Early Treatment Regimens Achieve Sustained Virologic Remission in Infant Macaques Infected with SIV at Birth

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Article

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Abstract

Early antiretroviral therapy (ART) in HIV-infected infants generally fails to achieve a sustained state of ART-free virologic remission, leading to rapid viral rebound, even after years of analytical treatment interruption. Our studies showed that in contrast to adults, skewing of viral integration was observed in neonatal macaques intravenously exposed to SIV at birth. Further, one month of ART regimen combined an integrase inhibitor, initiated at day 3 but not day 4 and 5 post infection, efficiently and rapidly suppressed viremia to undetectable levels. Finally, interventions initiated at day 3 post infection for 9 months achieved a sustained virologic remission in ~80% infants. Collectively, an early intervention strategy with a key time checkpoint and regimen may result in viral remission for neonatal SIV infection, which may be clinically relevant for optimizing treatment strategies for HIV-infected infants.

Introduction

Although the Berlin, London, and Düsseldorf adult patients appeared to have experienced a "sterilizing HIV cure"¹, the treatment regimen involved toxic chemotherapy followed by hematopoietic stem cell transplantation, which is simply not feasible for most patients due to the high risk and expense. In HIV-infected adult patients, proviral reservoirs seeded are extremely stable during long-term antiretroviral therapy (ART), and become the driving force for viral rebound after analytic treatment interruption (ATI)^{2, 3, 4, 5, 6}. Although the timing of viral genome integration is uncertain in acute HIV infection, adult nonhuman primate model of SIV suggest that proviral reservoirs are essentially established as early as 3 days post infection^{7, 8}. Mother-to-child HIV transmission either occurs *in utero* at late pregnancy, at the time of delivery, in the days or weeks after birth through breastfeeding^{9, 10}. Current interventions to prevent *in utero* HIV infection have been highly successful, yet ~150,000 children are still postnatally infected with HIV every year¹¹. Although early initiation of ART in infants may reduce the viral reservoir size^{12, 13}, there are no genuine cases of a sustained state of ART-free virologic remission or cure in pediatric AIDS clinical trials or case reports^{12, 14, 15, 16, 17, 18, 19}. Note that the presence of HIV genome integration in tissues of human infants at or near the initiation of ART is rarely confirmed due to sample collection limitations. Moreover, current pediatric treatment regimens (*e.g.*, dual nucleoside reverse transcriptase inhibitors/NRTI AZT and 3TC, plus proteinase inhibitors) do not prevent cells from viral genome integration, so these regimens may also permit new or continual proviral reservoir seeding. In addition, late treatment regimens even when combined with integrase inhibitor in infant macaques, initiated at 35 dpi or 12 weeks p.i., also show viral rebound after short-term ATI^{20, 21}. All of these suggest that integrated proviral reservoirs, once established, are difficult to eradicate by conventional cure strategies and remain the major obstacle towards a pediatric HIV cure.

Newborn infants possess a unique immune system, characterized by the "immature" systemic immune system and the "functional/mature" mucosal system^{22, 23}. Neonates and infants infected with HIV develop disease rapidly, which is accompanied by compromised immune development and function^{24, 25}. We thus speculate that proviral reservoir seeding in infants exposed to HIV may be distinct from adults

that have larger numbers of potential target cells in tissues²⁶. However, HIV/SIV genome integration and reservoir seeding in developing infants exposed to viruses remain unclear. In this study, we examined viral DNA integration in neonatal macaques inoculated with SIV at birth and then cART including dolutegravir (DTG) was initiated at 3-, 4- or 5-days post infection. The results showed that integrated provirus was only detected in one of five neonates examined at 3 days post infection. One month of treatment initiated at day 3, but not delayed one day beyond, rapidly and efficiently suppressed viral replication to undetectable levels. Remarkably, in an additional group, 9 months of treatment initiated at day 3 resulted in a sustained virologic remission in 4 of 5 infants, as indicated by undetectable viral load and CD4+ T cell preservation after analytical treatment interruption, and absence of viral rebound by further CD8+ cell depletion *in vivo*. These findings suggest that early interventions, by rationale timing and regimen, likely achieve a HIV remission for pediatric HIV infection.

