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RESEARCH ARTICLE

The Per-1 Short Isoform Inhibits *de novo* HIV-1 Transcription in Resting CD4+ T-cells

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Abstract: ***Background*:** Understanding of the restriction of HIV-1 transcription in resting CD4+ T-cells is critical to find a cure for AIDS. Although many negative factors causing HIV-1 transcription blockage in resting CD4+ T-cells have been found, there are still unknown mechanisms to explore.

***Objective*:** To explore the mechanism for the suppression of de novo HIV-1 transcription in resting CD4+ T-cells.

***Methods*:** In this study, a short isoform of Per-1 expression plasmid was transfected into 293T cells with or without Tat's presence to identify Per-1 as a negative regulator for HIV-1 transcription. Silencing of Per-1 was conducted in resting CD4+ T-cells or monocyte-derived macrophages (MDMs) to evaluate the antiviral activity of Per-1. Additionally, we analyzed the correlation between Per-1 expression and viral loads *in vivo*, and silenced Per-1 by siRNA technology to investigate the potential anti-HIV-1 roles of Per-1 *in vivo* in untreated HIV-1-infected individuals.

***Results*:** We found that short isoform Per-1 can restrict HIV-1 replication and Tat ameliorates this inhibitory effect. Silencing of Per-1 could upregulate HIV-1 transcription both in resting CD4+ T-cells and MDMs. Moreover, Per-1 expression is inversely correlated with viral loads in Rapid progressors (RPs) *in vivo*.

***Conclusion*:** These data together suggest that Per-1 is a novel negative regulator of HIV-1 transcription. This restrictive activity of Per-1 to HIV-1 replication may contribute to HIV-1 latency in resting CD4+ T-cells.

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1. INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is caused by infection with human immunodeficiency virus (HIV) [1, 2]. Unlike other retroviruses, HIV-1 replication is not cell cycle-dependent and requires host cells for proliferation; however, the virus can infect both non-dividing and dividing cells [3-6]. After reverse transcription, proviral HIV-1 DNA is integrated into the host genome and subjected to transcription by host RNA polymerase II (Pol II) [7-10]. Pol II-mediated transcription can be subdivided into several interconnected stages, including preinitiation, initiation, promoter clearance, elongation, and termination [11, 12]. The HIV-1-encoded Tat protein is essential for activating transcriptional elongation from the viral long terminal repeat (LTR) [11, 13-16]. In the absence of Tat, transcription by Pol II is efficiently initiated, but it generates only short abortive transcripts [17]. After Tat becomes available, it dramatically improves the efficiency of Pol II elongation from the LTR promoter to produce full-length viral transcripts for the purpose of viral progeny assembly.

When HIV-1 infects resting CD4+ T-cells, it integrates into the genome as a chromatin template to produce viral transcripts [18-20]. The low transcriptional and metabolic activity of resting CD4+ T-cells results in the lack of cellular substrates and raw materials, which is detrimental to HIV-1 transcription [21]. HIV-1 transcription from the LTR promoter is the immediate early phase of infection. It is stochastic and influenced by cellular factors from the LTR in resting

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CD4+ T-cells [22-26]. The blockage of HIV-1 LTR-mediated transcription in resting CD4+ T-cells can also be attributed to the limitations in transcription initiation, which depends on the host cell transcription factors, such as NF-κB and NF-AT, which recognize specific target sites in the viral LTR promoter. The presence of these barriers to the initiation of HIV-1 transcription also contributes to HIV-1 latency in resting CD4+ T-cells. Some negative regulators have also been reported to suppress viral LTR-initiated transcription in resting CD4+ T-cells, such as Murr1, in active NF-κB, among others [21, 27-29].

Although resting CD4+ T-cells are refractory to HIV-1 transcription, the viral load can remain at a stable low copy number in infected patients, and their cell-associated HIV-1 transcription also persists in both isolated peripheral blood mononuclear cells (PBMCs) and CD4+ T-cells despite the treatment with potent antiretroviral drugs [30-32]. In fact, even with optimal treatment, replication-competent HIV-1 persists in resting CD4+ T-cells. These latently infected resting CD4+ T-cells in patients on combination antiretroviral therapy (cART) do not spontaneously produce HIV-1 unless activated [33, 34]. Most of the proposed mechanisms for HIV-1 latency involve transcription, including proviral integration into sites that are unsuitable for transcription, histone modifications that inhibit transcription, the absence of host transcriptional activators necessary for HIV-1 expression, and the presence of transcriptional repressors [35-38]. Therefore, an understanding of the restriction of HIV-1 transcription in resting CD4+ T-cells is critical to finding the cure for AIDS.

Period circadian regulator 1 (Per-1) [39-41], a circadian rhythm-regulating protein, has been shown a tumor-suppressing effect in a number of recent studies [42-48], and the over-expression of Per-1 has been shown to induce great inhibition of growth and stimulated apoptosis in prostate cancer cell line via suppression of tumor-associated genes [48]. In the meantime, we observed much higher expression of Per-1 in primary resting cells than in stimulated CD4+ T lymphocytes, which encourages us to explore whether Per-1 can repress HIV-1 transcription in resting CD4+ T-cells.

In this study, we show that Per-1 is highly expressed in resting CD4+ T-cells, resulting in the suppression of *de novo* HIV-1 transcription. More importantly, the depletion of Per-1 in unstimulated CD4+ T-cells from HIV-1-infected individuals' upregulates viral transcripts *ex vivo*. Per-1 expression is inversely correlated with the viral loads in Rapid progressors (RPs), but not in long-term nonprogressors (LTNPs). Therefore, Per-1 is a negative regulator of HIV-1 transcription in resting CD4+ T-cells and is a potential target for a novel therapeutic strategy for HIV infection.

2. MATERIALS AND METHODS

2.1. Cells and Reagents

293T, Jurkat, and THP-1 human cell lines were cultured as described elsewhere [49]. Plasmids were transfected into 293T cells using Fugene 6 (Roche) or Lipofectamine 2000 (Invitrogen). Stealth-grade siRNA human genes and controls were purchased from Invitrogen. PBMCs obtained from healthy blood donors were purified by Ficoll–Hypaque gradient centrifugation. Resting CD4+ T-cells were isolated from PBMCs via negative selection with the human CD4+ T-cells Enrichment Cocktail (StemCell Technologies). The resting CD4+ T-cells were cultured at a density of 2×10^6 cells per mL in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco), glutamine (2 mM), and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin). To activate CD4+ T-cells, CD3/CD28 activator magnetic beads (Invitrogen) were added to the culture medium for 2 days together with IL-2 (50 U/mL; Biomol), according to the manufacturer's instructions. To obtain postactivation resting T-cells, the IL-2 concentration was gradually decreased, as indicated in Fig. (**3A**) [50]. The isolation and culture of monocytes, MDMs, and MDDCs were performed as described previously [51]. Briefly, monocytes were purified from total PBMCs after Ficoll gradient separation with CD14-positive enrichment. MDMs were generated via stimulation of monocytes with 50 ng/mL recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF; R&D) for 7 days. MDDCs were generated by incubating CD14-purified monocytes in IMDM medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin, 10 mM HEPES, 1% non-essential amino acids, 1 mM sodium pyruvate, 10 ng/mL GM-CSF, and 50 ng/mL IL-4 (Miltenyi Biotec). On day 4, two-thirds of the culture medium was replaced with fresh medium containing GM-CSF and IL-4. Immature MDDCs were harvested and used for experiments on day 6.

