SIV latency in primary rhesus macaque $CD4^+$ T cells. Our optimized cell culture conditions promote viral entry into $CD4^+$ T cells with minimal cellular activation or proliferation. After 12 days in culture, 1–3.3% of cells harbored SIV DNA, primarily as intact proviruses within central and transitional memory $CD4^+$ T cell subsets. These cells remained quiescent, exhibiting minimal spontaneous viral protein production, but could be reactivated by T-cell stimulation and benchmark latency-reversing agents. Collectively, this model generates SIV-latently infected cells that resemble predominant cellular reservoirs *in vivo*—quiescent memory $CD4^+$ T cells carrying inducible proviruses. This system provides a platform to investigate mechanisms of SIV latency, identify shared and virus-specific features of HIV and SIV persistence, and evaluate strategies to reactivate viral reservoirs.

Importance

HIV's ability to persist in a dormant state within $CD4^+$ T cells remains a major obstacle to developing a cure. Rhesus macaques infected with simian immunodeficiency virus (SIV) serve as valuable models for studying HIV, but research on viral persistence is limited by the low frequency of latently infected cells *in vivo*. While *in vitro* models have

advanced our understanding of HIV latency, comparable tools for studying SIV were not available. To address this gap, we established a model of SIV latency using rhesus macaque $CD4^+$ T cells, mirroring key features of natural reservoirs. This model provides a platform for studying how viral latency is established and maintained, conducting direct comparisons between HIV and SIV infections, and evaluating potential cure strategies.

Introduction

In vitro models of human immunodeficiency virus (HIV) latency have been instrumental in identifying viral and cellular factors that regulate latency (1). These models complement *ex vivo* studies on patient-derived latently infected cells, whose scarcity *in vivo* makes them difficult to analyze (2□4). Similarly, simian immunodeficiency virus (SIV)-infected rhesus macaques serve as valuable models of HIV persistence, enabling longitudinal assessment of tissue reservoirs and preclinical testing of therapeutic interventions. While HIV and SIV share key biological features, they have evolved host-specific adaptations, suggesting that different mechanisms may govern their latency (5, 6). However, primary cell-based *in vitro* latency models have not yet been developed for SIV. Adapting these models to SIV would allow direct comparisons of the mechanisms that maintain and reverse HIV/SIV latency, strengthening preclinical testing of cure strategies in rhesus macaques.

In vitro latency models aim to replicate key features of HIV reservoirs, including the activation state and metabolic profile of naturally infected cells (1, 7, 8). In both

antiretroviral therapy (ART)-treated humans and rhesus macaques, HIV/SIV reservoirs primarily consist of long-lived resting memory $CD4^+$ T cells (9□13). Early models of HIV latency relied on immortalized cell lines with integrated proviruses, providing crucial insights into the regulation of viral gene expression (14, 15). However, these cells' high metabolic activity and continuous proliferation did not accurately reflect the quiescent nature of resting $CD4^+$ T cells *in vivo*, limiting their relevance for investigating cell-intrinsic mechanisms of viral latency (1, 16).

To better reflect naturally infected cells, several groups developed HIV latency models using primary $CD4^+$ T cells. One such approach directly infects quiescent cells (1). These models share a major challenge: resting $CD4^+$ T cells are inherently resistant to retroviral infection and restrict key steps in virus replication, including host cell entry, reverse transcription, and nuclear import (17, 18). To overcome these barriers, direct infection models typically treat resting T cells with cytokines (e.g., IL-2, IL-7, IL-15) (19□21) or chemokines (e.g., CCL19, CCL21) (22, 23) to increase their permissiveness, followed by spinoculation to enhance virus-cell interactions (24, 25). While these models generate relatively few latently infected cells (1, 7), their responses to mitogens and latency reversal agents (LRAs) can resemble patient-derived $CD4^+$ T cells (7, 23). However, despite their utility in advancing HIV research, the lack of comparable SIV latency models has limited the ability to conduct parallel mechanistic studies between humans and rhesus macaques (1).

To address this gap, we developed an *in vitro* model of SIV latency using primary rhesus macaque $CD4^+$ T cells. This approach primarily established latent infections in resting memory $CD4^+$ T cells, which predominantly harbor HIV and SIV *in vivo* (12, 26). The cultured cells remained quiescent and contained mostly intact proviruses, which were inducible upon stimulation. Overall, this model bridges *in vitro* HIV latency models with *in vivo* SIV studies, providing a useful tool to investigate mechanisms of SIV latency, evaluate responses to candidate LRAs, and compare HIV and SIV persistence.

Results

Modeling SIV latency in primary rhesus macaque $CD4^+$ T cells

Our goal was to develop an *in vitro* method for generating latently infected cells using replication-competent SIV and primary rhesus macaque $CD4^+$ T cells, building on direct infection models of HIV latency (19, 22). Specifically, we aimed to: (1) increase the susceptibility of resting $CD4^+$ T cells to SIV infection, (2) limit cellular activation and proliferation, and (3) minimize spontaneous SIV production.

Our first objective was to prime resting $CD4^+$ T cells for SIV infection without inducing overt activation or proliferation. To achieve this, we sought to decouple viral permissivity from cellular activation by targeting the restriction factor sterile alpha motif and histidine-aspartate domain-containing protein 1 (SAMHD1). In quiescent T cells, SAMHD1 restricts infection by depleting cytoplasmic deoxynucleoside triphosphates (dNTPs), limiting their availability for viral DNA synthesis (17, 27). However, the antiviral activity of SAMHD1 is suppressed by the cyclin A1/Cdk1 complex after cells enter the G_1 phase of

the cell cycle (28). We aimed to exploit this regulatory network by reversibly transitioning $CD4^+$ T cells into G_1 phase through two complementary approaches: stimulating with IL-15 (19, 29) and inhibiting forkhead box protein O1 (FOXO1), a global transcription factor and positive regulator of cellular quiescence (30). Importantly, without additional proliferative signals, $CD4^+$ T cells that enter G_1 revert to cellular quiescence (ie., G_0 phase) once FOXO1 activity resumes (7, 31).