Results

Proviral reservoir seeding in tissues of neonatal macaques intravenously infected with SIV at birth. To determine the viral reservoir establishment and viral genome integration in infants once *exposed to HIV*, we used the pediatric SIV macaque model to evaluate these events above at precise time stage of early HIV infection, when neonates are exposed to virus from the maternal blood during labor and delivery. Newborn macaques were intravenously inoculated with identical doses of SIVmac251 within 6 hours after birth, and then euthanized at day 1, 2, 3, 5 and 7 post SIV inoculation for complete tissue collections (Fig. 1A). The results showed that plasma viral load was detectable in ~50% neonates at 1dpi and subsequent time points thereafter (Fig. 1B), consistent with the dynamics of cell-associated (CA) SIV RNA in PBMC, axillary lymph node (LN) and colon (Figs. 1C-1E). Likewise, total SIV DNA were detectable in these systemic and mucosal lymphoid tissues of neonates as early as 1dpi once exposed to SIV (Figs. 1F-1H). Notably however, the onset of integrated reservoir seeding did not appear until day 3 of infection, when integrated viral DNA was detected in a small amounts and specific tissues (axillary LN and tonsil only) from one of five animal. Strikingly, all neonates at 5 and 7dpi showed readily detectable CA integrated proviral DNA in tissues (Figs. 1I-1M). These findings suggest that proviral reservoirs are not fully seeded at 3dpi in the majority of neonates exposed to SIV at birth, which provides a critical window to block the establishment of proviral reservoirs for sustained viral remission even a cure in pediatric HIV infection by early treatment interventions.

Viral replication in neonates on treatment intervention initiated at day 3, 4 or 5 post SIV infection. To further compare the impact of early interventions on viral replication and viral reservoir eradication in infants infected with SIV, short-term cART (TFV/FTC/DTG) was initiated at either day 3, 4 or 5 after IV inoculation and continued for 28 days (n=3 per group) compared with untreated infants (n=9). In untreated infants, viremia plateaued at 7dpi, then maintained a high set point. In comparison, interventions initiated at 3dpi completely suppressed viral replication to undetectable levels by 7dpi and after, despite detectable blips of viremia in some infants at 5dpi (Fig. 2A). Unexpectedly, initiating treatment at day 3 post infection, compared with even one- or two-day delayed initiation (at 4 or 5dpi),

exhibited distinct outcomes in suppression of viral replication (Figs. 2A-2C). Delayed initiation of treatment consistently resulted in persistent viral replication as shown by persistent viremia at 7 and 14dpi in infants when cART was initiated at 4- or 5-days post infection (Figs. 2A-2D). At day 28, levels cell-associated SIV RNA in PBMC, axillary LN and rectum significantly decreased in infants when cART was initiated at 3dpi, compared to those in infant cohorts by 1- or 2-day delayed treatment. On the contrary, these cellular SIV RNA showed accumulation in these tissues in infants treated at 5dpi (Figs. 2E). Although treatment initiated at 4dpi also markedly reduced SIV RNA/DNA size in these tissues, intervention at 3dpi caused significant decay of both cellular SIV DNA to undetectable levels after one month of cART (Figs. 2F-2G). These data suggest that timing of early intervention may be key to containing virus replication and preventing integration in infants exposed to HIV/SIV.

Efficacy of intervention initiated at 3 days post infection on achieving sustained virologic remission in infants. To investigate outcomes of early interventions with prolonged cART in infants, an additional five newborn macaques were infected with SIV and treated daily with cART beginning at 3dpi for 9 months. Consistent with Figs. 1B and 2B, plasma viral load was detected in these infants prior to early treatment. cART initiation at 3dpi effectively suppressed viral replication in infants at scheduled timepoints throughout the 9-months of treatment, as shown by undetectable viremia and preserved CD4+ T cells thereafter under ART. After analytical treatment interruption, viral rebound was not detected in 4 infants (3 months for NT17 and NR90, 18 months for NG22 and NG90). However, one infant (NG24) exhibited viral rebound at 2 months after ATI, concomitant with CD4+ T cell depletion at this time point. Even so, viral rebound in NG24 was significantly delayed, compared to the viral recrudescence in SIV-infected adult macaques, which largely occurs between 10 and 21dpi when the same cART is initiated at 3 dpi ^{7,8}. Together, these data demonstrate that pediatric HIV remission is possible in a proportion of postnatally HIV-infected infants by early interventions, which has not been observed in HIV-infected human infant and adults, or infant macaques receiving the same ART combination while late treatment is initiated at 35 dpi or 12 weeks post infection ^{20,21}.

To track viral reservoirs and viral genome integration in these 5 infant macaques before and after ATI, we measured cell-associated SIV RNA and integrated SIV DNA in PBMCs and axillary LN biopsies, which were collected at day 3, month 1, 4, 9 and 11. The results indicated that cell-associated SIV RNA from both PBMCs and LNs was detectable at day 28 post infection when cART was initiated at 3dpi, and gradually diminished from month 4 to 9 after treatment (Figs. 3C and 3F). Further, although nonintegrated SIV DNA was detected in tissues as early as 1 day post SIV infection, early intervention initiated at 3dpi could efficiently eliminate these unstable nonintegrated SIV DNA from PBMCs and LNs by day 28 and beyond (Figs. 3D and 3G). Most importantly, proviral DNA was not detected in four infants except NG24 that had very low levels of integrated SIV DNA in axially LN at multiple timepoints throughout treatment (Figs. 3E and 3H), which likely contributed to viral replication and dissemination after analytical treatment interruption. These data were also consistent with *in situ* hybridization (RNAScope) analysis, as shown by detectable SIV RNA in both LN and rectal tissues of infant NG24 at 2

months post ATI, compared with undetectable SIV RNA in both tissues of the other four infants at this timepoint (Figs. 3I-3J).

