HIV-1 can infect northern pig-tailed macaques (*Macaca leonina*) and form viral reservoirs in vivo

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ABSTRACT

Viral reservoirs of HIV-1 are a major obstacle for curing AIDS. The novel animal models that can be directly infected with HIV-1 will contribute to develop effective strategies for eradicating infections. Here, we inoculated 4 northern pig-tailed macaques (NPM) with the HIV-1 strain HIV-1$_{NL4.3}$ and monitored the infection for approximately 3 years (150 weeks). The HIV-1-infected NPMs showed transient viremia for about 10 weeks after infection. However, cell-associated proviral DNA and viral RNA persisted in the peripheral blood and lymphoid organs for about 3 years. Moreover, replication-competent HIV-1 could be successfully recovered from peripheral blood mononuclear cells (PBMCs) during long-term infection. The numbers of resting CD4$^+$ T cells in HIV-1 infected NPMs harboring proviruses fell within a range of 2- to 3-log$_{10}$ per million cells, and these proviruses could be reactivated both ex vivo and in vivo in response to co-stimulation with the latency-reversing agents JQ1 and prostratin. Our results suggested that NPMs can be infected with HIV-1 and a long-term viral reservoir was formed in NPMs, which might serve as a potential model for HIV-1 reservoir research.

1. Introduction

In contrast to two other widely used macaque species, rhesus macaques and cynomolgus macaques, pig-tailed macaques (PTM) are the only type of macaque that can be infected with HIV-1 [1–9]. Our group and others identified a unique TRIM5-cyclophilin fusion gene isoform in PTM that replaces TRIM5α and lacks the ability to restrict HIV-1 infection [10–12]. This finding provided an explanation on why PTM are susceptible to HIV-1 infection and experience AIDS-like symptoms in response to infection with engineered simian-tropic strains of HIV-1 (stHIV-1) [13–16].

On the other hand, although some researches in early reports have a suspicion that HIV-1 might be hidden at some site remote from peripheral circulation, with a very low-level replication because of persistent detectable antibodies and provirus genes [5,8], few researches were carried out to evaluate the features of the hidden viruses in HIV-1 infected pig-tailed macaques, which is equally significant so as to reveal the other side of HIV-1 infection. Additionally, pig-tailed macaques (*Macaca nemestrina* group) are now divided into 3 species: Sunda pig-tailed macaque (*M. nemestrina*), northern pig-tailed macaque (*M. leonina,* NPM) and Mentawai macaque (*M. pagensis*) according to the current widely-accepted primate taxonomy [17,18]. The previously studied pig-tailed macaques were mainly Sunda pig-tailed macaques that originate in Sumatra and, to a lesser extent, Borneo [19]. In China the local pig-tailed macaque species is NPM, which has a different distribution range and morphological characteristics that are distinct from *M. nemestrina* [20]. We previously found that peripheral blood mononuclear cells (PBMCs) from NPMs lacking the TRIM5α gene [21] are amenable to HIV-1 replication in vitro, too [22].

In this study, we inoculated 4 NPMs with the HIV-1 strain HIV-$1_{NL4.3}$ and monitored the infection for about 3 years. The results showed that HIV-1 successfully established an infection in all 4 animals to produce a peak plasma viral load in the early stages of infection and persistent HIV-1 antibodies during the observation period. Although the initial viremia declined to undetectable levels during the post-acute infection stages, proviral genes and viral RNA, as well as HIV-1 particles, could be detected throughout the infection period. More importantly, upon treatment with a combination of the LRAs, JQ1 and prostratin, proviruses harbored in resting CD4$^+$ T cells of these animals could be reactivated ex vivo and in vivo even at 3 years after infection. These results suggested that HIV-1 can infect NPMs and form viral reservoirs in vivo.

2. Materials and methods

2.1. Animals and infection

The four northern pig-tailed macaques (*Macaca leonina*, NPM) used in this study were provided by Guangdong Landao Biotechnology, Co., Ltd., China. All applicable institutional and/or national guidelines for the care and use of animals were followed. The NPMs used were two adult males: 05001, 05003 and two adult females: 04002, 05004, aged 6- to 7 years old. The males and females weighed 7- to 8 kg and 4- to 5 kg, respectively. Nested-PCR analyses at the start of the study showed that all animals were free from simian immunodeficiency virus (SIV), simian type-D retrovirus, and simian T-lymphotropic virus type 1. Ethylenediaminetetraacetic acid (EDTA)-blood (5–10 mL) was collected from the animals at each time point and centrifuged to separate the plasma from the cellular fraction. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (GE Healthcare) gradient centrifugation. Plasma and PBMCs were frozen in aliquots at −80 °C before use.

HIV-$1_{NL4.3}$ virus stocks were generated from the supernatant of 293T cells transfected with pNL4.3 proviral plasmid, and expanded by brief passage in C8166 cells. PBMCs (1×10^7) from each animal were infected with HIV-$1_{NL4.3}$ particles at a multiplicity of infection (MOI) of 0.01. Three days later, autologous PBMCs (1×10^7) and 1 mL of cell free virus stocks containing 2 ng HIV-1 p24 antigen were intravenously (IV) inoculated into each animal.

2.2. Plasma viral load measurements

The levels of viral RNA in plasma were quantified by a real-time PCR method based on amplification of a HIV-$1_{NL4.3}$-derived Gag coding sequence described previously with slight modification [23]. Briefly, total RNA eluted in a 50-μL volume was extracted from 200-μL plasma using the High Pure Viral RNA Kit (Roche, Indianapolis, Indiana, USA). For each sample, three separate 10-μL aliquots of eluted RNA were reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen Carlsbad, California, USA) and the gene special reverse primer 84R (5′-TGCTTGATGTCCCCC CACT-3′). Each total cDNA template was subjected to real time PCR amplification in a 50-μL volume with 1 × PCR II buffer, 1 × ROX reference, 4 mM MgCl$_2$, 0.6 μmol/L primers, 0.1 μmol/L probe, 0.3 mmol/L dNTP, and 1.25 units AmpliTaq Gold DNA polymerase (Applied Biosystems). The primers were 6F (5′-CATGTTTT CAGCATTATCAGAAGGA-3′) and 84R (5′-TGCTTGATGTCCCCCCACT-3′), and the probe 5′-FAM-CCACCCCACAAGA-TTTAAACACCATGC TAA-TAMRA-3′. PCR reactions were performed on an ABI Vii7 Sequence Detection System under the conditions of 1 cycle of 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The quantitation limit was determined to be 50 copies/ mL by a standard curve constructed from serial dilutions of an appropriate RNA transcript.