2.2. Plasmids

The FLAG-Per-1 001 (ENST00000317276.8) and 002 (ENST00000354903.9) expression constructs were purchased from OriGene, Addgene. We transduced shRNA (pLKO.1-puro) based on lentivirus vectors by infecting 293T, Jurkat, THP-1, and activated CD4+ T-cells, followed by puromycin selection. Recombinant lentiviruses for the shRNAs (Per-1: TRCN0000074184; TRCN0000074185 and shCtrl; SHC002 nonmammal targeting) were generated via the transient transfection of shRNA vectors (Sigma) and packaging plasmids (pMD2.D and psPAX2; Sigma) in 293T cells, according to the manufacturer's instructions. The pNL4.3.Luc-Tat null vector was gifted by Dr. Guangxia Gao. The vectors producing VLP-Vpx were gifted by Dr. Landau [52], and 8 μg of $SIV3^+$ was cotransfected with 2 μg of VSV-G-encoding plasmid for VLP-Vpx production. VLP-Vpx treatment was performed for 3 h before infection by adding RPMI-diluted VLP-Vpx to the cells, as described previously [50, 51].

2.3. RNA Interference in Resting CD4+ T-cells

To achieve shRNA-mediated silencing of PER-1, CD4+ T-cells (4×10^6 cells per mL) were stimulated with activator CD3/CD28 magnetic beads (Invitrogen) and IL-2 (50 U/mL). On day 3, the activated cells were transduced by spinoculation with VSV-G pseudotyped lentiviral vectors carrying either pLKO.1-puro-control-shRNA or pLKO.1-puro-shPer-1 in medium containing 40 U/mL IL-2. On day 5, the transduced cells were pooled and cultured with 30 U/mL IL-2 and puromycin (0.5 μg/mL) at a concentration of 2×10^7 cells per 6 mL. On day 7, the IL-2 concentration was

reduced to 15 U/mL, and, on day 8, the puromycin-resistant, viable cells were purified by Ficoll gradient centrifugation and resuspended in 5 mL of fresh medium containing 15 U/mL IL-2. On day 10, 50% of the culture medium was replaced with fresh medium to reduce the final IL-2 concentration to 7.5 U/mL. On day 12, 50% of the culture medium was replaced with a fresh medium to reduce the final IL-2 concentration to 3.75 U/mL. On day 13, the IL-2 concentration was adjusted to 1.25 U/mL, and living cells were counted and equalized before HIV-1 infection. On day 14, the cells were spin-infected with HIV-$1_{NL4.3.Luc}$ (VSV-G). On day 16, the cells were lysed prior to luciferase assays, stained with PE-conjugated anti-CD69 and CD25 antibodies as well as CellTrace (proliferation), and finally analyzed by flow cytometry.

To achieve siRNA-mediated silencing of freshly isolated resting CD4+ T-cells (3×10^6 cells); they were directly electroporated with the Amaxa System using human T-cell Nucleofector Solution and program U-14, according to the manufacturer's protocol (Amaxa) [50].

2.4. HIV Infection

Before infection, HIV-1 producer 293T cells were washed twice with PBS to remove the transfected HIV-1 plasmid, and the viral stocks obtained were also treated for 1 h at 37 °C with DNaseI (Takara) [51]. DNA was extracted by the DNeasy Blood and Tissue Kit (QIAGEN).

2.5. Luciferase and Detection Assay

The luciferase activity (Promega) in the cell lysates was measured according to the manufacturer's instructions.

2.6. Western Blotting

Western blotting was performed using a standard method to detect pulled-down proteins. The antibodies used in this study included rabbit anti-GAPDH (Thermo), mouse monoclonal anti-FLAG (Sigma-Aldrich), and anti-rabbit and mouse Igs HRP (Abcam).

2.7. Quantitative Polymerase Chain Reaction

Total RNA was extracted from cells using Trizol (Invitrogen) according to the manufacturer's instructions. The RNA obtained was dissolved in 100 µL of DPEC-H_2O, and 1 µg of the purified RNA was treated with DNaseI (Invitrogen, Amplification Grade) for 10–15 min at the room temperature according to the manufacturer's instructions. The RNA was immediately primed with oligo-dT and reverse transcribed using the Superscript III Reverse Transcriptase (Invitrogen). Real-time PCR analysis was performed using the Δ-ΔCT method. The results were normalized against the amplification results obtained for an internal control (GAPDH). The primers used in this study are given in Table **S1**.

2.8. Alu–polymerase Chain Reaction

Quantitative Alu-PCR to quantify viral integrants was performed as described elsewhere [53, 54]. Briefly, the following primers were used for the preamplification of genomic DNA: Alu forward, 5′-GCC TCC CAA AGT GCT GGG ATT ACA G-3′; and HIV-1 gag reverse, 5′-GCT CTC GCA CCC ATC TCT CTC C-3′. Alu forward primer (100 nM), 600 nM of the gag reverse primer, and 1 µL of gDNA were used for every 20 µL of the PCR solution. The Thermocycler (Eppendorf Mastercycler Pro Cycler) was programmed to perform a 2 min hot start at 94 °C, followed by 30 rounds of denaturation at 93 °C for 30 s, annealing at 50 °C for 1 min, and extension at 70 °C for 1 min 40 s. To quantify HIV-1 integration, a second round of real-time qPCR was performed using 2 µL of the material from the preamplification step. The sequences of the primers used included LTR forward, 5′-GCC TCA ATA AAG CTT GCC TTGA-3′ and LTR reverse, 5′-TCC ACA CTG ACT AAA AGG GTC TGA-3′. All reactions were performed using the Applied Biosystems QuantStudio 3 Real-Time PCR system. The thermal program started with 2 min at 50 °C, followed by a 10 min hot start at 95 °C before 40 cycles at 95 °C for 15 s and 60 °C for 60 s. GAPDH was used as an internal control to normalize to total DNA.

2.9. RNA-seq Analysis

Freshly isolated CD4+ T-cells were cultured in R10 medium containing 10% inactivated FBS. Alternatively, CD4+ T-cells were activated with CD3/CD28 activators and IL-2 at 50 U/mL. Subsequently, rRNA-depleted total RNA was prepared using the Ribo-Zero rRNA Removal Kit (Epicenter). Libraries were prepared with Ithe llumina TruSeq and TruSeq Stranded total RNA sample prep kits and then sequenced with 50–60 million 23,100 bp paired raw passing filter reads on an Illumina HiSeq 2000 V3 instrument. The Illumina raw reads were trimmed using cutadapt to remove the adaptors and low-quality bases. These cleaned reads were then mapped onto the reference genome from the ENSEMBL database (http://www.ensembl.org/) using the TopHat2 (v. 2.0.14) program [55]. Cufflinks (v. 2.1.1) was subsequently applied to assemble the whole transcriptome and to identify all possible transcripts [56]. The transcript abundances (*e.g.*, fragments per kilobase of transcript per million fragments mapped) were estimated, and differential analysis was performed using the cuffdiff command [57, 58]. The mapping results in the BAM format, which were then converted into the TDF format using igvtools for visualization in the Integrative Genomics Viewer (IGV) [59]. The RNA-seq data are accessible through the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the reference number: PRJNA522052.