To implement this approach, we cultured resting $CD4^+$ T cells with IL-15 and the small-molecule FOXO1 inhibitor AS1842856, along with low-dose IL-2 to support cell survival. After five days, we infected the cells with clonal SIV via “magnetofection,” a technique that enhances virus-cell interactions by coupling virions to ferrous nanoparticles and applying a strong magnetic field (24). After infection, cells were cultured for two days without supplemental cytokines or antiretroviral drugs to foster proviral integration. At 2 days post-infection (dpi), we transitioned the cultures to an immunosuppressive medium containing the HIV/SIV protease inhibitor saquinavir to limit ongoing virus replication, along with TGF-β, IL-8, and IL-10 to suppress T cell activation and promote viral latency (32). After five additional days, we selectively depleted cells expressing activation markers CD25 or HLA-DR, yielding a homogeneous population of resting $CD4^+$ T cells (Fig. 1).

Culture conditions do not drive T cell differentiation

To determine whether our culturing conditions preserved physiologically relevant $CD4^+$ T cell populations, we characterized major $CD4^+$ T subsets using memory-associated

surface markers CD28, CD95, and CCR7 (33). In rhesus macaques, these markers distinguish naive (T_N; $CD28^-/CD95^-$), central memory (T_{CM}; $CD28^+/CD95^+/CCR7^-$), transitional memory (T_{TM}; $CD28^+/CD95^+/CCR7^-$), and effector memory (T_{EM}; $CD28^-/CD95^+$) $CD4^+$ T cell subsets. At the end of the culture period, the distribution of these subsets closely resembled that of $CD4^+$ T cells freshly isolated from peripheral blood (15-35% $T_{CM/TM}$, 1-10% T_{EM}, 30-75% T_N) (Fig. 2) (33). These findings indicate that the culture conditions preserved the native composition of $CD4^+$ T cell subsets, in line with previously described direct infection models of HIV latency (19, 34).

$CD4^+$ T cells are maintained in a resting state

To determine whether IL-15 signaling and FOXO1 inhibition induced proliferation, we examined cell division at three key time points: immediately after $CD4^+$ T cell isolation (day 0), at the time of infection (day 5), and at the end of the culture period following $CD25^+$ and HLA-DR^+ depletion (day 12). At each time point, we assessed DNA ploidy using the intercalating dye 7-AAD and monitored cell division based on BrdU incorporation over 20 hours. Cells were categorized as apoptotic ($BrdU^-/7\text{-}AAD^{low}$), dividing ($BrdU^+/7\text{-}AAD^{intermediate/high}$) ($S + G_2 + M$ phases), or non-dividing ($BrdU^-/7\text{-}AAD^{intermediate}$) ($G_0 + G_1$ phases) (Fig. S2A). At all three time points, ≥98.9% of live $CD4^+$ T cells remained non-dividing, indicated by the absence of BrdU incorporation (mean±SD: 99.5±0.4) (Fig. 3A-B; Fig. S2B). In contrast, activated $CD4^+$ T cell controls showed markedly higher BrdU incorporation and 7-AAD staining (Fig. S2A). To distinguish non-cycling T cells in G_0 and G_1, we measured intracellular Ki-67 expression, a marker of cellular proliferation expressed after G_1 entry (Fig. S2C). Ki-67

expression remained low and stable throughout the culture period (mean±SD: 0.9±0.1), with only 0.8±0.5% of live $CD4^+$ T cells expressing Ki-67 on day 12 (Fig. 3C; Fig. S2D). Together, these results indicate that IL-15 signaling and FOXO1 inhibition did not induce T cell proliferation, as the cells maintained resting phenotypes throughout the culture period, consistent with previous studies (35□38).

We next evaluated whether the culturing conditions induced T cell activation by measuring CD25 and HLA-DR expression at the same three time points. By day 5, $CD25^+$ frequencies increased significantly from day 0 (p=.004), though levels remained significantly lower than in activated controls (p=.003; Fig.3C). This ~7.8% increase aligns with previous reports of IL-15-mediated CD25 upregulation in human $CD4^+$ T cells (19) and does not exceed ranges previously reported for resting cells (20, 39□41). HLA-DR expression showed a modest, non-significant increase over the same period (p=.08, Fig. 3D). As expected, exposure to immunosuppressive cytokines followed by selective depletion of $CD25^+$ and HLA-DR^+ cells reversed these trends, returning to baseline frequencies by day 12 (Fig. 3C-D). Thus, while early culture conditions transiently upregulated activation markers, the final $CD4^+$ T cell populations exhibited activation levels comparable to freshly isolated $CD4^+$ T cells, supporting a resting cell phenotype (41).

Cells harbor SIV DNA primarily within $T_{CM/TM}$ cell subsets

To evaluate the efficiency of our infection procedures, we quantified cell-associated SIV DNA (CA-vDNA) in $CD4^+$ T cells at the end of the culture period using droplet digital

PCR (ddPCR) targeting SIV *gag* and normalized to cellular genome equivalents based on the housekeeping gene ribonuclease protein p30 (*rpp30)* gene. On average, cultures contained ~10,000 SIV *gag* DNA copies per million cell equivalents (eq.) (Fig. 4A) (mean±SD: 10,316±5,924; min-max: 1,630-18,674; 95% CI: 4,837-15,794), consistent with direct infection models of HIV latency (22, 23, 39). Infection rates varied between replicates and donors, reflecting both experimental and biological variability, similar to findings in primary cell models of HIV latency (7).

We next explored the distributions of latently infected cells across major $CD4^+$ T subsets by sorting live $CD4^+$ cells into three populations: T_N ($CD28^+$ $CD95^-$), $T_{CM/TM}$ ($CD28^+$ $CD95^+$), and T_{EM} ($CD28^-$ $CD95^+$) (Fig. S1A-B). After sorting, we quantified CA-vDNA in each subset. Consistent with *in vitro* models of HIV latency, SIV DNA was most abundant on a per-cell basis in T_{EM} cells (mean±SD: 9,451±7,084 *gag* copies per million cell eq.; min-max: 3,759-17,384) (Fig. 4B), likely reflecting their heightened metabolic activity. However, since T_{EM} cells constitute only a small fraction of peripheral $CD4^+$ T cells (Fig. 2A), they accounted for only ~6% of total infected populations (Fig. 4C). In contrast, T_N cells, which are abundant in the blood, harbored low frequencies of SIV DNA (mean±SD:139±136 copies per million cell eq.; min-max: 0-273) (Fig. 4B), representing only ~9% of total SIV-infected cells (Fig. 4C). As expected, the majority of latently infected cells resided within the $T_{CM/TM}$ subsets (mean±SD: 3,576±2,305 copies per million cell eq.; min-max: 1,635-6,124), representing ~86% of all SIV latently infected $CD4^+$ T cells, consistent with their contribution to latent SIV reservoirs in ART-suppressed rhesus macaques (Fig. 4B-C) (42).