Given distinct outcomes of infants on 9-months early cART, in which four infants showed viral remission while one showed viral rebound after ATI, the potential correlates of immunity were investigated. First, all infants studied were negative for *Mamu-A*01*, *Mamu-B*08* and *Mamu-B*017* (Suppl Fig. 1), which are associated with elite control in SIV-infected rhesus macaques²⁷. Second, neither anti-SIV gp140 Abs nor autologous neutralizing Abs against SIV Env were elicited in these SIV-infected infant macaques, except NG24 who displayed detectable anti-SIV gp140 Abs 2 months after ATI (Figs. 3K and 3L). To prove whether a viral remission or sterilizing cure was achieved, CD8⁺ cells were depleted in the four virus-controlling infants treated by rhesus anti-CD8 α Ab *in vivo* as previously described^{28,29}. As shown in Fig. 3M, CD8⁺ cells were completely depleted for up to 3 months in these infants after M-T807 Ab administration *in vivo*. Notably, no viral rebound or blips was detected during CD8⁺ cell depletion in these 4 infants. Combined, these findings demonstrate that a pediatric viral remission was achieved in these SIV-infected infants when early intervention combined with integrase inhibitor was initiated at day 3 post infection, which did not correlate with known protective MHC alleles, neutralizing Ab, or CTL responses.

Discussion

Utilizing the pediatric SIV macaque model of HIV, the data generated are informative for pediatric HIV remission by early interventions, based on our novel findings: 1) dynamic of tissue viral reservoirs and delayed onset of viral genome integration in neonates at the early time stage of SIV infection; 2) rapid suppression of viral replication and effective decay of nonintegrated viral DNA in infants by early ART combined with an integrase inhibitor at or near the timepoint of proviral reservoir seeding; and 3) complete eradication of reside reservoir and sustained virologic remission achieved by “long-term” intervention initiated prior to viral genome integration. The magnitude and persistence of proviral reservoirs, which are difficult to eradicate with current therapy, may determine the outcome of treatment interventions. If effective treatment is initiated prior to proviral reservoir seeding, sustained virologic remission or even a cure may be possible for pediatric HIV infection. In summary, ART-free viral remission may be achieved in a proportion of HIV-infected infants by rational early treatment regimens.

Given a unique immune system in developing neonatal infants, immune maturation, viral susceptibility, viral reservoir seeding in neonates exposed to HIV might differ from those in adults^{24,25}. Compared to unstable nonintegrated viral DNAs which may yield productive viral particles in a limited range of early HIV/SIV infection^{30,31,32,33,34,35} even the presence of integrase inhibitors^{34,35}. HIV genome integration is an essential hallmark for stable HIV replication and persistence, as integrated proviral DNA is the dominant template for persistent HIV replication³⁶, and difficult to eradicate by conventional cure strategies^{37,38,39}. Insight into the timing and onset of proviral reservoir seeding in infants exposed to HIV is critical for providing a clue in pediatric HIV therapy. Current scalable assays to measure replication-competent proviral reservoirs are difficult to reflect the *bona fide* functional provirus size and overall