2.10. Virus Infection

Infection of 293T, Jurkat, and stimulated CD4+ T-cells was performed by the addition of HIV-1 at an MOI of 1. Viruses were added to the cells, and the luciferase activity was measured after infection. Resting CD4+ T-cells were incubated with 100 ng of p24 virus centrifuged at 1200 ×*g* for 2 h at 25 °C, as previously described [20].

2.11. Lentiviral Vector-mediated Gene Silencing in Jurkat, THP-1, MDM, and Stimulated CD4+ T-cells

Lentiviruses carrying shRNAs were prepared using 293T cells, which were transfected with the expression plasmids for Gag-Pol and VSV-G using Lipofectamine 2000 (Invitro-

gen). The recovered lentiviral vectors were transduced into 293T, Jurkat, THP-1, MDMs, and stimulated CD4+ T-cells before selecting with puromycin at 0.5-1 μg/mL concentration.

2.12. Flow Cytometry

CD4+ T-cells were cultured and stained in fluorescence-activated cell sorting (FACS) buffer with αCD69-PE (BD Pharmingen), αCD25-BB515 (BD Horizon), or CellTrace (Thermo). Data were collected on an FACS LSRII (BD Biosciences), and analyses were performed using the FlowJo software.

2.13. ELISA

HBs ELISA was performed as described elsewhere [60].

2.14. Patients

HIV-1-positive patients who were not or were undergoing cART treatment and whose viral loads were <50 copies/mL as well as HIV-1-negative healthy individuals were enrolled in this study. Untreated HIV-1 patients were divided into two groups: LTNPs (CD4+ T-cell number remained >500 cells/μL after at least 8 years of infection) and RPs (CD4+ T-cell number < 350 cells/μL after 1-2 years of infection), as described previously [49]. Ethical approval for this study was obtained from the ethics review committee of the China Medical University, and written informed consent was obtained from all participants. PBMCs from these subjects were prepared by Ficoll–Hypaque density gradient centrifugation, and the cells were cryopreserved in fetal calf serum supplemented with 10% dimethyl sulfoxide and stored in liquid nitrogen within 8 h of collection. Total RNA extracted from thawed PBMCs ($2\text{-}8 \times 10^6$) was reverse transcribed using oligodT as the primer. The mRNA levels of Per-1 and GAPDH were measured by SYBR Green real-time PCR (Takara) in the Light Cycler 480 System (Roche). The levels of Per-1 mRNA were normalized to those of GAPDH mRNA.

2.15. Measurement of CD4+ T-cell Counts and Plasma HIV RNA

CD4+ T-cell counts were measured from whole blood and performed using the FACSCalibur Flow Cytometer (BD Biosciences, NJ, USA). The standard lyse/no-wash procedure was used with anti-CD4-FITC/CD8-PE/CD3-PerCP as staining reagents and TruCOUNT tubes (BD Biosciences, NJ, USA). TruCOUNT Control Beads were used for quality control. A Roche Amplicor Monitor Standard Assay (COBAS AmpliPrep/COBAS Taqman HIV Test; Roche, Switzerland) was used to detect plasma HIV RNA. The values were calculated according to the manufacturer's reference standards. The limit of detection was set at 20 copies/mL.

2.16. Statistical Analysis

Differences were assessed using two-tailed, unpaired Student's *t*-tests and were considered significant when $P < 0.05$.

3. RESULTS

3.1. The 002 Per-1 Isoform, but not 001 Isoform, Restricts *de novo* HIV-1 Transcription

Sleep disorders are common in patients with HIV/AIDS, which often results in poor quality of life. Notably, approximately 70% of adult patients experience sleep difficulties, such as insomnia and daytime sleepiness [61, 62]. Recently, cerebrospinal HIV Tat protein has been suggested to affect the circadian rhythmicity [63]. In addition, Per-1 has been reported to play an important role in the circadian clock via regulation of the mammalian suprachiasmatic nuclei [39-41, 64-66]. Per-1 is supposed to be expressed in the central nervous system and has recently been shown to induce a tumor-suppressing effect [42-48]; however, we unexpectedly detected a dramatically higher level of Per-1 expression in resting CD4+ T-cells than in stimulated ones after a high-throughput RNA-seq analysis (Fig. **S1A**). Therefore, acting on our curiosity regarding why resting CD4+ T-cells express such high levels of Per-1, we investigated whether Per-1 is involved in HIV-1/AIDS disease progression or interacts with HIV-1.

Initially, we examined the ectopic expression of human Per-1 in HIV-1-infected 293T cells. Based on the previous findings that full-size Per-1 isoform occurs principally in the cytoplasm and the shorter isoform can translocate into the nucleus [67]; therefore, we cloned both the long and short isoforms into a FLAG-tagged expression vector for use in this study considering their comparable transcript lengths (4707 and 3115 bps corresponding to 1290 and 859 amino acids, respectively). To determine whether Per-1 can suppress HIV-1 replication, we transfected the expression constructs of the long isoform or short isoform into 293T cells, which were infected with the VSV-G pseudo-type reporter virus $HIV\text{-}1_{NL4.3.Luc}$ after transfection. The short Per-1 isoform, but not the long isoform, significantly inhibited HIV-1 infection (Fig. **1A**). Moreover, viral integration was not significantly affected by the presence of either of the Per-1 isoforms, which suggests that inhibition by the short isoform occurred during a later phase of HIV-1 replication. To explore whether this inhibitory effect of the short isoform Per-1-002 is specific to HIV-1, we tested the Per-1 expression in conjunction with a CMV promoter-driven hepatitis B virus (HBV) replicon in 293T cells. Consequently, we observed that neither of the Per-1 isoforms produced any inhibitory effects on HBV replication (Fig. **1B**). This suggests a distinctive mechanism of short isoform inhibition in HIV-1 infection. To elucidate the potential mechanism of the short isoform Per-1-002 inhibition in HIV-1 infection, we first determined the levels of HIV-1 transcripts in the presence of Per-1-002. Viral Gag RNA was significantly decreased by the overexpression of Per-1-002 but not by that of Per-1-001, in both 293T and HeLa cells (Figs. **1C** and **S2**). Therefore, the short isoform Per-1-002 may function to downregulate the viral transcript levels during HIV-1 replication in host cells.

3.2. Tat Ameliorates the Inhibitory effect of Per-1 on HIV-1

Since Per-1-002 can suppress the HIV-1 transcript levels, we hypothesized that HIV-1 transcription may be inhibited.