SIV proviruses are primarily intact

Quantifying SIV *gag* alone provides valuable insights into infection rates but may overestimate the frequency of replication-competent infections by including unintegrated or defective proviruses, such as incomplete reverse transcripts or hypermutated genomes. To address this limitation, we employed the intact proviral DNA assay (IPDA), which selectively quantifies intact proviruses, excludes those with common defects, and corrects for sequence shearing during DNA extraction (43, 44). This assay offers a more comprehensive assessment of the proviral DNA landscape than measuring SIV *gag* alone, allowing us to distinguish between intact—potentially replication-competent proviruses—and defective viral genomes.

Our analysis revealed that cultured $CD4^+$ T cells contained approximately 16,000 intact proviral copies per million cell eq. (mean±SD: 16,420±7,211; min-max: 6,908-24,215; 95% CI: 4,945-27,894) (Fig. 5A). Intact proviruses accounted for ~66% of total SIV DNA (Fig. 5B), suggesting that high proportions of proviral genomes were replication-competent. Additionally, unintegrated 2-long terminal repeat (2-LTR) circles comprised ~9% of total SIV DNA (mean±SD: 2,247±1,219 copies per million cell eq.; min-max: 622-3,552; 95% CI: 307-4,187) (Fig. 5A-B). Defective proviral DNA—characterized by 5' or 3' deletions and/or hypermutations—comprised only ~24% of total SIV DNA (Fig. 5B). These findings highlight the robustness of our model in generating SIV-infected $CD4^+$ T cells with a low proportion of defective proviruses.

SIV latently infected cells contain reactivatable virus

While the SIV IPDA distinguishes between intact and defective proviruses, additional factors—such as proviral integration sites, epigenetic modifications, and host transcription factor availability—may influence viral reactivation (45). To determine whether the intact proviruses in our model were functional, we assessed their ability to produce viral proteins following cellular activation. We incubated cells either with anti-CD2/CD3/CD28-coated T cell activation beads for 36 hours or left them untreated as controls. We then measured intracellular SIV Gag using two monoclonal antibodies that target distinct epitopes, which enhanced both sensitivity and specificity of detection. Upon stimulation, 0.2–0.6% of live T cells expressed SIV Gag (Fig. 6B), a level comparable to HIV direct infection models (22, 23). In contrast, unstimulated cells showed minimal spontaneous virus production, with SIV Gag expression consistently below 0.12% of live T cells (Fig. 6A). These results demonstrate that our system generates SIV-infected cells that rarely express viral proteins when unstimulated, but readily reactivate in response to strong stimuli.

We next evaluated how our latently infected cells responded to benchmark LRAs with distinct mechanisms of action: IL-15 (PI3K/Akt activator), SAHA (histone deacetylase inhibitor), bryostatin-1 (protein kinase C agonist), tumor necrosis factor α (TNF-α; NF-κB activator), and ionomycin (calcium ionophore; NFAT activator). Cultured cells were exposed to individual LRAs in the presence of raltegravir, an integrase inhibitor that prevents ongoing viral replication, for 48 hours. SIV Gag levels in culture supernatants

were then quantified by ELISA and normalized to unstimulated (baseline) and bead-stimulated (maximum induction) controls [n=3] (Fig. 7).

IL-15, bryostatin-1, and ionomycin significantly increased virion production, whereas TNF-α and SAHA had minimal effects. Notably, the most potent LRAs activated transcription factors that recruit positive transcription elongation factor b (p-TEFb) to the viral promoter. Specifically, bryostatin-1 activates NF-κB, ionomycin modulates NFAT, and IL-15—the strongest inducer—can affect both pathways, leading to p-TEFb-mediated transcription of viral genes (19, 46, 47). Despite activating NF-κB, TNF-α was ineffective, possibly due to low TNF-α receptor expression on peripheral memory $CD4^+$ T cells, as reported in primary cell cultures and HIV-infected individuals (7, 48). These findings align with HIV direct infection models and highlight the central role of NF-κB and NFAT signaling in SIV reactivation (7, 49, 50). Taken together, our *in vitro* model generates SIV latently infected cells that respond differentially to LRAs, demonstrating its utility for studying mechanisms of SIV latency and testing latency reversal strategies.

Discussion

In this study, we describe an *in vitro* model of SIV latency using primary rhesus macaque $CD4^+$ T cells, bridging existing HIV latency models and *in vivo* macaque studies. Our approach reliably establishes latent infections in resting memory $CD4^+$ T cells, recapitulating key features of *in vivo* HIV/SIV reservoirs. These cells predominantly harbor intact proviruses that remain inducible upon stimulation. Altogether, this model provides a valuable platform for investigating mechanisms of viral

latency and persistence, as well as for evaluating HIV cure strategies before advancing to *in vivo* studies in rhesus macaques.