tissue distribution in the body^{40, 41, 42, 43, 44, 45, 46, 47, 48}. For example, a small proportion of proviruses might exist in the body during long-term treatment, not limited to peripheral blood and lymphoid tissue samples. Viral reservoirs examined by Quantitative Viral Outgrowth Assays (QVOA) or Tat/rev Induced Limiting Dilution Assays (TILDA) may not fully reactivated by stimulators, especially when the proviruses are located in the "gene desert" sites of chromosomes enriched in repressive chromatin marks^{45, 49, 50, 51}. Near full-length Individual proviral sequencing (FLIPS) and intact proviral DNA assay (IPDA) may also not accurately distinguish the functional proviral reservoir, as indicated by existence of intact proviral "quasispecies" and viral polymorphism^{52, 53}, difficulties to predict production of replication-competent virions in the process of viral life cycle of HIV/SIV, functionality of viral genomes by various depletions and mutations^{47, 54, 55, 56}, or potential functional revertant mutation from deficient viral genome^{57, 58}, thereby lack of absolute gold standard approach to assess the genuine size of proviral reservoirs in the body⁵⁹. Although qPCR may overestimate the viral reservoirs because of defective proviruses, total proviral DNA levels still reflects approximate size of intact viral genome, as intact viral genome accounts for ~11.7% in HIV+ patients and exists with higher fraction in SIV+ macaques on cART^{60, 61}. Here we combined a ddPCR-based total viral RNA assay with nested qPCR for proviral DNA quantification targeting multiple repetitive *Alu* DNA islands on macaque chromosomes, providing a rapid and sensitive approach to generate comparable data in the assessment of proviral reservoir seeding and size from various tissues. Our data show that cell-associated SIV DNA/RNA is essentially detected in systemic and lymphoid tissues as early as 1dpi in neonates once infected with SIV at birth, concomitant with detectable plasma viral load in ~50% neonates at 1dpi. These data suggest that detectable plasma viral load at very early SIV infection is irrelevant to contamination of virus inoculation due to rapid viral replication. Unexpectedly, viral genome integration confers distinct patterns, as indicated by integrated proviral DNA is not detected until day 3 of infection, and only detectable in one of five infant animals (axillary LN and tonsil only in same infant) at this time point, suggesting routine PBMC samples may not be adequate to assess the proviral reservoir seeding. In such a scenario, nonintegrated viral DNA is able to produce virus particles^{31, 32, 33}, which was also confirmed by our *in vitro* studies, lacking integrated SIV DNA in presence of integrase inhibitor. All these findings suggest that early interventions at a key time checkpoint, combined with an INSTI prior to proviral reservoir seeding, can potentially block viral genome integration, thereby likely achieving sustained virologic remission and even a cure in infants exposed/infected with to HIV.

All children exposed to HIV before, during, and after birth should receive early antiretroviral (ARV) drugs to reduce the risk of HIV acquisition, morbidity, and mortality^{12, 16, 25, 62, 63}. Although nucleoside reverse transcriptase inhibitors (NRTIs) block viral RNA conversion into double strand viral DNA in the viral life cycle, current early ART regimen with a combination of dual-NRTI (AZT+3TC) plus protease inhibitors (LPV/r) may not effectively prevent new or continual viral integration in infants infected with HIV. Existence of nonintegrated viral DNA predisposes the high risk to the viral genome integration in absence of INSTI application. In such situation, the regimen combined with integrase inhibitors necessitate to prevent potential proviral reservoir seeding for early pediatric HIV therapy. Of integrase inhibitors, DTG, a

very well-tolerated, highly effective, and affordable INSTI drug in low- and middle-income countries ⁶⁴, achieves more rapid and complete viral suppression compared to raltegravir ⁶⁵. Although one study in Botswana shows that DTG used in the early first trimester of pregnancy may be associated with the incidence of neural tube defects (NTDs) in newborns ⁶⁶, whereas additional data from this cohort also indicate the risk may only be slightly greater over any non-DTG ART ⁶⁷. Worldwide pharmacovigilance data do not provide strong support concerning the relationship between NTDs and DTG ^{68, 69} and irrelevant to HIV-infected infants. DTG could be combined with two NRTIs (TFV and FTC) in an early intervention in pediatric HIV infection.

Early ART in infants may restrict viral reservoirs and reduce HIV proviral DNA ^{12, 13, 16, 62, 63, 70, 71, 72, 73, 74}. However, there is no reported case to eventually achieve an ART-free sustained virologic remission or cure in the HIV+ infants treated by early ART (immediate treatment to several years of age) in the Pediatric AIDS Clinical Trials and cases, including Mississippi and Canadian babies, South African Children with HIV Early Antiretroviral Therapy (CHER) trial, IMPAACT, Pediatric Early HAART and Strategic Treatment Interruption Study (PEHSS), Botswana and European Pregnancy and Pediatric HIV Cohort Collaboration (EPPICC) study. Unfortunately, the eventual viral rebound is observed during the follow-up of analytical treatment interruption ^{13, 14, 15, 18, 25, 71, 75, 76, 77, 78}. Notably, the status of viral genome integration is not fully examined in various tissues of these infants owing to limited sample collection at the initiation of ART. Also, conventional regimen administered to infants generally lacks integrase inhibitors combination, likely resulting in viral DNA integration in infants during pediatric HIV therapy. Building on patterns of proviral reservoir seeding in infants exposed to SIV at birth, intervention initiated at day 3, but not one day or two days delayed when viral genome integration essentially occurs in tissues, can rapidly and efficiently suppress viral replication to undetectable levels. These unexpected results are very interesting, yet which are consistent with onset of proviral reservoir seeding in general in newborn macaques exposed to SIV, suggesting the timing of early intervention initiation is a critical factor for blockade of viral integration and ultimate ART-free virologic remission in pediatric HIV infection. Although our data regarding early proviral reservoir seeding may not rule out the possibility that viral genome integration may occur in other unexamined tissues at 1-3dpi, however, early proviral reservoirs seeding in majority of systemic and lymphoid tissues essentially reflect their emergence, existence and distribution in neonates upon intravenously exposed to SIV, as directly supported by further investigation that nine months of treatment intervention initiated at day 3dpi indeed achieved pediatric viral remission in 4 of 5 infants. Pediatric early intervention but not prevention could be considered, as both plasma viral load and cell-associated viral RNA/DNA were detectable at the timepoint of cART initiation, yet ultimate viral rebound was not observed after CD8+ cell depletion *in vivo* when these early intervention is initiated at 3dpi for 9 months. Together, these findings might be associated with successful blockade of viral integration and viral reservoir eradication by rational early interventions, since a small viral reservoirs in anatomic tissues are high risk of viral rebound after ATI ^{16, 79, 80, 81, 82}.