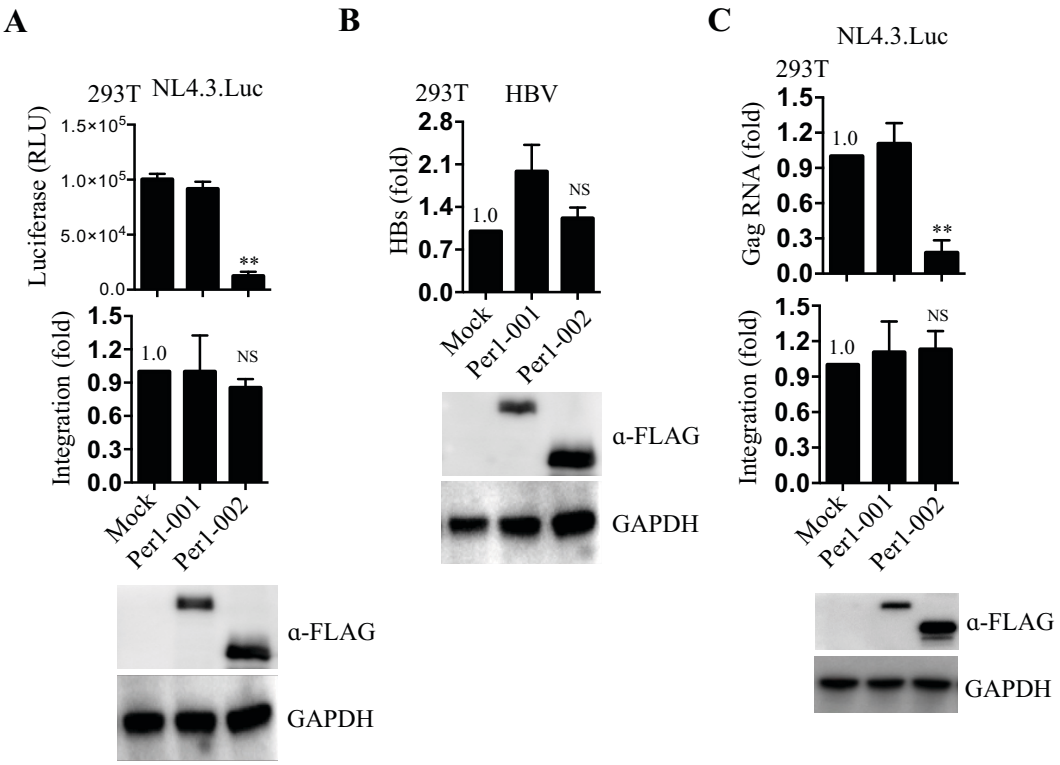

Fig. (1). The short isoform Per-1-002 restricts HIV-1 replication in 293T cells. (**A**) 293T cells were transfected with a construct of FLAG-tagged Per-1-001 or Per-1-002 and Mock or with an HBV replicon (CMV promoter) (**B**). At 24 h after HIV-$1_{NL4.3.Luc}$ (VSV-G) infection or HBV replicon transfection, the cells were lysed to measure the luciferase activity and an HBs ELISA was performed on the culture supernatants to assess the secretion of HBV particles. Western blotting was conducted to detect the FLAG-tagged Per-1 isoform or GAPDH expression using anti-FLAG or anti-GAPDH antibodies. (**C**) The 293T cells were transfected with a construct of FLAG-tagged Per-1-001 or Per-1-002 and Mock. At 24 h after transfection cells were washed and infected with HIV-$1_{NL4.3.Luc}$ (VSV-G). At 24 h after HIV-$1_{NL4.3.Luc}$ (VSV-G) infection, total RNA and genomic DNA were extracted for the assessment of the levels of HIV-1 transcripts normalized to GAPDH (qPCR) and of HIV-1 integration (Alu-PCR). Western blotting was conducted to detect FLAG-tagged Per-1 isoform or GAPDH expression using anti-FLAG or anti-GAPDH antibodies. RLU, relative luminescence units. $^{**}P < 0.01$ (*t*-test). NS, not significant. Data are presented as mean ± S.E.M. from three independent experiments.

Thus, we employed luciferase reporter constructs based on the HIV-1 LTR (LTR-Luc) or CMV promoters (CMV-Luc) to examine the short isoform Per-1-002 antiviral activity. Per-1-002 caused a significant reduction in LTR, but not in CMV-mediated luciferase expression (Fig. **2A**), which suggests that the short isoform can directly suppress HIV-1-promoter-initiated transcription in order to inhibit HIV-1 replication in host cells. Notably, Tat can dramatically promote HIV-1 LTR promoter activity [17, 68, 69]. We were therefore curious about whether Tat can influence the inhibitory effect of the short isoform on HIV-1 transcription. We examined the short isoform expression using Tat present pNL4.3.Luc or absent pNL4.3.Luc-Tat Null vectors and found that HIV-1 inhibition increased from 5.28- to 12-fold in the absence of Tat (Fig. **2B**), thereby indicating that Tat can ameliorate the short isoform Per-1-002-mediated suppression of the HIV-1 promoter. We then cotransfected 293T cells with Per-1-002 vectors and an HIV-1 LTR-Luc reporter construct, without or with an HA-tagged Tat expression vector. Subsequently, Per-1-002 inhibition of the LTR promoter gradually decreased with the increasing levels of Tat protein (Figs. **2C** and **D**). Therefore, Tat can counter Per-1-002 inhibition of HIV-1 transcription. Interestingly, the Per-1 expression is dramatically reduced during CD4+ T-cell activation, and we also employed quantitative (q)PCR to independently corroborate this result in activated or resting CD4+ T-cells isolated from two independent healthy donors (Fig. **S1B**). Then, we were prompted to explore the relationship between endogenous Per-1 and HIV-1 transcription in resting CD4+ T-cells.

3.3. Per-1 Restricts HIV-1 Replication in Resting CD4+ T-cells

To investigate the physiological roles of Per-1, we first examined its expression profile in various HIV-1 host cells. Per-1 was highly expressed in resting CD4+ T-cells, monocytes, monocyte-derived macrophages (MDMs), and differentiated THP-1 cells. However, it was barely expressed in stimulated CD4+ T-cells, 293T, Jurkat, HeLa, and undifferentiated THP-1 cells (Fig. **2E**). Thus, the anti-HIV activity of Per-1 may occur in resting CD4+ T-cells, MDMs, or stimulated THP-1 cells. First, we directly evaluated the capacity of the antiviral properties of Per-1 in resting CD4+ T-cells and employed two RNAi strategies to silence the Per-1 expression. These primary CD4+ T-cells were activated to allow the effective transduction of lentiviral vectors carrying shRNAs for Per-1 (Fig. **3A**). The IL-2 concentration was then gradually reduced, and, after the cells returned to a quiescent state, they were analyzed to detect HIV-1 infection by