2.3. Flow cytometry

Antibodies and reagents were obtained from BD Biosciences (BD, Franklin Lakes, NJ, USA). Surface staining for lymphocyte immunophenotyping was performed as previously described. Briefly, 100 μL of whole blood was lysed with FACS Lysing Buffer for 10 min at room temperature. After washing with DPBS containing 2% newborn calf serum and 0.09% sodium azide (staining buffer), the residual leukocytes were resuspended in staining buffer followed by staining with the relevant mAbs for 30 min on ice, and then fixed in PBS containing 4% paraformaldehyde. For Ki67 staining, 1×10^5 leukocytes were first stained with relevant surface marker mAbs, fixed and permeabilized in Cytofix/Cytoperm solution, washed in Perm/Wash Buffer, and incubated with anti-Ki67 PE mAb in Perm/Wash Buffer. After washing once, the cells were fixed with PBS containing 2% paraformaldehyde and then resuspended in a staining buffer. Flow cytometric data acquisition was performed on a BD FACS-Verse cytometer driven by FACSuite software (version 1.0.3; BD). FlowJo software (version 7.6.1; TreeStar) was used for data analysis.

The surface marker mAbs used were: anti-CD3-PE/-APC-Cy7 (clone SP34-2), anti-CD4-FITC/-PerCP-Cy5.5 (clone L200), anti-CD8α-PE-Cy7 (clone RPA-T8), anti-CD20-PerCP-Cy5.5 (clone 2H7), anti-CD14-APC (clone M5E2), anti-HLA-DR-APC (clone L243), anti-CD28-APC (clone CD28.2), anti-CD95-FITC (clone DX2), anti-Ki67-PE (clone B56), Anti-CD38-FITC (clone AT-1), anti-CD21 APC (clone B-ly4) and anti-CD25 (BC96).

2.4. Anti-HIV-1 antibody

HIV-1-specific antibodies were measured by ELISA using the Anti-HIV antibody ELISA kit (WANTAI Bio-pharm, Beijing, China). Briefly, plasma at the relevant time points was serially diluted and distributed into 96-well plates and the anti-HIV IgG antibody titers were determined according to the manufacturer's protocol.

2.5. Cell-associated HIV-1 RNA and DNA

PBMCs ($\sim 4 \times 10^6$) or resting CD4$^+$ T cells ($\sim 4 \times 10^5$) were split into two equal parts to measure cell-associated HIV-1 DNA and RNA. Total cellular DNA was extracted using the QIAmp DNA Blood Mini Kit (Qiagen, German) and eluted in a 100 μL volume. HIV-1 DNA was quantified by qPCR using the same primers and probes for HIV-1 RNA quantification mentioned above, and a pNL4.3 plasmid with known copies as quantified by spectrophotometric analysis was prepared to generate a standard curve. Meanwhile, cell numbers were assessed in parallel by genomic CCR5 DNA that was quantified with primers 5′-AGCCAGGACGGTCACCTT-3′ (forward) and 5′-ATGATTCCTGGGAGAGACGC-3′ (reverse) and the sequence-specific probe 5′-FAM-AACACAGCCACCACCCAAGT GATCA-3′-TAMRA. Each reaction contained 10-μL total DNA in a 25-μL reaction volume. Reaction mixtures and conditions were as mentioned above and the quantitative limit was proposed to be 5 copies per reaction. HIV-1 DNA levels were normalized as copy numbers relative to CCR5 genomic DNA copies per equivalent sample.

Cell-associated RNA was extracted by Trizol (Invitrogen) reagent and dissolved in 100-μL volume. A 10-μL aliquot was quantified for HIV RNA by the same procedures as described above for plasma viral RNA. The lower limit of detection was determined to be 5 copies of viral RNA per qRT-PCR reaction. Cell-associated HIV-1 RNA is presented as the number of HIV-1 RNA copies per sample relative to CCR5 genomic DNA copies per equivalent sample measured above.

2.6. Virus isolation and sequence analysis

For virus isolation, PBMCs isolated from the inoculated animals at given time points were depleted of CD8$^+$ cells (CD8-depleted PBMCs) using CD8 MicroBeads (Miltenyi). CD8-depleted PBMCs (2×10^6) were cultured in 2 mL medium containing 10 μg/mL Con A. Meanwhile, HIV-1 sero-negative human PBMCs were depleted of CD8$^+$ cells using the same methods and stimulated with 5 μg/mL PHA-P. Three days later, the medium was completely replaced and 1×10^6 human CD8-depleted PBMCs were added. All cultures were maintained in RPMI-1640 with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 U/mL IL-2. Co-cultures were split 1:2 every 3 to 4 days and new stimulated human CD8-depleted PBMCs were added every 7 days. HIV-1 expression was monitored after 21 days by HIV-1 p24 antigen ELISA (ZeptoMetrix, Buffalo, NY, USA).

HIV-1 *gag* and *env* sequences were amplified by nested PCR from PBMC DNA isolated using the QIAmp DNA Blood Mini Kit (Qiagen). Nested PCR was performed with previously described primers, DNA polymerase, and conditions [15]. Single-genome sequencing of HIV-1 *env* was performed as previously described [24]. Briefly, cellular DNA was serially diluted and distributed in the wells of 96-well plates. The aforementioned nested PCR procedure for the *env* gene was carried out in each well. A dilution where the positive ratio was <30% was used for at least 8 more positive amplifications. The amplicon can be considered to be derived from a single DNA molecule. Phylogenetic trees using the resulting DNA sequences were generated by MEGA5 and hypermutation analysis using Hypermut 2.0 (http://www.hiv.lanl.gov/content/sequence-/HYPERMUT/hypermut. html).

2.7. Viral reservoir reactivated ex vivo and in vivo

Two latency reversing agents were used: prostratin (Santa Cruz, Dallas, Texas, USA), JQ1 (Selleck, USA). PHA-P (Sigma-Aldrich, USA), and Con A (Sigma) were used as positive controls. PBMCs or resting CD4$^+$ T cells isolated from the inoculated animals at various time points were isolated as mentioned above. PBMCs (1×10^6) were treated with the indicated agents (JQ1, 250 nmol/L; PHA, 5 μg/mL; ConA 10 μg/mL or prostratin, 250 nmol/L) alone or with JQ1 and prostratin combined for 48 h; DMSO was used as a negative control. The cells were collected and cellular RNA was extracted with Trizol (Invitrogen). Cell-associated HIV-1 RNA or DNA levels were determined as mentioned above.

For viral reservoir in vivo, JQ1 and prostratin were diluted in 5-mL normal saline and used to intravenously (IV) inject the NPMs with a combined dose of 100 μg/kg and 80 μg/kg, respectively. Three weeks later, the compounds were again inoculated into the animals at the same dosages. One of the animals, 05001, received a third dose at 9 weeks after the second dose. This animal was sacrificed 48 h after LRA treatment and the PBMCs, ileum, colon, inguinal lymph nodes (ILN), mesenteric lymph nodes (MLN), and spleen were isolated at necropsy.

2.8. Isolation of resting CD4$^+$ T cells

Gut lymph nodes (mesenteric and colonic), peripheral lymph nodes (axillary and inguinal) and spleen tissue were minced and pressed through nylon mesh screens to obtain single-cell suspensions of lymphocytes. PBMCs were separated from whole blood. Lamina propria mononuclear cells (LPMCs) from ileum (LPMCile) and colon (LPMCcol) tissues were isolated as described [25]. In brief, intestinal tissues were cut into 1 cm pieces and digested with collagenase and DNase. The LPMCs were pressed through nylon mesh screens and purified with Ficoll gradient centrifugation to obtain >85% purity as tested by flow cytometry analysis.