A major challenge in developing this model was overcoming the intrinsic resistance of quiescent $CD4^+$ T cells to SIV infection. Although both HIV and SIV can infect resting T cells (29, 51), cellular quiescence presents multiple barriers to infection, including a rigid actin cytoskeleton and low levels of cytosolic dNTPs and ATP, which collectively hinder viral entry, reverse transcription, and nuclear import (17, 49, 52□55). Treatment with IL-15 and the FOXO1 inhibitor AS1842856 can overcome these barriers and enhance retroviral infection of resting cells (19, 31, 56, 57). Among these restrictions, a key block occurs during reverse transcription in quiescent cells, where SAMHD1 continuously depletes cytosolic dNTPs (17). During G_1 phase entry, the cyclin A2/Cdk1 complex phosphorylates and inhibits SAMHD1 activity, increasing dNTP availability for viral DNA synthesis (28). Both IL-15 signaling and FOXO1 inhibition have been shown to enhance Cdk1 activity in primary human T cells, potentially explaining their ability to support SIV infection in resting rhesus macaque $CD4^+$ T cells (19, 31). Additionally, IL-15 likely also acts through the mTOR pathway to boost cellular metabolism and expand dNTP pools needed for reverse transcription (58). Furthermore, these treatments may promote viral entry and nuclear trafficking by upregulating host cell surface receptors, increasing ATP production, and remodeling the actin cytoskeleton (29, 37, 57, 59).

With these optimized conditions for establishing latent infections in resting T cells, our model provides a platform for investigating SIV latency and mechanisms of reactivation

in primary cells. The maintenance and reversal of retroviral latency depend on multiple factors, including proviral integration sites, chromatin accessibility, epigenetic modifications, transcription factors, and the viral protein Tat (45). When we tested our model's response to various LRAs, we found that compounds modulating host transcription factors effectively reactivated SIV, whereas the histone deacetylase (HDAC) inhibitor SAHA had minimal effects. These differential responses, consistent with previous studies (7, 60, 61), indicate that transcription factors such as NF-κB and NFAT play a more prominent role in reversing SIV latency than epigenetic remodeling alone. However, combining HDAC inhibitors with transcription factor modulators often produces synergistic effects on viral reactivation—an approach that may further enhance SIV reactivation in our system (60□64). Notably, the LRA response profile of latently SIV-infected cells in our model resembled those observed in HIV direct infection models and patient-derived latently infected cells (7, 65), demonstrating our model's relevance for future comparative studies between HIV and SIV latency.

Intriguingly, the latently infected cells generated by our *in vitro* model could potentially be used to establish SIV reservoirs of defined size and composition in rhesus macaques. By infusing these cells into SIV-naïve animals, this approach would allow the creation of viral reservoirs with known quantities of intact proviruses. When testing LRAs, such an approach would enable direct comparisons of the same cell populations in both laboratory and host environments. This controlled system could reveal how tissue microenvironments, immune factors, and other *in vivo* conditions influence viral persistence and reactivation patterns.

Despite its strengths, our study has some limitations. One challenge is that the latently infected cells generated by our model contain relatively few effector memory T cells, which can be a sizable component of *in vivo* reservoirs (66). This outcome was anticipated, as peripheral $CD4^+$ T cells—the source for our cultures—are mainly comprised of naïve and central memory T cells (33). Enriching for memory $CD4^+$ T cells at the start of the culture period could help address this limitation; however, rhesus macaques lack naïve T cell markers equivalent to human or murine CD45RA, complicating the selective depletion of naïve cells (33). Additionally, we did not specifically quantify all forms of unintegrated vDNA, such as 1-LTR circles or linear intact proviruses. However, these proviral forms are unlikely to substantially impact our findings, as 1-LTR circles are rarely intact due to their mode of formation, and unintegrated linear proviruses decay rapidly (67□69). Assuming that SIV follows decay kinetics similar to HIV in direct infection models, over 85% of linear proviruses are lost within three days of infection (68), making it unlikely that substantial amounts persisted after five days of culture. Future refinements of our model—such as improving effector memory T cell representation—could enhance its utility for investigating mechanisms of viral latency and reactivation.

In conclusion, this study describes a direct infection model of SIV latency using primary rhesus macaque $CD4^+$ T cells, offering a physiologically relevant system for studying viral persistence *in vitro*. Our approach generates infected cells that closely resemble *in vivo* latent reservoirs, with latency predominantly established in resting $CD4^+$ $T_{CM/TM}$

cells harboring intact, inducible proviruses. By enabling direct comparisons between HIV and SIV latency, this model may serve as a valuable tool for identifying both shared and virus-specific mechanisms of persistence. Furthermore, it provides a preclinical platform for evaluating novel LRAs across species, potentially enhancing the translational relevance of SIV/rhesus macaque models for HIV cure research.

Materials & Methods

Animal handling & care

Rhesus macaques (*Macaca mulatta*) were maintained at the Wisconsin National Primate Research Center (WNPRC) in accordance with guidelines approved by the University of Wisconsin-Madison Graduate School Institutional Animal Care & Use Committee (IACUC protocol G006049) and recommended in the National Research Council's Guide for the Care and Use of Laboratory Animals' Weatherall Report. Staff administered anesthesia and/or fluid replacement therapies as needed.

Production of SIV stocks

We generated SIV_{mac239} stocks from hemi-genome plasmids (NIH AIDS Research and Reference Reagent Program), as described previously (70). Briefly, we seeded 5 x 10^5 Vero cells into individual wells of a 6-well tissue culture plate and cultured them overnight in R10 media (RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution, and 1% L-glutamine; all from Cytiva except the FBS, which was from Corning) at 37˚C with 5% CO_2. The following day, we transfected the Vero cells with SIV_{mac239}-encoding plasmids using Lipofectamine (Invitrogen) and added

CEMx174 cells to the cultures 24 hours later. After two additional days, we transferred the CEMx174 cells to T75 flasks and monitored them for syncytia formation. Upon observing widespread syncytia, we harvested cell-free supernatants daily for three consecutive days. The virus-containing supernatants were then divided into 1mL aliquots and stored in liquid nitrogen.