It might be difficult to mediate viral reservoir clearance by prolonged early cART even beginning 4-9 d post SIV infection, as viral reservoirs including proviruses are essentially established in adult macaques as

early as 3dpi^{7,8} and tissue viral reservoirs are still detectable at 28 day old of infants on continuous cART initiated at 4 or 5dpi. In SIV-infected macaques on 16-months therapy, there is still significantly higher frequency of intact SIV proviral genomes compared to ART-treated HIV-infected humans⁶¹. In fact, intact HIV genomes decline more rapidly within first seven years yet then more slowly afterwards, and the half-life of the reservoirs is ~4.0 years until year seven and ~18.7 years thereafter^{4,5,83}. Also, residue HIV reservoirs still exist when cART is initiated during primary HIV infection⁸⁴. To date, only a rare HIV-infected individuals remain healthy without treatment, in which long-term treatment pressure drives proviruses to accumulate in human inactive gene regions of host chromosomes, likely stymieing new virus production^{85,86}. All these studies suggest that viral genome integration highly correlates with outcomes of viral remission. It is reported that broadly neutralizing antibody (bnAb) therapy given at 30 h or cART regimen at 48 h in one-month-old macaques after oral exposure to low pathogenic SHIVSF162P3^{87,88} could clear tissue viral reservoirs^{89,90}, in which all animals show aviremic outcomes over time treatment, inconsistent with our results that plasma viral load is essentially detectable in neonates at 24-48 h post intravenous SIV inoculation. It is also unknown whether viral genome integration occur in these infants and how bnAb treatment beginning at 1 day SHIV exposure could block virus replication, integration and production in SHIV-infected cells and completely eradicate productive/latent reservoirs established in immune developing infant, since rapid cell-associated viral RNA and nonintegrated viral DNA definitely emerge as early as 2 h *in vitro* infection (SIV RNA, ~1.5·10⁵ copies/10⁶ cells, nonintegrated SIV DNA, ~2.8·10³ copies/10⁶ cells) and 24-48 h in tissues of animals exposed to virus. Our data show that cART initiated as early as 3dpi for 1-month cannot eliminate cell-associated SIV RNA in various tissues examined, it is not understood how 21-day cART regimen beginning at 48 h could result in cell-associated viral RNA/DNA eradication and no viral rebound in infants after treatment is discontinued, especially when an amount of viral reservoirs probably exist in tissues in the body. However, our results may explain the efficacy of this early cART at 48 h p.i., as proviral reservoirs basically have not been established at this time point. To this end, multiple factors, including proviral reservoir seeding, timing of prolonged intervention, route of perinatal infection as well as pathogenic HIV/SIV/SHIV strains, are likely determine ultimate outcome in infants on early treatment.

The distinct proviral reservoir seeding and discrepant outcomes of early intervention in infants likely evolves immune maturation, ontogeny, susceptibility, host genetics, or other maternal factors²⁵. It remains unclear why infant animals show delayed proviral reservoir seeding, albeit identical SIV inoculum. Also, there are still tremendous fundamental gaps in the pediatric HIV infection and efficacy by early treatment, e.g., some infants infected with HIV/SIV show rapid AIDS development but not for others. HIV remission is proposed to be associated with multiple factors including initial viral suppression rate and rapidity with early ART⁹¹. The latter is confirmed by this study that timing and regimen of early ART initiation likely determines the suppression of viral replication, decay of viral reservoirs and clinical outcomes. However, it seems a challenge to evaluate the status of proviral reservoir establishment from various tissues (besides blood sample). Conceivably, proviral reservoirs once seeded in the body, *e.g.*, by intrauterine infection, is likely difficult to fully eradicate by early interventions, thus predisposing to a high

risk of viral persistence and viral rebound once treatment is discontinued. A small proportion of the HIV-infected infants develop *de novo* potent and broadly neutralizing antibodies (bnAbs) that respond to different and perhaps new epitopes of HIV-1 Env as early as one year post infection, compared with bnAbs development in HIV+ adults after 2-3 years of infection ⁹². However, neutralizing Abs against SIV Env were not detected in any of five SIV-infected infant macaques on early ART, likely because SIV Env does not effectively elicit nAbs ⁹³. Interestingly, neither nAbs nor CTLs are responsible for protection against SIV in the four infant macaques with viral remission, the mechanisms behind discrepant proviral reservoir seeding in infants at very early infection need to be further investigated.