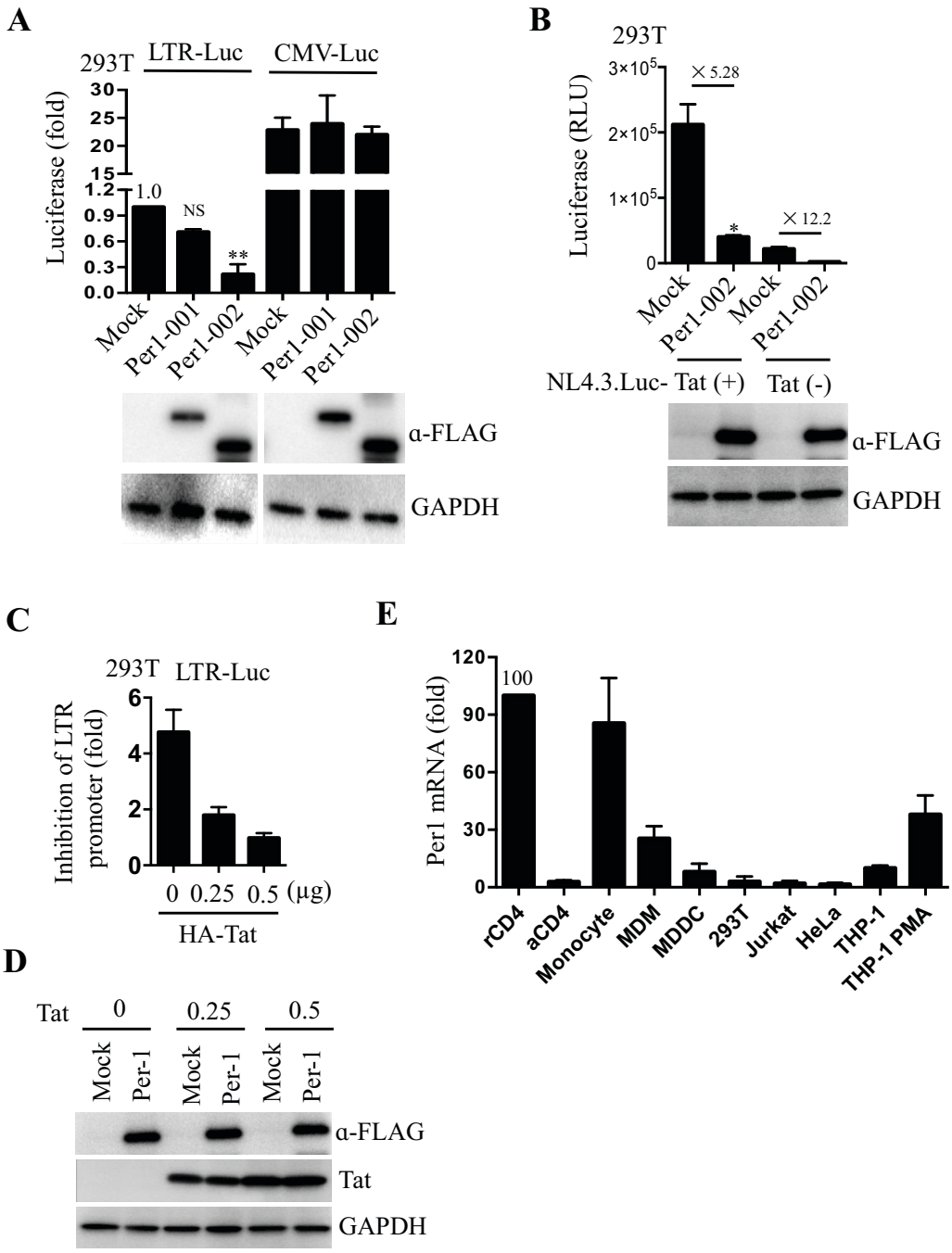

Fig. (2). Tat ameliorates the inhibitory effect of Per-1 on HIV-1 transcription. (**A**) 293T cells were cotransfected with a construct containing FLAG-tagged Per-1-001, Per-1-002, or Mock along with HIV-1 LTR (LTR-Luc) or CMV (CMV-Luc) promoter-driven luciferase reporter constructs. After 48 h of cotransfection, the cells were lysed to measure the luciferase reporter levels. Western blotting was conducted to detect FLAG-tagged Per-1 isoform or GAPDH expression using anti-FLAG or anti-GAPDH antibodies. (**B**) 293T cells were cotransfected with a construct of FLAG-tagged short isoform Per-1-002 or Mock and with or without Tat in the form of a pNL4.3.Luc construct. After 48 h of cotransfection, the cells were lysed to measure the luciferase reporter levels. Western blotting was conducted to detect FLAG-tagged Per-1-002 or GAPDH expression using anti-FLAG or anti-GAPDH antibodies. (**C**) 293T cells were cotransfected with a construct of FLAG-tagged short isoform Per-1-002 or Mock and an LTR-Luc construct in the presence or absence of an HA-Tat expression vector. After 48 h of cotransfection, the cells were lysed to measure the luciferase reporter levels. The inhibition of viral promoter LTR-mediated luciferase expression was accordingly calculated between Per-1-002 and Mock. Western blotting was conducted to detect FLAG-tagged short isoform Per-1-002, HA-Tat, or GAPDH expression using anti-FLAG, anti-HA, or anti-GAPDH antibodies (**D**). (**E**) Total RNA was extracted from resting and stimulated CD4+ T-cells, monocytes, MDMs, MDDCs, 239T, Jurkat, HeLa, and THP-1 as well as differentiated THP-1 cells and used for qPCR to assess Per-1 transcripts normalized to GAPDH. RLU, relative luminescence units. $^{**}P < 0.01$ (*t*-test). NS, not significant. Data are presented as mean ± S.E.M. from three independent experiments.