In vitro latency model

Overview: We developed a 12-day protocol to generate latently SIV-infected $CD4^+$ T cells isolated from rhesus macaque peripheral blood, consisting of cell isolation and priming, virus preparation, SIV infection via magnetofection, immunosuppressive conditioning, and depletion of activated cell populations.

i. $CD4^+$ T cell isolation and priming: We obtained PBMCs from whole blood by density-gradient centrifugation with Ficoll-paque PLUS (Cytiva). $CD4^+$ T cells were isolated from PBMCs using a nonhuman primate $CD4^+$ T cell isolation kit (Miltenyi Biotec) according to manufacturer's instructions. $CD4^+$ T cells were resuspended in Priming Media at 5 x 10^6-8 x 10^6 cells/mL and seeded in flat-bottom 48-well tissue culture plates (Corning) at 1 mL per well. Priming Media consisted of R10 supplemented with recombinant human IL-2 (10U/mL; R&D Systems), recombinant human IL-15 (10ng/mL; R&D Systems), and FOXO1 inhibitor AS1842856 (500nM; Sigma Aldrich). After 2-3 days, we replaced ≥500 µL in each well with fresh Priming Media. Cells were maintained at 37˚C with 5% CO_2 throughout the 12-day culture period.

ii. Virus stock purification: Before use, we purified SIV stocks using a 20% sucrose cushion to remove residual proteins (70). Briefly, virus stocks were layered onto 100 µL of 20% sucrose solution and centrifuged at >50,000xg for 1.5-3 hours at 4°C. After discarding supernatants, virus-containing fractions (~30-100µL) were diluted with ~150 µL of phosphate-buffered saline (PBS) at room temperature.

iii. SIV infection: On day 5, we replaced Priming Media with equal volumes of R10 and infected cultured cells with SIV by magnetofection (24). First, 150-250 µL purified virus in PBS was added to 30 µL Viromag R/L transduction reagent (OZ Biosciences) at room temperature. After 15 minutes, this mixture was added to cultured cells and centrifuged at 670xg for 5 minutes. We then incubated the pelleted cells on Super Magnetic Plates (OZ Biosciences) for 1-3 hours at 37°C before resuspending.

iv. Immunosuppressive conditioning: At 2 days post-infection (day 7), we replaced R10 with equal volumes of Immunosuppressive Media, consisting of R10 supplemented with saquinavir mesylate (5µM; Sigma Aldrich), recombinant human TGF- β1 (10ng/mL; R&D Systems), recombinant human IL-8/CXCR8 (50ng/mL; R&D Systems), and recombinant human IL-10 (10ng/mL; Peprotech). Between days 9 and 10, we replenished ≥500 µL of Immunosuppressive Media in each well.

v. Depletion of cells expressing activation markers: To obtain final populations, we depleted activated [ie., $CD25^+$ or $HLA\text{-}DR^+$] subsets on day 10 or 12 of the culture period. Briefly, we incubated cells with biotinylated anti-CD25 (clone BC96; Biolegend)

and anti-HLA-DR (clone LN3; Biolegend) monoclonal antibodies for 15 minutes at 4˚C. Once labeled, cells were incubated with anti-biotin microbeads (Miltenyi Biotec) for 15 minutes at 4˚C and passed through LS columns (Miltenyi Biotec) according to manufacturer's instructions. Final populations were collected from negative fractions.

Flow cytometry

i. General: To detect cell surface markers, we stained cells with viability dye and fluorophore-conjugated antibodies at room temperature for 30-45 minutes. After two PBS washes, cells were fixed with 2% paraformaldehyde (PFA) in PBS for >10 minutes at room temperature. For intracellular staining, fixed cells were subsequently washed with FACS buffer (2% FBS in PBS) and permeabilized in Medium B Permeabilization Buffer (Invitrogen) with corresponding antibodies for 20 minutes at room temperature. Cells were then washed twice with FACS buffer and fixed with 2% PFA. For cell cycle analysis, we added 7-amino-actinomycin (7-AAD) intercalating dye after the final wash, according to manufacturer's instructions. Data were collected on a Becton Dickinson (BD) FACSymphony A3 flow cytometer with FACSDiva acquisition software and analyzed with FlowJo v10 (TreeStar Inc.). Gating strategies are shown in Fig. S1.

ii. Cell surface staining: Cells were labeled with Near-IR fixable dead cell dye (Invitrogen) and antibodies against the following surface markers: CD3-Alexa Fluor 700 (clone SP34-2, BD), CD4-APC (clone OKT4, Biolegend), CD28-PE (clone CD28.2, BD), CD95-PE-Cy5 (clone DX2, BD), CCR7-Brilliant Violet 605 (clone G043H7, Biolegend),

CD25-Brilliant Violet 711 (clone M-A251, Biolegend), and HLA-DR-PE/Dazzle594 (clone L243, Biolegend).

iii. Cell cycle analysis: We assessed BrdU uptake, DNA ploidy, and activation marker expression on days 0, 5, and 12 of the protocol. For comparison, primary $CD4^+$ T cells were cultured in parallel with recombinant human IL-2 (70U/mL; R&D Systems) and nonhuman primate T cell activation beads (Miltenyi Biotec) at 0.6 beads/cell (Fig. S2A/C). Prior to each time point, we cultured 1x10^5–1x10^6 cells with and without BrdU for 20 hours. Cells were then stained with Near-IR fixable dead cell dye (Invitrogen) and antibodies against CD4-APC (clone OKT4, Biolegend), CD25-Brilliant Violet 421 (clone M-A251, Biolegend), HLA-DR-PE/Dazzle594 (clone L243, Biolegend), Ki-67-Brilliant Violet 421 (clone B56, BD), as well as BrdU-FITC and 7-AAD (both from the FITC BrdU Flow Kit, BD). Data represent three mock infections of live $CD4^+$ T cells (Fig. S2 A–B) from a single donor.

iv. Intracellular SIV Gag staining: On day 10.5, final populations were divided into stimulated or unstimulated groups and cultured at <8 x 10^6 cells/mL for the remainder of the culture period. For unstimulated groups, we replaced culture media with fresh Immunosuppressive Media without saquinavir to permit virus replication. Stimulated groups were cultured in R10 with raltegravir (50µM; SelleckChem) and T cell activation beads (Miltenyi Biotec) at 0.6 beads/cell. After 36 hours at 37˚C with 5% CO_2, cells were labeled with viability dye and antibodies against surface markers as well as anti-SIV

Gag p27-FITC (clone 55-2F12) and anti-HIV Gag p24-Pacific Blue (clone 183-H12-5C), which recognize distinct epitopes on SIV Gag p27. The anti-HIV Gag p24 monoclonal antibody was isolated from hybridoma (ARP-1513; NHP AIDS Reagent Resource) supernatants and conjugated to Pacific Blue using a commercial kit (Invitrogen), following manufacturer's instructions. The cells were gated on singlets/lymphocytes/live/$CD3^+$/$p24^+$/$p27^+$.