Cell populations were then enriched for resting CD4+ T cells by magnetic bead separation. Briefly, biotin-conjugated monoclonal antibodies against HLA-DR (LN3, BioLegend, San Diego, CA, USA) and CD25 (BC96, eBioscience, San Diego, CA, USA) were added into a biotin-antibody cocktail from the CD4+ T Cell Isolation Kit (non-human primate, Miltenyi Biotec, Inc. Auburn CA, USA) to deplete cells expressing CD25, HLA-DR, CD8, CD11b, CD16, CD20, CD56, and CD66abce, and leaving a population of HLA-DR-CD25- CD4+ T cells. FACS analysis showed that resting CD4$^+$ T cells were present at >95% purity.

2.9. Immunohistochemistry

Tissues were paraformaldehyde-fixed, paraffin-embedded, and consecutively cut into 5 μm sections that were mounted on glass slides for hematoxylin and eosin (HE) staining and immunohisto-chemistry analysis. Antigen retrieval was performed by heating sections at 100 °C for 6 min three times in saline sodium citrate buffer (SSC), followed by cooling to room temperature (RT) and washing with phosphate-buffered saline containing 0.05% Tween-20 (PBST). The sections were then incubated with 0.1% TritonX-100 for 10 min at RT, washed, treated with 3% hydrogen peroxide for 10 min at RT, washed, and then blocked with 10% bovine serum albumin (BSA) at 37 °C for 60 min. The primary antibody anti-HIV-1 p24 mAb P6F1 generated by our laboratory [26] or mouse anti-LPS core (WNA 22-5; Hycult Biotech, PA, USA) were diluted in BSA and incubated with the sections overnight at 4 °C. Thereafter, the sections were washed and secondary antibodies conjugated with horseradish peroxidase (HRP) were added. After incubation in BSA for 60 min at RT, 3′-diaminobenzidine tetrahydrochloride (DAB) was used as a substrate to yield a deep brown color, and counterstained with hematoxylin. Sections were mounted on a Leica DMI 4000B (Leica Microsystems, Wetzlar, Germany).

2.10. Statistics

Significance between paired data of 4 macaques was determined by paired *t* test. Difference between LRA-treatment groups was calculated by one-way analysis of variance. Correlations were determined by the Spearman rank method. For all tests, a *P* value less than 0.05 was considered statistically significant.

2.11. Study approval

Macaques were housed in the ABSL-3 laboratory at the Kunming Primate Research Center, Kunming Institute of Zoology, Chinese Academy of Sciences in accordance with the regulations of the American Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures were performed according to the guidelines approved by the Ethics Committee of the Kunming Institute of Zoology (approval ID: SYDW-2011009). Efforts were made to minimize suffering of the macaques. For invasive procedures such as intravenous injections and blood collection, macaques were anesthetized by intramuscular injection 2 mg/kg of xylazine hydrochloride. For euthanasia, macaque 05001 was injected intravenously 80 mg/kg of pelltobarbitalum natricum combined intramuscularly 10 mg/kg of ketamine hydrochloride.

3. Results

3.1. Establishment of HIV-1$_{NL4.3}$ infection in northern pig-tailed macaques (NPM)

We have shown that HIV-1 could replicate in PBMCs of NPMs [22]. Here, 4 juvenile NPMs, two males and two females, were

inoculated intravenously with HIV-1$_{NL4.3}$. Infection was successfully established in all 4 animals, and a peak plasma viral load of 10^4 to 10^5 copies of viral *gag* RNA (vRNA) per mL was reached at 1 week after inoculation. Thereafter, the viremia decreased gradually, and by 6 weeks post-infection (wpi), there were fewer than 50 copies/mL (the detectable limit) of vRNAs in all 4 macaques. Sporadic blips of plasma vRNAs were detected between 6 and 12 wpi, thereafter, vRNAs cannot be detected in plasma during the observed times (Fig. 1a). A mild but temporary decline in the number of CD4$^+$ T cells in peripheral blood was observed during primary HIV-1 infection (5 to 14 wpi) but after this period the numbers recovered. However, these decline of CD4$^+$ T cell numbers can hardly be attributed to HIV-1 replication, for the lack of other haematological data and contemporaneous analyses of co-housed uninfected macaques (Fig. 1a, b).

To confirm HIV-1 infection, antibodies against whole HIV-1 antigens were measured by ELISA. All infected animals developed HIV-1 antibodies that were detectable at 4 wpi. Antibody titers peaked at 8 wpi and then remained at high levels. However, the patterns of antibody responses differed among the 4 animals. For example, animal 05001 generated robust antibody titers and peak values over 3000, which was consistent with the high B cell levels seen in this animal (Fig. S1). Meanwhile, the other three animals mounted 10- to 100-fold lower antibody responses relative to animal 05001 during the course of the infection (Fig. 1c).

We also evaluated immunological changes in NPM after HIV-1 infection by analyzing the numbers of CD8$^+$ T and B cells in peripheral blood (Supplementary Fig.1a, b), the subpopulation of CD4$^+$ T cells (Fig. S1c), the abilities of CD4$^+$ and CD8$^+$ T cells to express Th1 cytokines (IFN-γ, IL-2 and TNF) and Th2 cytokines (IL-4 and IL-13) (Fig. S1d-g), and the activation status of the CD4$^+$, CD8$^+$ T cell and B cells (Fig. S2). All of these parameters showed few changes during the course of infection. The animals experienced no fever, weight loss, or diarrhea. Together these data implied that HIV-1$_{NL4.3}$ infection caused few immunological perturbations in NPM.

Thus, HIV-1 could infect NPM without high and persistent viremia or pathogenicity. Yet the persistent antibodies to HIV-1 observed during the course of infection might support the possibility that the HIV infection persists in vivo for 3 years and that the virus might be replicating at a low level.

3.2. A stable viral reservoir was formed in HIV-1$_{NL4.3}$ infected NPMs

Although viral RNA in plasma could not be detected in infected NPM at 12 wpi, nested PCR assays showed the presence of the proviral genes *gag* and *env* in PBMCs taken from 4 infected animals at various time points during the infection. The exception was a negative result for *env* in animal 04002 at 137 wpi (Table S1). Considering that the half-life of unintegrated, cytoplasmic provirus is roughly 1 day [27,28], these results suggested that the viruses were not eliminated, but were instead consistently integrated in

the host genome. Subsequently, detailed information concerning provirus sequences provided by single viral genome amplification and sequencing (SGA) revealed that a few, but not accumulated, G → A mutations were present in the env sequences (Fig. S3). These mutations might have been induced by host APOBEC3 proteins [29,30]. Of note, there were some sequences that were identical to that of the inoculated virus, suggesting that the proviruses, to a certain degree, consisted of original viral genomes that were established upon primary infection. However, because of the limited numbers of the *env* sequences in each monkey (Fig. S3), whether these proviruses resulted from low-level HIV-1 replication or had spread during the acute infection phase is difficult to determine.