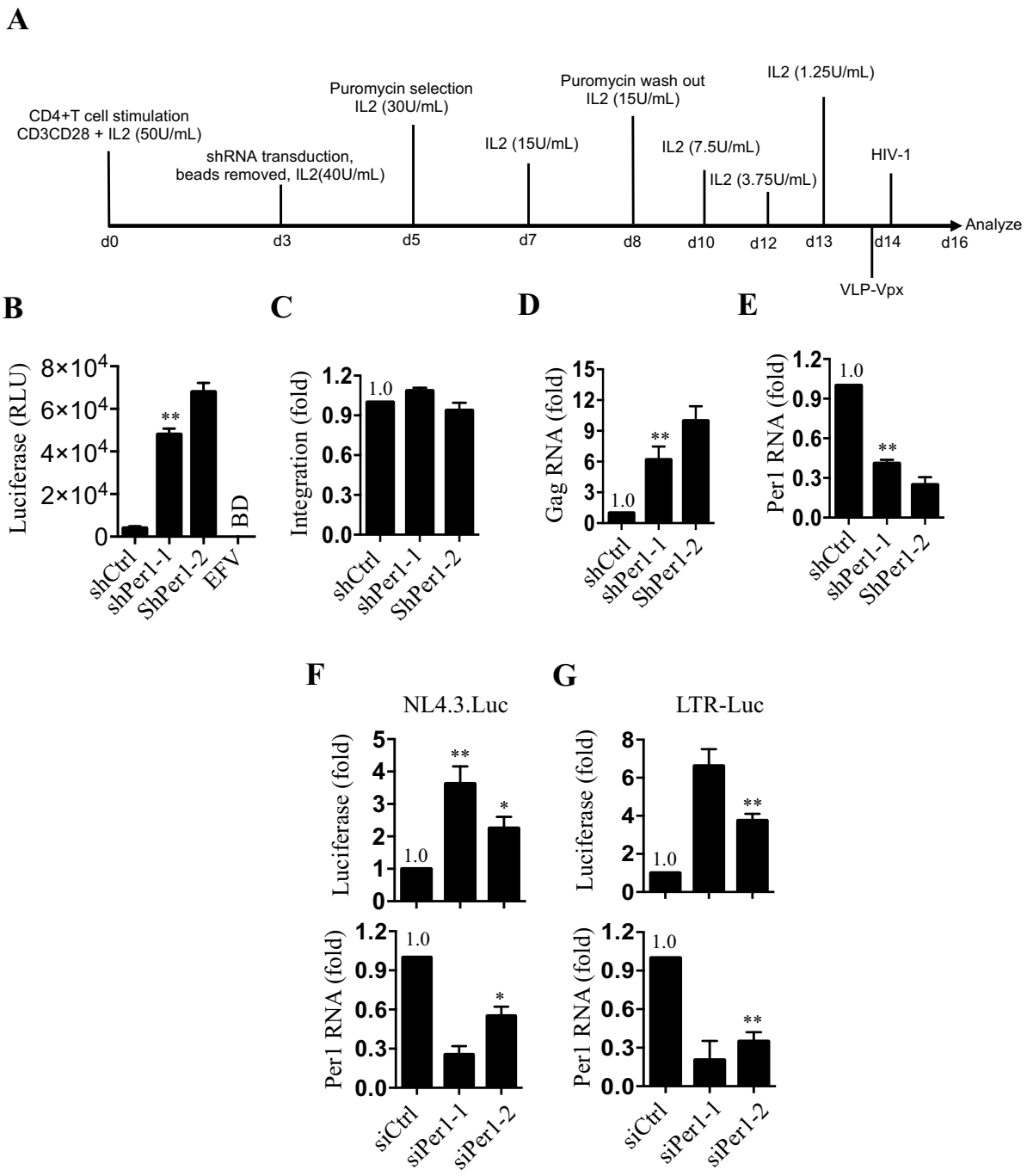

Fig. (3). Per-1 suppresses HIV-1 transcription in post-activated resting CD4+ T-cells. (**A**) Schematic representation of the experimental design. CD4+ T-cells were stimulated with CD3/CD28 activator magnetic beads and IL-2 for 48 h and transduced with shRNA against Per-1 or control lentivirus in the presence of puromycin selection. CD4+ T-cells were cultured with gradual dilutions of IL-2 to transform them into the resting state (*i.e.*, CD69, CD25, and cell proliferation were measured by flow cytometry as shown in Fig. S3). The resting cells were further treated with VLP-Vpx for 6 h and spinoculated with HIV-$1_{NL4.3.Luc}$. At 48 h after infection, the cells were lysed to measure the luciferase reporter levels (**B**). Genomic DNA and total RNA were extracted for qPCR to assess HIV-1 integration by Alu-PCR (**C**) and viral Gag (**D**) and Per-1 (**E**) transcripts normalized to GAPDH. (**F**) Resting CD4+ T-cells were electroporated with siRNAs against Per-1 or control along with pNL4.3.Luc or CMV-Luc vectors and cultured for 2 days before lysis and then measured for the luciferase reporter levels, which were normalized between the pNL4.3.Luc and CMV-Luc samples. Total RNA was extracted for qPCR to assess Per-1 transcripts normalized to GAPDH. (**G**) Resting CD4+ T-cells were electroporated with siRNAs against Per-1 along with LTR- or CMV-Luc vectors and then cultured for 2 days. The cells were lysed to measure the luciferase reporter levels, which were normalized between the LTR- and CMV-Luc samples. Total RNA was extracted for qPCR to assess Per-1 transcripts normalized to GAPDH. RLU, relative luminescence units. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). NS, not significant. Data are presented as mean ± S.E.M. from three independent experiments.