Cell sorting

To isolate distinct memory and naive T cell populations (33), we labeled the final cell preparations with Near-IR fixable dead cell stain (Invitrogen) and antibodies against CD4-APC (clone OKT4, Biolegend), CD28-PE (clone CD28.2, BD), and CD95-PE-Cy5 (clone DX2, BD) antibodies for 30 minutes at room temperature. After two PBS washes, we sorted live $CD4^+$ cells into three subsets using a BD FACSJazz Cell Sorter: naïve (T_N; $CD28^+$/$CD95^-$), central/transitional memory ($T_{CM/TM}$; $CD28^+$/$CD95^+$), and effector memory (T_{EM}; $CD28^-$/$CD95^+$) (Fig. S1A-B).

DNA extraction

We extracted genomic DNA from cultured $CD4^+$ T cells one of two Qiagen kits: the DNeasy Blood & Tissue Kit for SIV *gag* quantification or the QIAmp DNA Mini Kit for the IPDA, as previously described (43, 71). Excess sample eluent was removed using the Vacufuge Plus System (Eppendorf), and DNA concentrations were measured using a Nanodrop One spectrophotometer (ThermoFisher).

SIV *gag* ddPCR

Cell-associated viral DNA (CA-vDNA) was measured by SIV *gag* ddPCR. To prepare for the assay, we digested sample DNA with EcoRI-HF (New England Biolabs) per the manufacturer's instructions. SIV *gag* and host *rpp30* were quantified in separate singleplex ddPCRs on the Bio-Rad QX200 ddPCR System, as previously described (71, 72) . Briefly, each 20 µL reaction contained 3.2 µL digested DNA (≤1,000ng), ddPCR Supermix for Probes (no dUTP) (Bio-Rad), 900nM primers, and 250nM probes (all from Integrated DNA Technologies (IDT)) (Table S1).

To prevent oversaturation of positive droplets during *rpp30* amplification, we diluted sample DNA for this target separately at fixed ratios to yield 0.5-2 ng/µL before transferring equal volumes to each 20 µL reaction. Dilution factors were then applied to *rpp30* frequencies to represent equivalent DNA input across all reactions.

For thermal cycling conditions, SIV *gag* reactions, droplets were cycled as follows: 95°C for 10min; 40 cycles of 94°C for 30s and 58°C for 60s; 98°C for 10min; 4°C hold. For singleplex *rpp30* ddPCRs: 95°C for 5min; 40 cycles of 94°C for 15s, 55°C for 30s, 72°C for 30s; 72°C for 5min; 4°C hold. Droplets were analyzed on the QX200 Droplet Reader with Quantasoft Studio Software (Bio-Rad). Data represent duplicate wells averaged across 2-3 replicates. Negative controls included no template controls and uninfected DNA from each macaque. As a positive control, we extracted DNA from 3D8 cells, an SIV-infected cell line (NHP AIDS Reagent Resource).

IPDA

Intact and defective SIV DNA were quantified using the previously described intact proviral DNA assay (IPDA) as previously described (43, 73). Briefly, the SIV IPDA utilizes three duplexed ddPCR reactions targeting the following amplicons: (1) SIV *pol* and *env*(44), (2) SIV 2-LTR junctions (74) and *env*, and (3) two regions of the *rpp30* housekeeping gene (Table S2). For each reaction, corrected data were averaged from duplicate wells across ≥3 replicates. Intact proviruses were defined as pol^+/env^+ sequences without 2-LTR junctions, while total SIV DNA was calculated as the sum of pol^+/env^+, pol^+/env^-, and pol^-/env^+ sequences, irrespective of 2-LTR junctions (43, 75).

SIV reactivation with LRAs

To assess the responsiveness of our latently infected cells to latency reversal agents (LRAs), we compared SIV Gag p27 production in culture supernatants after exposure to benchmark LRAs for 48 hours. Briefly, final populations were resuspended in R10 with raltegravir (50µM; SelleckChem, Houston, TX) and divided equally (1.2-2.5 x 10^6 cells/well) into individual wells of a 96-well plate at 225 µL/well. Parallel cultures were left unstimulated or exposed to individual LRAs for 48 hours, then SIV Gag p27 was quantified in culture supernatants using a commercial ELISA kit (Zeptometrix) according to the manufacturer's instructions. Each replicate compared SIV p27 produced by unstimulated controls to the following stimuli: SAHA (250nM; MilliporeSigma), recombinant human IL-15 (25ng/mL; R&D Systems), bryostatin-1 (25ng/mL; R&D Systems), recombinant rhesus macaque TNF-α (10ng/mL; R&D Systems), ionomycin (750nM; Research Products International), and T cell activation beads (0.6 beads/cell).

To normalize across infection replicates, baseline (unstimulated) SIV p27 concentrations were subtracted from treatment values, and the differences were divided by maximum response (T cell activation beads) to yield percent reactivation. Data represent duplicate wells averaged across three infection replicates [n=3] from a single donor.

Statistical Analysis

Data were analyzed using Prism 10.4 software (GraphPad) by one- or two-way repeated measures (RM) ANOVA with multiple comparisons. All data passed preliminary tests for single-pooled variances. For the analysis of Ki67, CD25, and HLA-DR expression, multiple comparisons were calculated with and without activated controls. Pairwise comparisons to activated controls were performed using the entire data set. For all other comparisons, activated controls were excluded from the analysis to increase statistical power for detecting differences between experimental groups.