In summary, our data suggest that onset of proviral reservoir seeding in tissues, timing and regimen of early intervention may determine the outcome of pediatric HIV therapy. These findings demonstrate that sustained HIV remission and even a cure are possible in infants postnatally exposed to HIV by early treatment regimens, providing a rational and translatable pediatric cure strategies to the clinic.

Methods

Ethics statement

All animals in this study were housed at the Tulane National Primate Research Center in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International standards. All studies were reviewed and approved by the Tulane University Institutional Animal Care and Use Committee under protocol number P0401. Animal housing and studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH, AAALAC #000594) and with the recommendations of the Weatherall report; "The Use of Non-Human Primates in Research". All clinical procedures were carried out under the direction of a laboratory animal veterinarian. All procedures were performed under anesthesia using ketamine, and all efforts were made to minimize stress, improve housing conditions, and to provide enrichment opportunities (*e.g.*, objects to manipulate in cage, varied food supplements, foraging and task-oriented feeding methods, interaction with caregivers and research staff).

Animals and virus

A total of thirty-seven newborn, Indian-origin rhesus macaques (*Macaca mulatta*) were utilized in this study. Newborn macaques were intravenously inoculated with 100 TCID₅₀ SIVmac251 on the day of birth (<6h, day 0). To examine viral DNA integration in tissues, fourteen neonatal macaques were euthanized for complete tissue collection at day 1 (n=3), 2 (n=3), 3 (n=5), 5 (n=2) and 7 (n=1) post SIV inoculation. An additional nine infant macaques received combined antiretroviral treatment (cART) with 3 anti-HIV drugs [tenofovir (TFV), 20 mg/kg/day; emtricitabine (FTC), 30 mg/kg/day; and dolutegravir (DTG), at 2.5 mg/kg/day] initiated at day 3 (n=3), 4 (n=3) or 5 (n=3) post SIV infection for 28 days of treatment. For a long-term intervention, 5 additional animals were infected and treated with cART initiated on day 3 and continued daily for 9 months. TFV and FTC were kindly provided by Gilead Inc. (Foster city, CA), and DTG

was kindly provided by ViiV Healthcare (Research Triangle, NC). Blood was collected weekly to monthly, and lymph node and rectal biopsies were collected at 1 month on cART, and 2 months after analytic treatment interruption (ATI). Animals were euthanized for tissue collection if viral rebound after treatment interruption was observed. Plasma and single-cell suspensions were prepared to examine plasma viral load, cell-associated SIV RNA/DNA, and for flow cytometry analysis. Archived plasma and PBMC samples from untreated infant macaques (n=9), which were infected with the same SIV lot, dose, and route were used to compare plasma viral load and the percentages of peripheral CD4⁺ T cells.

Tissue collection and phenotyping

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-treated venous blood by density gradient centrifugation with Lymphocyte Separation Medium (MP Biomedicals, Santa Ana, CA) or lymphocytes from other tissues, and then stained and analyzed by flow cytometry as we previously reported ⁹⁴. Cells were stained with: CD3 (SP34), CD4 (OKT4, BioLegend), CD8 (SK1) and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Grand Island, NY). Isotype-matched controls were included in all experiments. All antibodies and reagents were purchased from BD Biosciences Pharmingen (San Diego, CA) unless otherwise noted. Samples were resuspended in BD Stabilizing Fixative (BD Biosciences) and acquired on a FACS FORTESSA (Becton Dickinson, San Jose, CA). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Genomic DNA and total RNA extraction

Fresh PBMC and lymphocytes ($\sim 10^7$ per samples) were processed to extract total genomic DNA and cellular RNA with a AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Viral RNA in plasma was directly isolated using the QIAamp Viral RNA Mini Kit (Qiagen). The extracted DNA and RNA samples were stored at -80°C until further analysis.

Quantification of plasma viral load and cell-associated SIV RNA and DNA

Plasma viral load and cell-associated SIV RNA/DNA were measured by specific primer sets and probes as previously described ⁹⁵. Briefly, the extracted RNA was reverse-transcribed into cDNA using a SuperScript III first-strand synthesis system (Invitrogen) in a thermocycler at 25.0°C for 5 min and 50.0°C for 60 min, followed by an enzyme inactivation step at 70.0°C for 15 min. cDNA from cell-derived RNA was further used to quantify total SIV transcripts by digital droplet PCR (QX100 Droplet Digital qPCR system, Bio-Rad). Samples were run in duplicate in a 20 μ L volume containing Supermix, 250 nM primers, 900 nM probe and 2 μ L undiluted cDNA under the following cycling conditions: 10 min at 95°C, 40 cycles of 94°C/30s and 63°C/60s, and a final step at 98°C for 10 min. Droplets were analyzed by the QuantaSoft software in absolute quantification mode. Copies of SIV RNA, expressed as copies per 1 million cells, were measured and normalized to cellular input, as determined by copies of genomic CCR5 (single-copy rhesus macaque CCR5 DNA per cell) ⁹⁶. The limit of detection (LOD) was based on three or more replicates and calculated using GenEx 5 (www.multid.se).