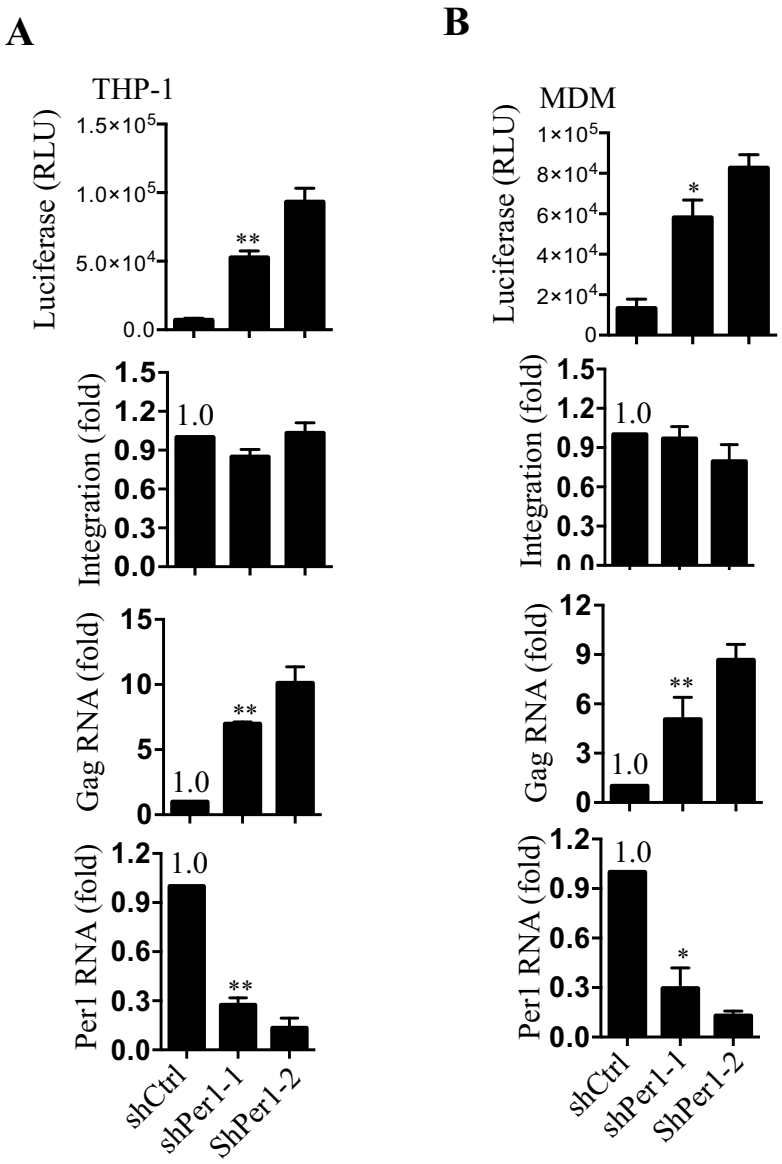

Fig. (4). Per-1 suppresses HIV-1 transcripts in MDMs. (**A**) THP-1 cells were stimulated with PHA for 2 days and then transduced with shRNA against Per-1 or a control. After puromycin selection for 3 days, the cells were treated with VLP-Vpx and infected with HIV-$1_{NL4.3.Luc}$ for 2 days and lysed to measure the luciferase reporter levels. Genomic DNA and total RNA were extracted to assess HIV-1 integration by Alu-PCR and viral Gag and Per-1 transcripts by qPCR, normalized to GAPDH. (**B**) MDMs were transduced with shRNA against Per-1 or a control. After puromycin selection, the cells were treated with VLP-Vpx and infected with HIV-$1_{NL4.3.Luc}$ for 2 days and then lysed to measure the luciferase reporter levels. Genomic DNA and total RNA were extracted to assess HIV-1 integration by Alu-PCR and viral Gag and Per-1 transcripts by qPCR, normalized to GAPDH. RLU, relative luminescence units. *$P < 0.05$, **$P < 0.01$ (Student's *t*-test). NS, not significant. Data are presented as mean ± SEM from three independent experiments.

measuring the CD69 and CD25 levels and cell proliferation (Fig. **S3**); the cells were then challenged with HIV-$1_{NL4.3.Luc}$ infection for 48 h. Per-1 showed an inhibitory effect on HIV-1 transcripts in resting CD4+ T-cells (Fig. **3B-E**), but it did not affect the viral integration (Fig. **3C**). However, the same Per-1 shRNA transduced into Jurkat or stimulated CD4+ T-cells did not affect HIV-1 infection, suggesting their specific usages in resting CD4+ T-cells (Figs. **S4A** and **B**).

We also directly electroporated siRNA against Per-1 into resting CD4+ T-cells and observed that Per-1 knockdown could substantially restore HIV-1 transcripts that were driven by the viral LTR promoter (Fig. **2F**). In addition, the depletion of Per-1 also increased LTR-driven luciferase reporter expression in resting CD4+ T-cells (Fig. **2G**). In contrast, the same Per-1 siRNA did not affect HIV-1 infection in stimulated CD4+ T-cells (Fig. **S4C**), which indicated the specific usage of siRNA for Per-1 in resting CD4+ T-cells. Cumulatively, these results indicate that Per-1 is a negative regulator that restricts the HIV-1 promoter activity, and, therefore, HIV-1 replication in resting CD4+ T-cells; this mechanism may contribute to the resting CD4+ T-cells being refractory to HIV-1 infection.

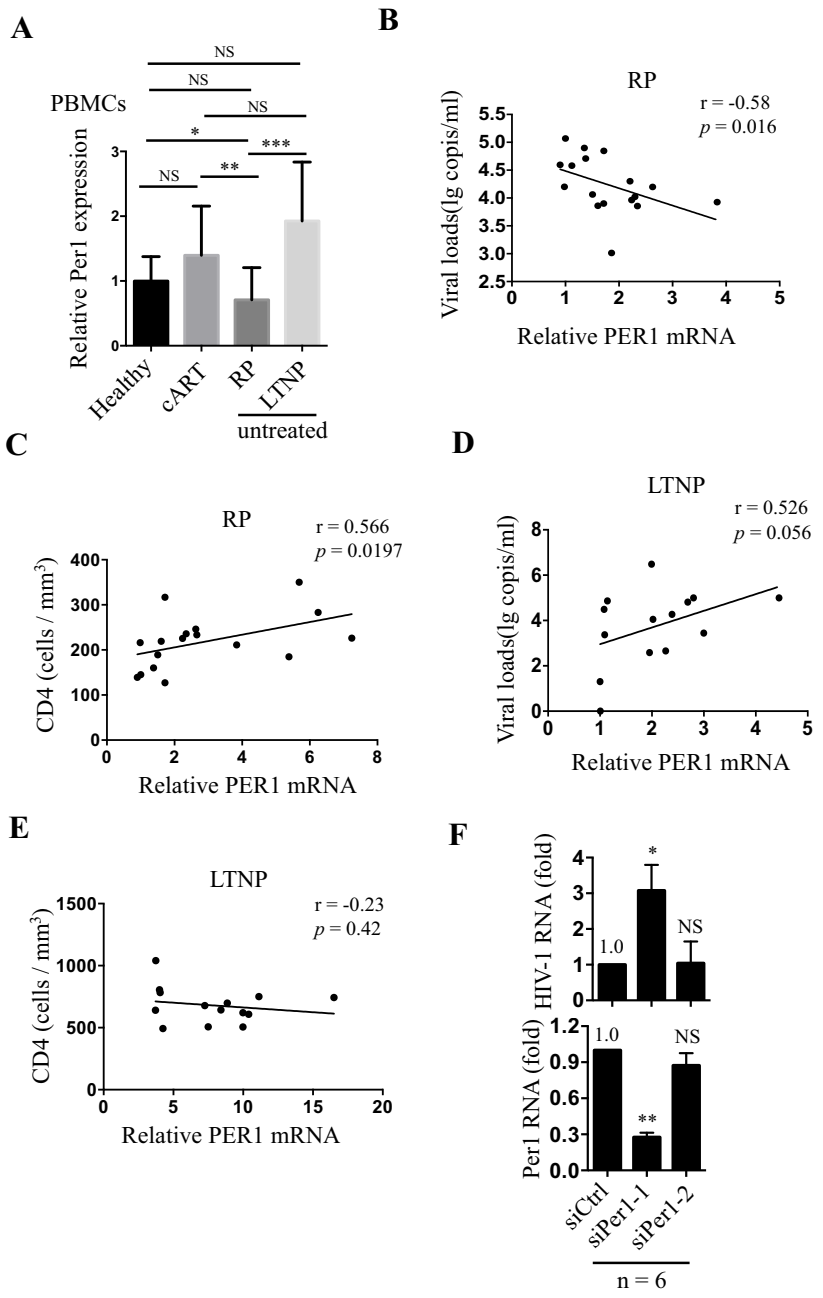

Fig. (5). Per-1 expression is inversely correlated with viral loads in RPs. (**A**) Total RNA was extracted from PBMCs isolated from healthy donors (n = 16) and from cART-treated (n = 28, viral loads <50 copies/mL) or cART-untreated HIV-1-positive individuals, 17 of whom were RPs and 14 LTNPs. The samples were analyzed by qPCR to measure the Per-1 transcripts, which were normalized to GAPDH. (**B**) The correlation between PBMC Per-1 expression and viral load as well as the number of CD4+ T-cells, with RPs (**B** and **C**) or LTNPs (**D** and **E**). The Spearman correlation was used for correlation analysis. (**F**) Total CD4+ T-cells isolated from six untreated HIV-1-infected individuals were electroporated with siRNA against Per-1 or control and then cultured for 48 h in the absence of stimulation. Total RNA was extracted for qPCR analysis for Gag and Per-1 transcripts normalized to GAPDH. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Student's t-test). NS, not significant. Data are presented as mean ± S.E.M.