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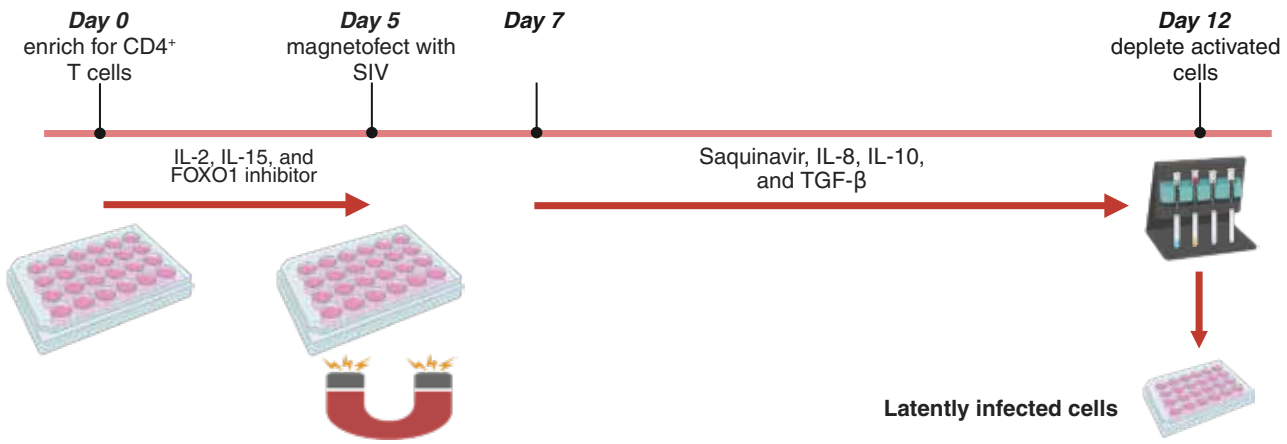

Fig 1 Establishment of an in vitro SIV latency model in primary CD4+ T cells. CD4-expressing cells were enriched from PBMC and cultured from day 0 to 5 in R10 media supplemented with IL-15, IL-2, and a FOXO1 inhibitor (AS1842856). On day 5, cells were infected with SIV via magnetofection and cultured for 48 hours in standard R10 media. On day 7, the medium was replaced with R10 containing the protease inhibitor saquinavir and an immunosuppressive cytokine cocktail (IL-8, IL-10, and TGF-β). On day 12, cells expressing activation markers HLA-DR or CD25 were depleted via magnetic bead separation to generate a homogeneous population of resting CD4+ T cells. Created with https://BioRender.com.

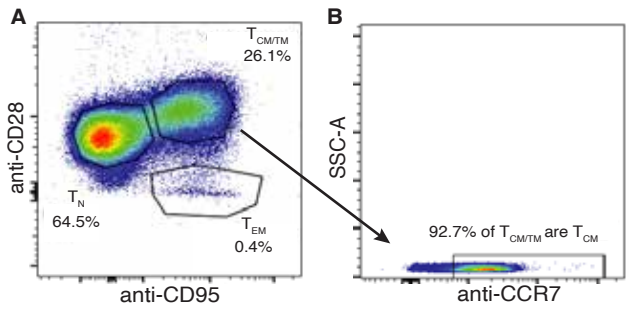

Fig 2 $CD4^+$ T cell memory and naive subsets after 12 days in culture. (A) Representative flow cytometry plots showing the gating strategy for defining naive (T_N; $CD28^+/CD95^-$), effector memory (T_{EM}; $CD28^-/CD95^+$), and central/transistional memory (T_{CM}/T_{TM}; $CD28^+/CD95^+$) T cells. (B) CCR7 expression was used to differentiate between T_{CM} ($CD28^+/CD95^+/CCR7^+$) and T_{TM} ($CD28^+/CD95^+/CCR7^-$) subsets.

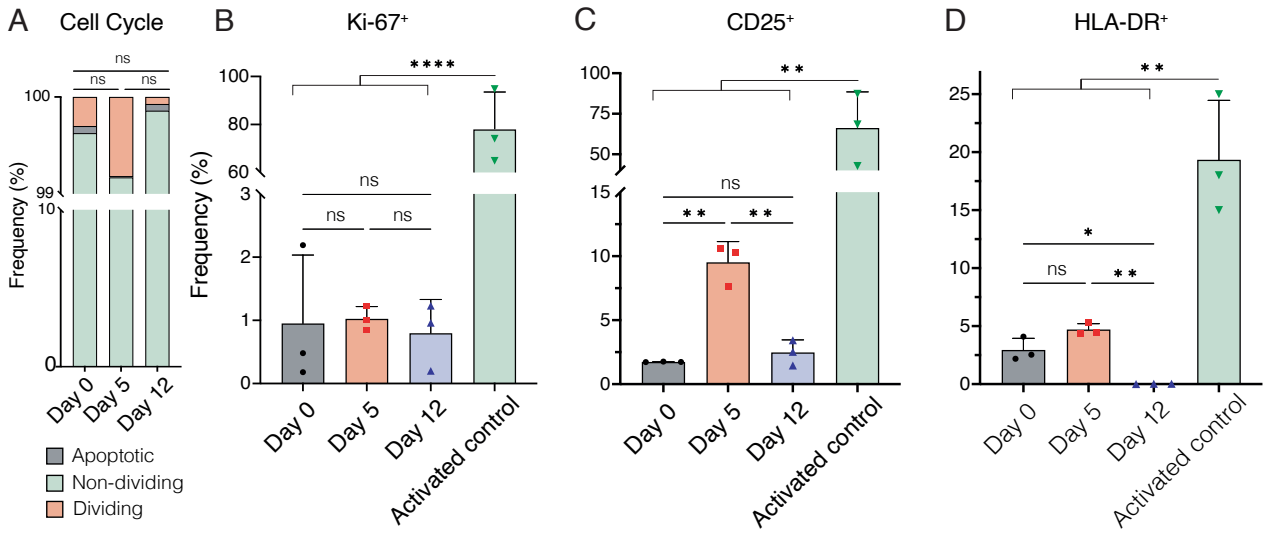

Fig 3 Cultured cells exhibit quiescent, non-activated phenotype. Cell cycle status and activation marker expression were assessed by flow cytometry at three timepoints: after $CD4^+$ T cell isolation (day 0), at infection (day 5), and at the end of culture (day 12). (A) Cell cycle progression was determined by 7-AAD staining and BrdU incorporation over 20 hours to identify apoptotic ($BrdU^-/7\text{-}AAD^{low}$), non-dividing (G0 + G1 phase; $BrdU^-/7\text{-}AAD^{intermediate}$), or dividing (S + G2 + M phases; $BrdU^+/7\text{-}AAD^{intermediate/high}$) cells. (B-D) Expression of activation markers Ki-67 (B), CD25 (C), and HLA-DR (D) at each time point. Data represent mean ± SD from three independent replicates from the same donor. For all panels, statistical analysis was performed using repeated-measures ANOVA: two-way for panel A and one-way for panels B-D, followed by Tukey's post-hoc test. *$p < 0.05$; **$p < 0.01$; **** $p < 0.0001$.