To quantify SIV DNA, nested PCR was run in parallel to quantify total viral DNA and integrated proviral DNA. To measure integrated proviral DNA, briefly, the pre-amplification reactions were performed using SIV long terminal repeat primer and outward primers targeting multiple *Alu islands* on a 7900HT Sequence Detection System (Life Technologies). The reaction conditions were performed as follows: each 25 µL of reaction mix, contained 1X PCR buffer, 0.2 mM dNTPs, 2 mM MgCl₂, 0.8 µM of each primer and 0.5 U Taq DNA polymerase (Invitrogen Life Technologies) and was run for a 5-minute hot start at 95°C, followed by 20 cycles of denaturation at 95°C for 30 seconds, annealing at 63°C for 30 seconds and extension at 72°C for 3 minutes. Next, 2.5 µL of each amplicon was further amplified in triplicate by real-time PCR reaction using 40 cycles at 95°C for 15 seconds and 63°C for 1 minute. Highly reproducible calibration curves were generated by plotting C_q values against log-transformed concentrations of serially diluted standard. Internal standard curves were generated using known copy numbers of target plasmids (1-500 copies) diluted in cellular DNA from SIV naïve rhesus macaques. The calibration curves and internal regression curves were used for interpolating initial copies of each target in unknown samples. A non-template control (NTC) and extracted cellular DNA from the HUT78/SIVmac239 cell line (positive control) were included in the qPCR reactions. Quantification of integrated proviral DNA was expressed as copies per 1 million cells (Limit of detection, 1 copy in equivalent 2.4·10⁵ cells), in which cell numbers were determined by copies of genomic CCR5 DNA per cell. Nonintegrated SIV DNA was calculated by subtracting integrated viral DNA from total SIV DNA.

Detection of SIV RNA by in situ hybridization

To identify the SIV RNA in LN and rectal biopsies, formalin-fixed, paraffin-embedded sections (5-µm thickness) were cut and adhered to glass slides. After deparaffinization in xylene, and serial dehydration with 50%, 70% and 100% ethanol, sections were subjected to an RNAscope assay with RNAscope2.5 HD Detection Reagent-Red (ACD) following the manufacturer's instructions. The slides were denatured at 60°C for 1 h and incubated with pre-warmed (60°C) SIV gag-pol sense probe (ACD) with an additional incubation for 4 h at 40°C. The signal was then amplified by Amp1 to Amp6 and identified on tissue sections using a Zeiss digital slide scanner system.

In vivo CD8⁺ lymphocyte depletion

To deplete CD8⁺ lymphocytes (CD8⁺ T cells and NK cells), rhesus macaques received a single dose of 50mg/kg anti-CD8α CDR-grafted rhesus IgG1 antibody MT807R1 (NIH NHP Reagent Resource Program) by intravenous route as described⁹⁷. Absolute CD8⁺ T cell counts, and viral loads were measured following MT807R1 infusion.

ELISA

ELISA plates were coated with SIVmac239 gp140 (Immune Tech, New York, NY) at 5 µg/mL in PBS at 4°C overnight. After blocking with 1% BSA in PBS at 37°C for 1 hour, serially diluted plasma was incubated on plate at 37°C for 1 hour. Horseradish peroxidase (HRP)-conjugated goat anti-human IgA+IgG+IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added at 37°C for 1 hour. All volumes

were 100 μ L/well, except 200 μ L/well for blocking. Plates were washed between each step with 0.1% Tween 20 in PBS, developed with 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories), and read at 450 nm.

SIV neutralization antibody assay

SIV neutralization was measured using single-round infection of TZM-bl cells with Env pseudoviruses as described⁹⁸. Briefly, 40 μ L of virus was incubated for 30 min at 37°C with 10 μ L of serially diluted plasma in duplicate wells before the addition of TZM-bl cells. To maintain consistent assay conditions, sham medium was used in place of plasma in specified control wells. Plasma dilutions were defined at the point of incubation with virus supernatant. Virus infection levels were determined after 2 days by a luciferase assay (Promega, Madison, WI). Neutralization curves were fitted by the 5-parameter nonlinear regression built in Prism 8.0 (GraphPad Software, La Jolla, CA), and 50% inhibitory dilutions (ID₅₀) were defined as the plasma reciprocal dilutions required to inhibit viral infection by 50%.