We then used quantitative PCR to further assess the numbers of viral *gag* DNA and RNA in PBMCs from the infected animals. PBMC-associated viral DNA was consistent with the PCR amplification results, with a peak at 2 wpi and then maintenance of about 100 copies per million PBMCs (Fig. 2a). However, by 134 wpi the viral RNA in PBMCs declined slowly but consistently from 10,000 copies per million PBMCs to about 100 copies per million PBMCs (Fig. 2b). Interestingly, in the presence of viremia, the plasma viral load significantly correlated with PBMC-associated viral RNA ($r = 0.78$, $P = 0.001$; Fig. 2d) but not DNA ($r = 0.15$, $P = 0.39$; Fig. 2c), suggesting that a relatively stable viral reservoir was formed during the initial infection, even though virus replication was impeded at this stage. Meanwhile, during the whole process of infection, viral RNA and DNA in PBMCs had a strong positive correlation ($r = 0.25$, $P = 0.035$), suggesting that some proviruses were consistently activated (Fig. 2e).

In the absence of viremia, PBMC-associated HIV-1 RNA levels represented the numbers of HIV-1 transcripts, which could provide a useful alternative biomarker to plasma viral RNA in vivo [31]. The apparent stabilization of HIV DNA and the persistence of HIV RNA transcription in PBMCs implied that HIV-1 was viable and might replicate at a low level in the 4 infected NPMs.

To address whether or not the putative proviruses could be reactivated, we isolated CD8-depleted-PBMCs from infected animals and co-cultured these cells with CD8-depleted-PBMCs from healthy human donors in the presence of Con A and IL-2. In contrast to previous reports in which HIV could not be recovered during late infection stages in infected pig-tailed macaques [9], here we showed that HIV-1 particles could be successfully amplified even at 137 wpi in all 4 NPMs (Table S1, Fig. 2f). This result confirms that a persistent HIV-1 infection was established and replication-competent viruses could be recovered after potent stimulation of PBMCs with a Con A/IL-2 mitogen cocktail ex vivo.

3.3. HIV viral reservoir can be induced ex vivo by JQ1 and prostratin

The relative homeostasis of HIV-1 DNA and the decline in HIV-1 RNA levels in PBMCs at longitudinal post-infection time points
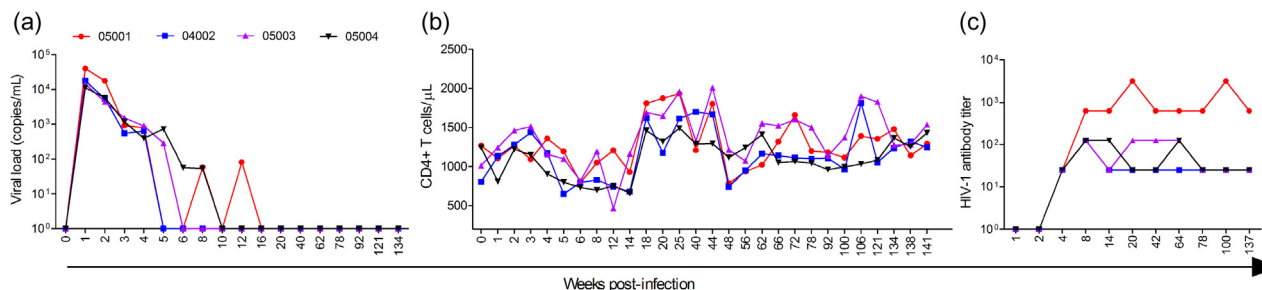

Fig. 1. (Color online) HIV-1$_{NL4-3}$ infection of northern pig-tailed macaques (NPMs). Four NPMs were inoculated intravenously with HIV-1$_{NL4-3}$ and monitored the infection for approximately 3 years. (a) Plasma viral load measurements; (b) CD3$^+$ CD4$^+$ T-cell counts in peripheral blood; (c) HIV-1 special IgG antibody titers by ELISA.
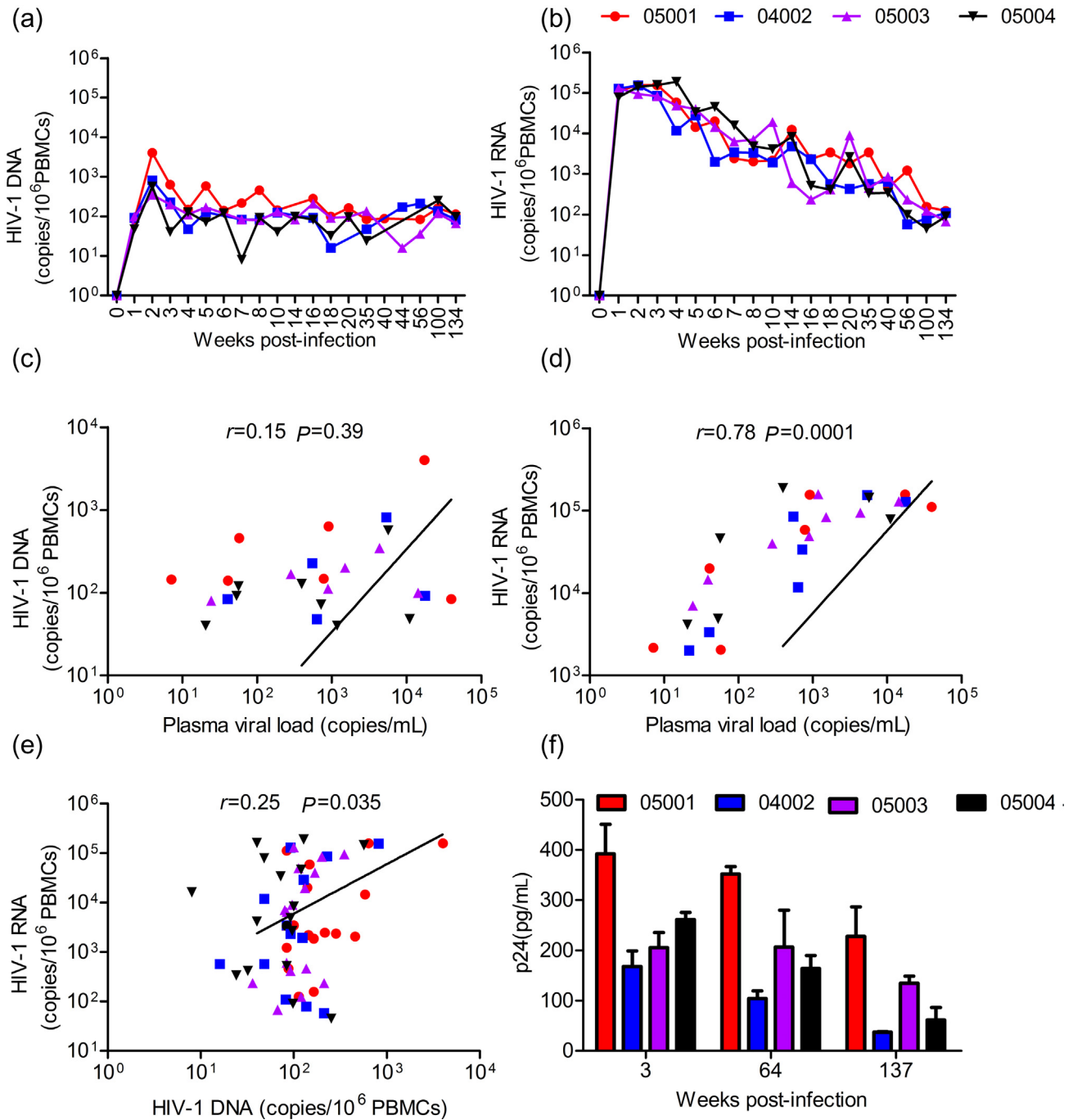
Fig. 2. (Color online) HIV-1 transcription can be detected and HIV-1 particles can be recovered from four infected NPMs. Longitudinal analysis of PBMC-associated (a) DNA and (b) RNA levels in HIV-1$_{NL4.3}$ infected NPMs. (c) Correlation of plasma viral load with PBMC-associated DNA and (d) RNA. (e) Correlation of PBMC-associated RNA with PBMC-associated DNA. P values were calculated by Spearman rank method. (f) Virus isolation by co-culturing CD8-depleted PBMCs from HIV-1-infected NPMs with human PBMCs. HIV-1 replication in the supernatant was detected by p24 ELISA at 21 days post co-culture.