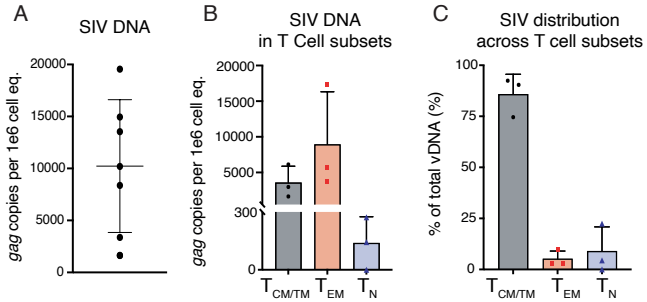

Fig 4 SIV preferentially infects memory CD4⁺ T cells. (A) Total cell-associated viral DNA (vDNA) in CD4⁺ T cells. Each point represents an independent infection replicate (n=7) from three rhesus macaques. (B and C) Live CD4⁺ T cells were sorted into central/transitional (T_{CM}/T_{TM}), naïve (T_N), and effector memory (T_{EM}) populations based on CD28 and CD95 expression. (B) vDNA content of each CD4⁺ T cell subset. (C) Relative contribution of each CD4⁺ T cell subset to the total vDNA pool based on both infection frequencies and relative abundance. Data represent the mean from three infection replicates from a single donor rhesus macaque. vDNA measurements were normalized to cell equivalents (eq) using the rhesus macaque *rpp30* gene. All ddPCR reactions were performed in duplicate with ≥3 technical replicates; error bars indicate standard deviations (SD).

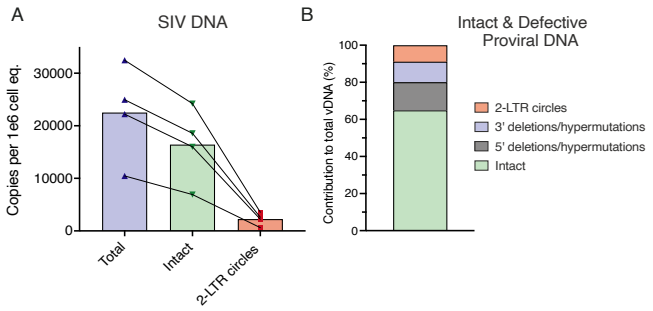

Fig 5 Intact SIV proviral DNA predominates in latently infected cells. (A) Quantification of viral DNA (vDNA) forms using the intact proviral DNA assay (IPDA). Data show total vDNA, intact vDNA, and 2-LTR circles from $CD4^+$ T cells across three independent infection replicates from the same donor. (B) Relative percentage contribution of each vDNA form to the total vDNA detected. vDNA measurements were normalized to cell equivalents (eq.) using the rhesus macaque *rpp30* gene. All ddPCR reactions were performed in duplicate with ≥3 technical replicates.

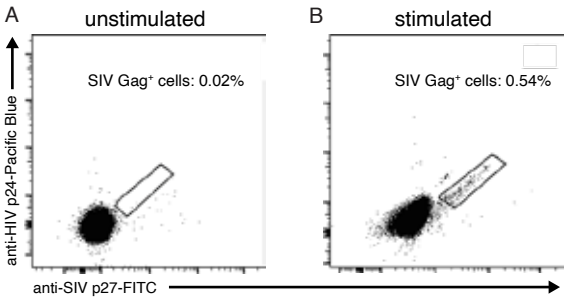

Fig 6 T cell activation triggers SIV reactivation from latency. We collected cells on day 10.5 of the cultures after depleting cells expressing activation markers CD25 or HLA-DR. Cells were either (A) left unstimulated or (B) stimulated with anti-CD2/CD3/CD28-coated beads for 36 hours. Flow cytometry dot plots show intracellular SIV Gag detection using two antibodies binding distinct epitopes: anti-SIV p27-FITC (clone 55-2F12, x-axis) and anti-HIV p24-Pacific Blue (clone 183-H12-5C, y-axis). These representative analyses used cells from a single *in vitro* latency culture, with gating on singlets/lymphocytes/live/CD3$^+$.

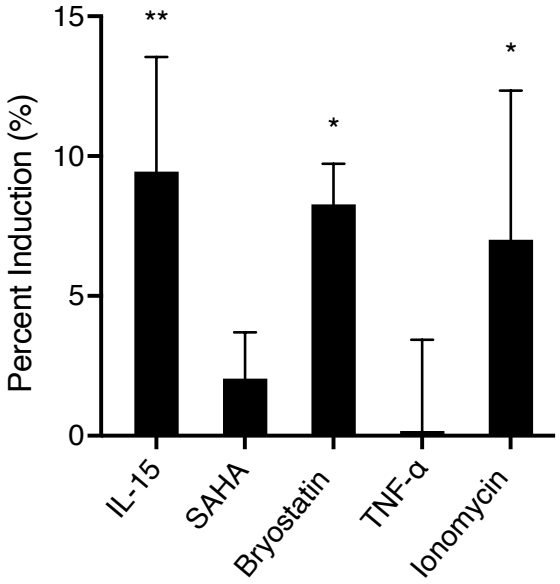

Fig 7 Latency reversal agents induce SIV reactivation. We collected cells on day 10.5 of the cultures after depleting activated cells and treated them with benchmark LRAs plus the integrase inhibitor raltegravir. After 48 hours, we measured extracellular SIV p27 in culture supernatants by ELISA. Data are normalized across three independent infection replicates from a single donor, with unstimulated controls set as 0% and anti-CD2/CD3/CD28 bead stimulation defined as 100% activation. Statistical significance was calculated by 1-way ANOVA with Dunnett's multiple comparisons test comparing each LRA to unstimulated controls. Error bars represent standard deviation. *, $p<0.05$; **, $p<0.01$.