3.4. Per-1 Restricts HIV-1 Replication in MDMs

Per-1 was also expressed at a comparably higher level to resting CD4+ T-cells in monocytes and MDMs as well as in THP-1 cells (Fig. **2E**). We first transduced Per-1 shRNA into PMA-treated THP-1 cells to deplete endogenous Per-1. After selection with puromycin, these cells were treated with Vpx and then infected with HIV-$1_{NL4.3.Luc}$. The depletion of endogenous Per-1 increased the levels of the luciferase reporter and viral transcripts, but did not affect viral integration (Fig. **4A**). To determine whether Per-1 can suppress HIV-1 transcripts in MDMs, primary MDMs that have been transduced with Per-1 shRNA were treated with Vpx and infected with HIV-$1_{NL4.3.Luc}$. HIV-1 transcripts, but not viral integration, which also increased in the absence of Per-1 (Fig. **4B**), indicating that Per-1 also suppresses the viral transcript levels in MDMs.

3.5. Per-1 Plays *in vivo* Roles in HIV-1-infected Individuals

After we identified that Per-1 protects against HIV-1 infection in primary resting CD4+ T-cells and MDMs, we investigated any potential anti-HIV-1 roles *in vivo*. Thus, we accordingly assessed the Per-1 expression in PBMCs from healthy donors and cART-treated (viral loads < 50 copies/mL) and untreated HIV-1-infected individuals, who were divided into RPs and LTNPs (Table **S2**) [49, 70]. The Per-1 transcript levels were significantly lower in PBMCs from untreated RP patients as compared with those from cART-treated patients or healthy donors (Fig. **5A**). *In vivo* HIV-1 infection-induced stimulation of CD4+ T-cells and immune systems probably accounts for this downregulation of Per-1 transcripts in RPs. Nevertheless, the Per-1 expression in LTNPs was not significantly different between cART-treated patients and healthy donors. More importantly, we aimed to determine the existence of a possible correlation between the Per-1 expression and plasma levels of HIV-1 RNA *in vivo*. We found that the Per-1 transcript levels were inversely correlated with viral loads (Fig. **5B**) and positively correlated with the number of CD4+ T-cells (Fig. **5C**) in RPs, but not in LTNPs (Fig. **5D** and **E**), which suggests that Per-1 plays a role in the control of HIV-1 infection and disease progression in RPs. This finding also supports the hypothesis that Per-1 is a negative regulator in the control of HIV-1 infection *in vivo*.

For this purpose, we sought to examine the anti-HIV activity of Per-1 in CD4+ T-cells *ex vivo*. We directly electroporated Per-1 siRNA into total CD4+ T-cells isolated from six untreated HIV-1-infected patients (Table **S3**) and found that the depletion of Per-1 results in the upregulation of CD4+ T-cell-associated viral transcripts (Fig. **5F**), thereby demonstrating that Per-1 possibly suppresses HIV-1 transcription *in vivo*.

4. DISCUSSION

Profound physiological differences exist between resting and stimulated CD4+ T-cells, and HIV-1 5′-LTR transcription initiation in resting cells is inhibited by the lack of key cellular transcription factors, including NF-κB, NF-AT, Sp1, and AP1, which can be activated in stimulated CD4+ T-cells. Although several factors that inhibit HIV-1 transcription have been described previously [29, 71], there are only a few reports on restriction factors of HIV-1 transcription in resting CD4+ T-cells [21, 28]. In this study, we identified Per-1-002 as a negative regulator to restrict HIV-1 *de novo* transcription, but not HBV replication, in various host cells. Interestingly, this restriction could be ameliorated by viral Tat protein, which is indicative of the important role of Tat in activating HIV-1 transcription. Until the present study, it was unknown whether endogenous Per-1 can restrict HIV-1 transcription in resting CD4+ T-cells. The knockdown of Per-1 could substantially upregulate HIV-1 replication and the transcript levels, whereas viral integration was unaffected in postactivation resting CD4+ T-cells. Moreover, the direct depletion of Per-1 in resting CD4+ T-cells using siRNA oligonucleotides resulted in an increase in HIV-1 transcripts and in viral LTR promoter activity. These data suggest that Per-1 physiologically inhibits HIV-1 promoter activity in resting CD4+ T-cells, which may lead to resting CD4+ T-cells becoming refractory to HIV-1 replication. Because of the limited activity of Tat and the significantly higher expression levels of Per-1 in resting CD4+ T-cells, HIV-1 transcription could be heavily inhibited. In contrast, stimulated CD4+ T-cells are highly permissive to HIV-1 transcription, which may be partially due to the dramatically lower expression of Per-1 and the higher activity of viral Tat. In addition, we observed that Per-1 can also actively repress HIV-1 replication in MDMs; therefore, our results indicate that Per-1 is also a negative regulator of HIV-1 replication in both differentiated THP-1 and primary MDMs, suggesting that restriction by Per-1 is not limited to resting CD4+ T-cells.

Most importantly, we utilized a cohort of HIV patients to investigate the potential anti-HIV activity of Per-1 *in vivo*. We found that the Per-1 expression was inversely correlated with viral loads and positively correlated with the number of CD4+ T-cells in RPs, thereby suggesting its potential role in controlling HIV-1 replication and disease progression. Nevertheless, no relationship was noted between the Per-1 expression in LTNPs, which suggests that Per-1 may play different roles in contributing to the disease progression in LTNPs and RPs. Finally, to demonstrate the anti-HIV-1 roles of Per-1 *in vivo*, we depleted endogenous Per-1 in CD4+ T-cells isolated from patients who were not being treated with cART and noted a significant restoration of viral transcript levels in the absence of Per-1. Therefore, we propose that Per-1 plays an anti-HIV-1 role *in vivo*.

CONCLUSION

Taken together, our data suggest that Per-1 is a novel negative regulator of HIV-1 transcription in resting CD4+ T-cells. This restrictive activity of Per-1 to HIV-1 replication may induce HIV-1 latency in resting CD4+ T-cells; however, additional studies are required to determine whether Per-1 plays a direct role in this process.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval for this study was obtained from the ethics review committee of the China Medical University, China.

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All humans research procedures were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10 ethics/ 10helsinki/).

CONSENT FOR PUBLICATION

Written informed consent was obtained from all participants.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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AUTHOR CONTRIBUTIONS

G. Liang directed the overall study. L. Zhao, M. Liu, J. Ouyang., Z. Zhu, W. Geng, J. Dong, Y. Xiong, S. Wang, X. Zhang, Y. Qiao, H. Ding, H. Sun, and X. Han performed the experiments and analyzed the data. H. Shang and X. Han provided intellectual advice regarding the experimental design. G. Liang wrote the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

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