suggested that the transcription of a significant number of proviruses was gradually impeded, which raised the possibility that in macaques a large proportion of viral DNA was maintained in a dormant state during the course of infection. Therefore, to determine whether these viruses can be reactivated at late infection time points, we isolated PBMCs from infected animals at 130 wpi and stimulated them with the latency-reversing agents (LRAs) JQ1 and prostratin. Compared with baseline levels, a single JQ1 (250 nmol/L) or prostratin (250 nmol/L) dose modestly increased HIV-1 gag expression by about 5.0 and 5.8-fold, respectively. Given that prostratin can boost HIV-1 transcription initiation by

activating NF-κB [32,33], and that JQ1 affects HIV-1 transcription elongation by promoting the release of PTEF-b from BRD4 [34,35], the combination of these two LRAs could potently increase latent *gag* RNA expression, as evidenced by the ~28- fold (21.5–39.7) increase in expression in NPM PBMCs. This synergistic effect was comparable to the increase in expression produced by PHA (25.0-fold), and was slightly weaker than that of Con A (70.5-fold) (Fig. 3a). Of note, the JQ1-prostratin combination did not induce global T-cell activation in parallel co-cultures (Supplemental Fig. 4). Together these results show that the combination of JQ1 and prostratin could robustly reactivate the latent viruses ex vivo.
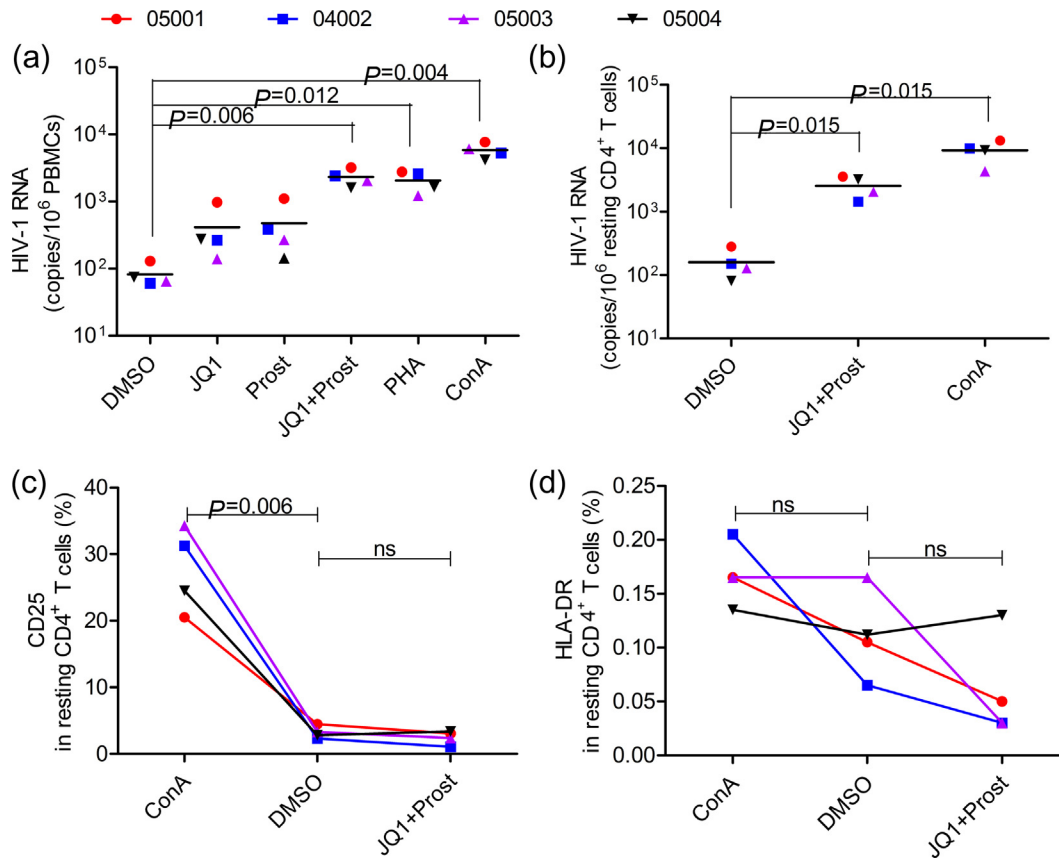
Fig. 3. (Color online) HIV-1 reactivation by ex vivo stimulation. (a) PBMCs from the infected NPMs at 130 wpi and (b) Resting CD4^{+} T cells from the infected NPMs at 134 wpi were treated with JQ1 and prostratin. After a 48 h co-culture, cell-associated HIV-1 RNA was quantified by RT-PCR. DMSO served as the negative control, and PHA and Con A were the positive controls for both treatments. The differences between LRA-treatment groups were assessed by one-way analysis of variance. The activation levels of resting CD4^{+} T cells were evaluated by surface staining of (c) CD25 and (d) HLA-DR in parallel. The P values between treatment and non-treatment groups of activation levels were determined by paired t test.

Considering that the resting CD4^{+} T cells harboring latent, replication-competent HIV-1 DNA can act as a stable viral reservoir and present a major obstacle to eradicating HIV-1 [36], we next determined whether latent virus harbored in resting CD4^{+} T cells can be reactivated by JQ1 and prostratin. We isolated resting CD4^{+} T cells from PBMCs of each inoculated NPM at 134 wpi by depleting other cells with antibody-coated magnetic beads (Fig. S5), and evaluated the changes of HIV-1 *gag* expression after treatment with the same concentration of JQ1 and prostratin used to stimulate PBMCs. We found that JQ1 and prostratin could robustly activate latent *gag* transcription in resting CD4^{+} T cells (Fig. 3b) without altering CD25 and HLA-DR expression (Fig. 3c, d). An optimum expression level of resting CD4^{+} T cell-associated HIV RNA (rc RNA) emerged 48 h after JQ1 and prostratin exposure, and was increased by about 16-fold relative to pre-treatment levels, which was consistent with recent studies that showed stimulation of latent virus in resting CD4^{+} T cells isolated from human patients on long-term antiretroviral therapy [34,37]. Together, these results demonstrated that HIV-1 was latent in infected NPM and was similar to human patients in that latent infection could be potently reactivated ex vivo from both PBMCs and resting CD4^{+} T cells. As such, resting CD4^{+} T cells from NPM might serve as a potential primary cell model to assess LRAs and latency-reversing strategies.