Statistical analysis

Statistical analyses were performed by GraphPad Prism 7.0 Software (GraphPad Software, San Diego, CA). Statistical comparison between groups at different time points was analyzed using Mann-Whitney test. A nominal α level of 0.05 was used to define statistical significance and the data are presented as mean and SEM. The relationship of integrated viral reservoir size in tissues with the timing after viral inoculation was analyzed using a nonlinear regression curve fitting model.

Declarations

Author Contributions: X.Wang, E.V., S.S., K.T., H.L., R.B., X.Wu, M.W., J.S., W.Z., L.D.M., K.E.R. contributed to data collection. R.P.B., R.S.V. contributed to data interpretation and revisions. X.W. and H.X. designed the experiments and wrote the manuscript.

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Figures

Figure 1

Figure 1

Early viral DNA integration in systemic and lymphoid tissues in neonatal macaques intravenously infected with SIV at birth. (A) Study outline for infant macaques sampling and early interventions. (B) Plasma viral load in staggered sample collection from neonates after SIV infection. *, p < 0.05, compared with 1dpi. Total cell-associated SIV RNA (C-E) or SIV DNA (F-H) in PBMC, axillary LN and colon. **, p < 0.05, compared with 2dpi. Statistical comparison between groups was analyzed using Mann-Whitney test. (I-

M) Onset of viral DNA integration in PBMC, auxiliary LN, mesenteric LN, colon and tonsil in neonates with the days after SIV infection. Tissue lymphocytes were collected from euthanized neonatal macaques at 1dpi (n=3), 2dpi (n=3), 3dpi (n=5), 5dpi (n=2) and 7dpi (n=1). Cell-associated integrated proviral DNA were measured by *Alu*-based nested qPCR. Data were analyzed by curve fitting using nonlinear regression.

Figure 2

Figure 2

Suppression of viral replication in SIV-infected infant macaques on short-term early interventions initiated at 3-, 4- or 5-days post infection. Newborn macaques were intravenously inoculated with SIVmac251 within 6 hours after birth. Plasma viral load in staggered samples of infant macaques on early intervention initiated at 3 (**A**, n=3), 4 (**B**, n=3), or 5dpi (**C**, n=3) till 28 days of age. *, p<0.001, compared with the timepoint prior to treatment. (**D**) Statistical analysis of plasma viral load in infant cohorts by early intervention initiated at -3, -4 or -5dpi, compared plasma viral load in untreated infant macaques (n=9). (E-F) Levels of total SIV RNA, noninterated SIV DNA and integrated SIV DNA in lymphocytes isolated from PBMC, Axillary LN and rectum in infant macaques at day 28 of age. *,** and ***, p<0.001, compared with the 3dpi. #, p<0.001, compared with animal cohorts on early interventions at 3dpi. Note that early interventions initiated at 3dpi, compared to interventions with one or two days delayed (initiated at 4 or 5dpi), conferred rapid and efficient suppression of viral replication.

Figure 3

Figure 3

Efficacy of 9-months early interventions on the sustained virologic remission in SIV-infected infant macaques when intervention is initiated at 3 days post infection. (A) Plasma viral load in five SIV-infected infants on early intervention initiated at 3 dpi for 9 months (colorful line, n=5) or without treatment (solid dark line, n=9). Infant NG24 was euthanized at 2 months post ATI when viral rebound was observed. Four infants on early intervention showed viral remission throughout the study (up to 18 months for NG22 and

NG90 or 3 months for NT17 and NR90 off-ART to date). **(B)** Changes in peripheral CD4⁺ T cells in the SIV-infected infants on early intervention (colored line, n=5), compared with those of age-matched SIV-infected and untreated (solid dark line, n=9). Peripheral CD4⁺ T cell loss was observed in infant NG24 at 2 months post ATI. Levels of cell-associated total SIV RNA, nonintegrated viral DNA, and integrated SIV DNA in PBMCs **(C-E)** and axillary LN biopsies **(F-H)** from 5 infant macaques on early intervention or untreated infant cohort post SIV infection (dark line, n=4). *In situ* SIV RNA⁺ cells in axillary LN **(I)** and rectal **(J)** biopsies in infant macaques at 2 months post ATI. SIV RNA was detected in both LN and rectal tissue of infant NG24 only. Bar, 100µm. **(K)** Anti-SIV gp140-specific antibodies in the infants before and after ATI. Anti-SIV gp140 antibodies were detected in infant NG24 only at 2 months post ATI when viral rebound was observed. **(L)** Autologous neutralizing antibody against SIV Env in infants after 2 months of ATI. Neutralizing antibodies were not elicited in these infants. **(M)** CD8⁺ T cell numbers after anti-CD8α Ab administration and viral rebound after CD8⁺ cell depletion *in vivo*, in which plasma viral load or viral blips were not detected in the 4 infants up to 3 months thus far.

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