3.4. Viral reservoir in peripheral blood can be reactivated by JQ1 and prostratin in vivo

Because the combination of JQ1 and prostratin could potentially induce HIV-1 gene transcription ex vivo, we evaluated the reactiva-

tion of HIV-1 replication in vivo. At 138 wpi each macaque was treated with both JQ1 (100 µg/kg) and prostratin (80 µg/kg) by intravenous injection. As early as 6 h after administration, animals 04002 and 05004 showed a rapid rebound of virus replication. At 24 h post-injection, HIV-1 viral RNA could be detected in the plasma of all the macaques at a mean of 119 copies/mL. The plasma viral loads peaked at 48 h post-injection and varied significantly among the test animals, ranging from 10^2 to 10^3 viral RNA copies/mL. The viral RNA levels then decayed rapidly, and within 24 h of injection were near (animal 05001) or below (the other 3 animals) the limit of detection at 72 h post-injection, thus indicating that the effect of LRA co-stimulation was transient (Fig. 4a). Additionally, an increase in the expression of HIV-1 *gag* RNA in PBMCs was detected in these animals after exposure to JQ1 and prostratin. Consistent with the reappearance of plasma virus, PBMC-associated viral RNA increased 7-fold (range 4.4–11.0) 6 h after administration and peaked at 59-fold (range 34.8–82.6) 48 h after administration relative to the baseline. Thereafter, the levels decreased and within 72 h of treatment returned to pre-treatment levels (Fig. 4b). However, PBMC-associated DNA levels did not change significantly during JQ1 and prostratin treatment (Fig. 4c). The level of cell-associated viral DNA represents the size of proviral DNA genome, and can partly act as a surrogate indicator of the HIV-1 reservoir. Thus, these observations indicated that some proviruses could be reactivated from a relatively stable HIV-1 reservoir in these infected NPM.

All macaques tolerated the LRA dose used and showed no obvious clinical side effects. Meanwhile, the main subpopulation and activation status of immune cells in each animal fluctuated but
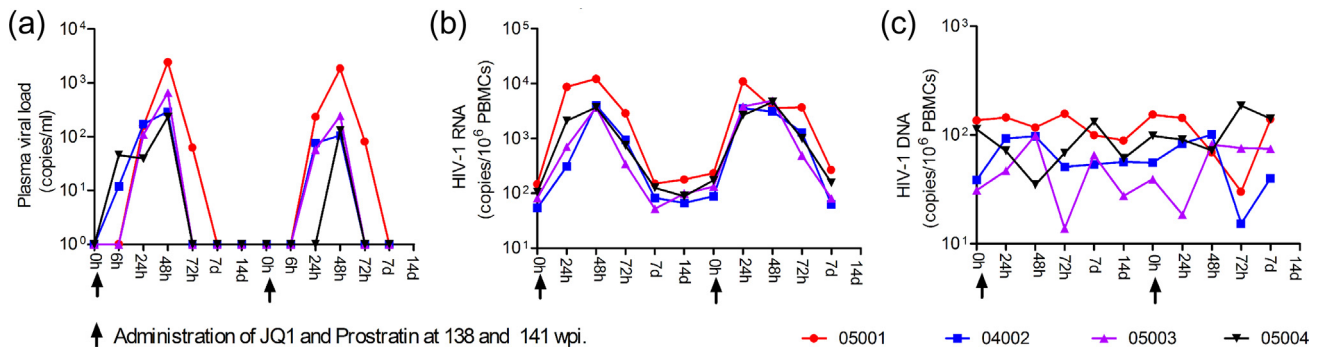
Fig. 4. (Color online) HIV-1 replication rebound after in vivo administration of JQ1 and prostratin. Infected NPMs were injected intravenously twice with JQ1 and prostratin at 138 and 141 wpi. The (a) plasma viral load, (b) PBMC-associated viral **RNA**, regular and (c) PBMC-associated viral **DNA**, regular were analyzed by real-time PCR. Dose 1 and 2 indicate the first and second administration of JQ1 and prostratin, respectively.

returned to pre-treatment levels within 7d (Figs. S6 and S7). Thus, a second injection of JQ1 and prostratin at the same dose was given to each macaque 3 weeks after the first dose, at 141 wpi. The changes in plasma HIV RNA, PBMC-associated viral RNA and DNA were consistent with those produced by the first treatment (Fig. 4), confirming that the viral reservoirs in infected macaques could be reactivated in vivo.

3.5. Viral reservoir harbored in peripheral blood can be reactivated in vivo

Because the levels of plasma and PBMC-associated viral RNA in vivo peaked 48 h after JQ1 and prostratin injection, we isolated circulating resting CD4$^+$ T cells from PBMCs in each animal at this time point, and evaluated the viral RNA and DNA by real-time PCR. In agreement with the ex vivo results, HIV-1 replication was elevated in resting CD4$^+$ T cells after the two doses of JQ1 and prostratin. Compared with the baseline, resting CD4+ T cell-associated RNA (rc-RNA) was greatly upregulated by 46-fold (range 13.1–76.2) and 32-fold (range 18.8–57.1) after the first and second dose, respectively (Fig. 5a). However, neither treatment produced a significant change in resting CD4$^+$ T cell-associated DNA (rc-DNA) ($P > 0.05$) (Fig. 5b).

3.6. Viral reservoir harbored some lymphoid tissues can be reactivated in vivo

The lymphoid organs are primary sites of HIV-1 replication and contain more than 98% of CD4$^+$ T cells in humans [38]. To evaluate the extent of HIV-1 latency in lymphoid tissue and the degree of LRA activation, at 150 wpi, or 9 weeks after the second dose, animal 05001 was injected with a third dose of JQ1 and prostratin. The animal was sacrificed 48 h after LRA treatment and the PBMCs, ileum, colon, inguinal lymph nodes (ILN), mesenteric lymph nodes (MLN), and spleen were isolated at necropsy. In resting CD4$^+$ T cells isolated from the PBMC and ILN, LRA stimulation produced no significant change in viral DNA levels, which was consistent with the results for doses 1 and 2. The viral DNA levels in the colon and MLN were higher than in other organs, suggesting that these organs might be viral sanctuaries in vivo (Fig. 5c). These results were similar to data obtained from simian immunodeficiency virus (SIV)-infected pig-tailed macaques after HAART [39]. On the other hand, the levels of resting CD4$^+$ T-cell-associated viral RNA from PBMC and ILN were up-regulated by 29- and 17-fold, respectively (Fig. 5d). Because we did not have baseline values for the other organs, we could not determine whether and to what extent viral reservoirs at other sites were activated.

To further confirm that latent viruses were activated by JQ1 and prostratin, ILN and colon tissues from animal 05001 were

subjected to pathological analysis. HE staining showed that these organs had no obvious pathological changes, and there were no significant microbial translocations as indicated by LPS staining (Supplemental Fig. 8). However, HIV-1 p24 staining revealed high expression levels in these organs after stimulation, which might be due to viral reservoir reactivation (Fig. 6).

4. Discussion and conclusion

The discovery in 1992 that pig-tailed macaques (*M. nemestrina*) were susceptible to infection by HIV-1 [1] increased the enthusiasm for developing a NHP model to study pathogenic HIV-1 infection. Thus, many studies during the 1990s concentrated on the following four aspects: (1) optimizing inoculation routes and doses [2,3,6,7], (2) selection of various viral strains [3,4,7], (3) in vivo passage of HIV-1 in pig-tailed macaques [8,9], and (4) introduction of minimally modified HIV-1 strains to evade innate immune sensing [13–16]. Here, we showed that HIV-1 could replicate at a low level and form a long-term viral reservoir in northern pig-tailed macaques (*M. leonina*, NPM), which extends earlier findings and broadens the understanding of HIV-1 latency in non-pathogenic animal models [40].

Early reports indicated that HIV-1 could only form a weak primary infection in *M. nemestrina*; in our studies, we initially attempted to establish an optimizational model of HIV-1-infected *M. leonina*, and to our disappointment, although the initial infection became viremic and seroconverted, HIV-1 replication diminished markedly within 12 weeks after infection and did not rebound thereafter, consistent with that of *M. nemestrima*. However, the continued presence of HIV-1- specific antibodies, the detectable HIV-1 genes and successful re-isolation of HIV-1 particles after approximately 3 years of monitoring documented herein were much more progresses than previously observed using HIV-1 in *M. nemestrina*, which might result from distinct genetic backgrounds between *M. leonina* and *M. nemestrima*, or different methods for genes amplification and virus isolation, the reason needed further examinations.

There were several clues that supported viral persistent replication in NPMs. (1) A mild viral replication at early stages of infection, a controlling viremia but persistent HIV-1 antibodies were found during 3 years of infection. (2) Persistent cell-associated HIV-1 DNA and RNA were detected in this model. (3) The viral reservoir (cell-associated DNA) in NPMs was stable through longitudinal post-infection even at the strict control of immunologic mechanisms.

The precise definition of the nature of HIV-1 viral reservoir is still debated, but it hallmarks as the presence of a small population of resting CD4$^+$ T cells harboring little, or non-expressing, integrated HIV proviruses which can be induced to produce
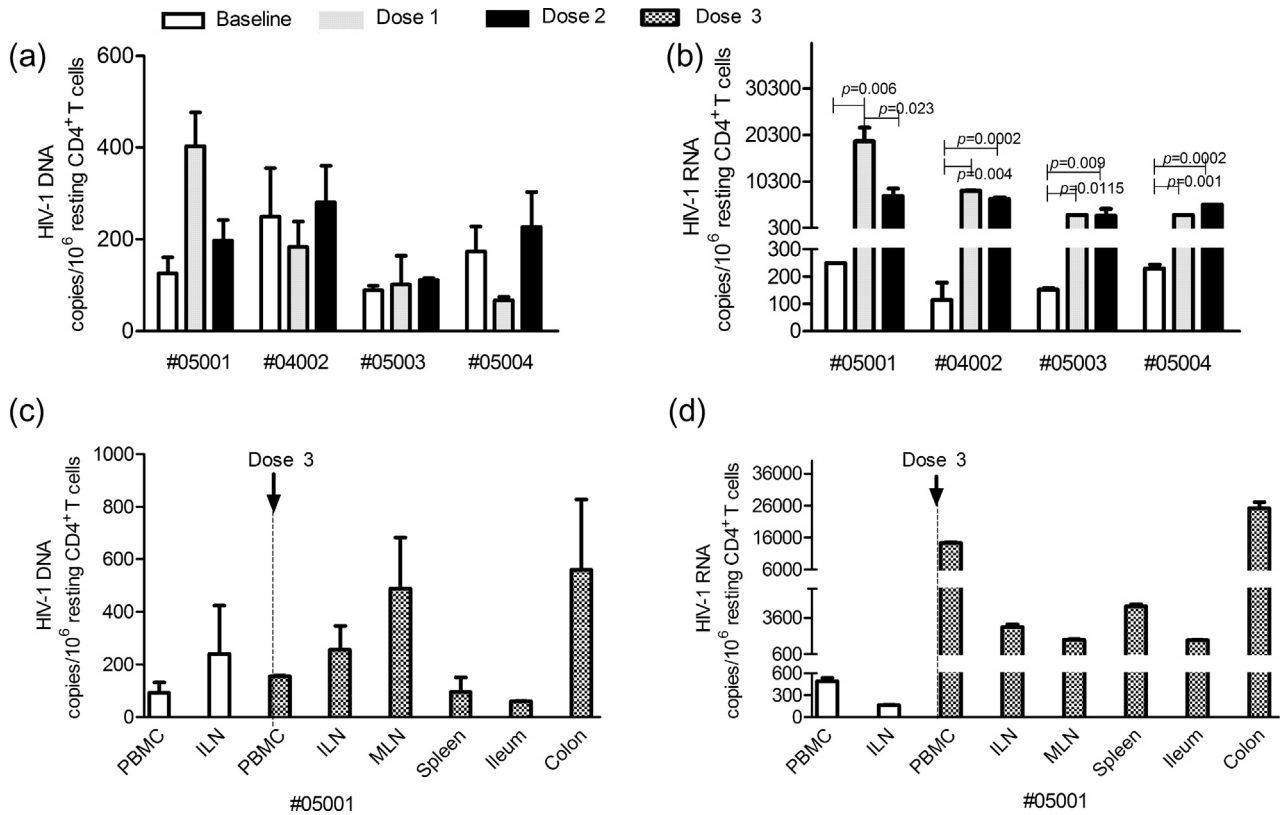
Fig. 5. HIV-1 replication in resting CD4+ T cells up-regulated by in vivo administration of JQ1 and prostratin. Two dose of JQ1 and prostratin was given to 4 HIV-1 infected animals at 138 and 141 wpi, at 48 h after treatment, resting CD4+ T cells were isolated from peripheral blood of infected NPMs. (a) Cell-associated viral DNA (rc-DNA) and (b) Cell-associated viral RNA (rc-RNA) were analyzed by quantitative PCR. Nine weeks after the second stimulation, a third treatment of JQ1 and prostratin was given to animal 05001 on 150 wpi. The animal was sacrificed 48 h after treatment, and the resting CD4+ T cells were isolated from several lymphoid tissues. (c) The rc-DNA and (d) rc-RNA in purified CD4+ T cells was analyzed by quantitative PCR. The data were presented as mean ± SEM. P values were determined by paired t test.
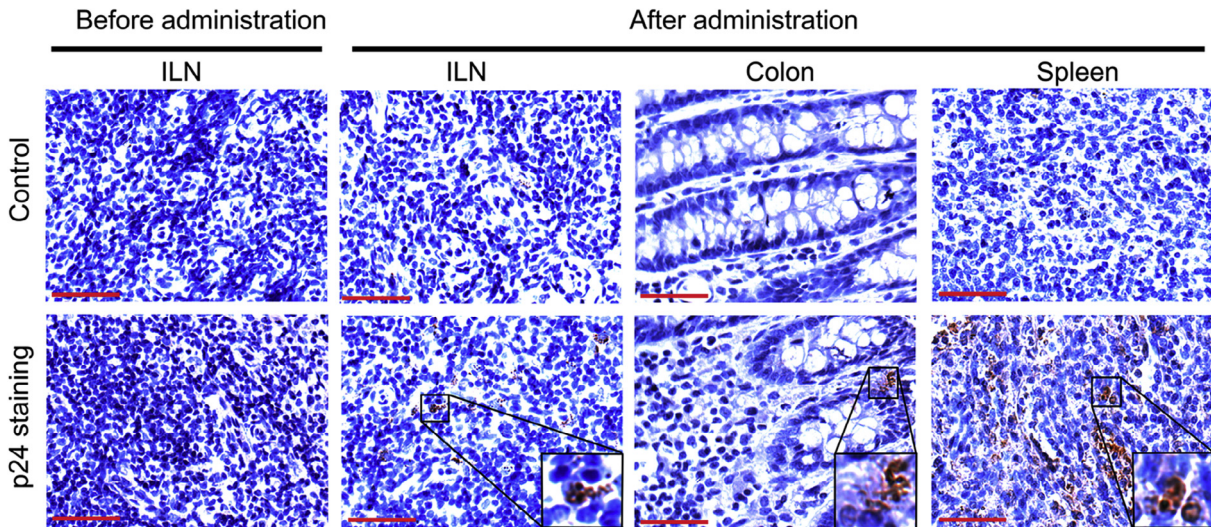

Fig. 6. Immunohistochemistry of HIV-1 replication in lymphoid tissues. Animal 05001 was injected with JQ1 and prostratin and sacrificed 48 h later. Immunohistochemistry was performed with anti-p24 antibody on paraffin sections of lymph nodes (ILN), spleen, and colon. Scale bars, 50 μm.

replicationcompetent viruses upon stimulation [41]. Several lines of evidence from our study support that, similar to those found in humans, HIV-1-infected NPM likely have HIV-1 reservoirs: (1) resting CD4+ T cells in both peripheral blood and lymphoid tissues carried a small number of HIV proviruses (ranging from 2 to 3 $\log_{10}$ per million resting CD4+ T cells), and the proviruses in PBMCs could be partly recovered even at late time points of infection; (2) HIV proviruses in resting CD4+ T cells could be reactivated in vitro and in vivo in response to strong stimulatory signals provided by LRAs; and (3) plasma viremia transiently rebounded, and tissue-distributed viruses could be reactivated by LRA treatment.

In this study, HIV-1 transcription and replication in NPMs were greatly reactivated by LRAs JQ1 and prostratin. The small molecule JQ1, with its high specificity for BRD4, can interfere with BRD4 binding to transcription factor pTEFb, then pTEFb was released from HIV-1 promoter, and interacts with Tat to promote HIV-1 transcription elongation [30,31]. Prostratin, an activator of protein kinase C, can promote the translocation of the transcription factor NF-κB into cell nucleus, where it can bind to the HIV LTR and initiate HIV-1 transcription [42]. Although JQ1 and Prostratin have shown potential ability to reverse HIV-1 latency in vitro, concerns regarding toxicities have impeded their clinical studies. JQ1 has demonstrated good tolerability of twice-daily administration at intraperitoneal dose of 100 mg/kg in mice when it was applied in cancer researches [43]. However, no data were presented in other animals, such as macaques. As for Prostratin, studies were conducted in CD2F1 mice using both the intraperitoneal route at a dose of 1.9 mg/kg and the intravenous route at a dose of 0.76 mg/kg, no obvious toxicities were observed. The maximum tolerated dose (MTD) in Fisher rats ranged from 0.2 to 0.4 mg/kg. In rhesus macaques, a single intravenous dose of either 0.1 or 0.4 mg/kg was well tolerated. At the 0.6 mg/kg dose, the monkey particular exhibited severe hepatotoxicity with 25- to 50-fold increases in level of ALT and AST over baseline measurements. The monkey injected with 0.8 mg/kg prostratin died of a respiratory failure [44]. In this study, to reactivate the latent HIV-1 in vivo, we selected a dose of JQ1 (100 μg/kg) and prostratin (80 μg/kg) combination by intravenous injection to each NPM. All macaques tolerated well and showed no obvious clinical side effects. This study could provide some valuable information for the preclinical toxicity evaluation of JQ1 and prostratin.

To date, there is little evidence showing that LRAs can upregulate in vivo viral RNA levels in resting CD4$^+$ T cells. Vorinostat and romidepsin, belonging to histone deacetylase inhibitors, were the only two LRAs that can produce marginal increases in viral RNA in clinical trials [45,46], whereas other LRAs showed inconsistent or negative results [47–49]. Thus, more robust LRAs either alone or in combination should be tested in the future [37,50], and preclinical animal models are urgently needed for further evaluations. The HIV-1-infected NPMs in this study represent an animal model of the rebound in HIV-1 replication both ex vivo and in vivo after stimulation with a combination of JQ1 and prostratin, which recapitulated the main features of HIV-1 latency reversing ex vivo model in humans [34,37]. Therefore, these in vivo results could give some clues for clinical study of LRAs.

Although long-term HIV infection in NPMs indeed showed a viral reservoir that recapitulated some features of HIV-1 latency in humans and was responsive to LRA stimulation, expanding this model for use as a testing platform for HIV-1 latency-reversing strategies should be carefully examined in future studies. The temporary viral replication and rebound implied a strong repression of immunological control in NPMs, suggesting that the HIV-1 infection in these animals was partly permissive. It may be due to the antagonism of host antiretroviral factors such as APOBEC3 and tetherin, or the restriction of CD8$^+$ cells and major histocompatibility complexes to HIV-1 [16]. Thus, the HIV-1 viral reservoir formed in NPMs is likely to be mediated by different mechanisms than those in elite controllers or patients under HAART treatment. Further improvements of this model as well as a detailed understanding of viral latency mechanisms could be achieved by systemic comparison of LRA efficiencies in NPMs and in human patients, and through inoculation with stHIV-1 recombinants or SIV that have more robust replication capacity in NPMs [51].

In summary, HIV-1 can infect NPMs and a long time viral reservoir was formed in vivo. LRAs JQ1 and prostratin could reactivate this viral reservoir, indicating that NPMs are a potential model to be developed for HIV-1 reservoirs research.

Conflict of interest

The authors declare that they have no conflict interests.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.scib.2017.09.020.

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