Cell-Associated HIV-1 DNA and RNA Decay Dynamics During Early Combination Antiretroviral Therapy in HIV-1-Infected Infants

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Background. The decay of human immunodeficiency virus type 1 (HIV-1)-infected cells during early combination antiretroviral therapy (cART) in infected infants is not defined.

Methods. HIV-1 DNA, including 2-long terminal repeat (2-LTR) circles, and multiply spliced (ms-) and unspliced (us-) HIV-1 RNA concentrations were measured at 0, 24, 48, and 96 weeks of cART in infants from the IMPAACT P1030 trial receiving lopinavir-ritonavir-based cART. The ratio of HIV-1 DNA concentrations to replication-competent genomes was also estimated. Linear mixed effects models with random intercept and linear splines were used to estimate patient-specific decay kinetics of HIV-1 DNA.

Results. The median HIV-1 DNA concentration before cART at a median age of 2 months was 3.2 log10 copies per million PBMC. With cART, the average estimated patient-specific change in HIV-1 DNA concentrations was −0.040 log10/week (95% confidence interval [CI], −0.05, −0.03) between 0 and 24 weeks and −0.017 log10/week between 24 and 48 weeks (95% CI, −0.024, −0.01). 2-LTR circles decreased with cART but remained detectable through 96 weeks. Pre-cART HIV-1 DNA concentration was correlated with time to undetectable plasma viral load and post-cART HIV-1 DNA at 96 weeks; although HIV-1 DNA concentrations exceeded replication-competent HIV-1 genomes by 148-fold. Almost all infants had ms- and usRNA detected pre-cART, with 75% having usRNA through 96 weeks of cART.

Conclusions. By 2 months of age, a large pool of HIV-1-infected cells is established in perinatal infection, which influences time to undetectable viral load and reservoir size. This has implications for informing novel approaches aimed at early restriction of HIV-1 reservoirs to enable virologic remission and cure.

Keywords. early combination antiretroviral therapy; HIV-1 DNA decay; HIV-1 RNA decay; perinatal HIV-1 infection; reservoir.
needed to support this notion [8]. Most HIV-1-infected infants, however, do not have the opportunity to start cART within hours of birth, as HIV-1 infection is typically identified between 1 and 3 months of age [9], which is considered early treatment and is life-saving [10].

The propensity to achieve low reservoir size in later childhood and adolescence with early long-term cART from infancy is established [11]. However, the concentration of HIV-1-infected cells before cART and its decay dynamics following early cART during the first 2 years of life is unknown. Previously, we showed that the resting CD4+ T-cell latent HIV-1 reservoir is established by two months of age and decayed with a half-life of 11 months between 24 and 96 weeks of cART [12] among a subset of infants enrolled in the International Maternal, Pediatric, and Adolescent AIDS Clinical Trials Network (IMPAACT) P1030 study [13, 14]. In the P1030 trial, the mean reservoir size was 0.32 infectious units per million resting CD4+ T cells (IUPM) at 96 weeks of cART [12]. However, the study of latent reservoirs was limited by a lack of analyses of the total HIV-1-infected cell concentration using proviral DNA measurements in circulating blood before and during cART. Here, we estimate the effects of early cART on the kinetics of HIV-1 DNA and cellular RNA concentrations over time using a nested, longitudinal study of infants in the IMPAACT P1030 clinical trial [13, 14].

MATERIALS AND METHODS

Study Participants

This substudy consisted of 19 of the 31 perinatally infected infants between the ages of 14 days and 6 months who were enrolled in the IMPAACT P1030 clinical trial (Figure 1), a multicenter, Phase I/II, open-label trial to establish the pharmacokinetic properties of lopinavir/ritonavir (LPV/r) for treatment of HIV-1-infected infants [14]. As part of the original study design, infants were stratified by age at treatment (< or ≥ 26 weeks of age). Peripheral blood was collected before (“week 0”) and 24, 48, 96 weeks post-cART, from which PBMCs were isolated and stored. Infants were included in the present substudy if they had an effective virologic response to cART defined as a ≥2-log10 decrease in pVL by 24 weeks of cART, an undetectable pVL of <400 copies/mL by 48 weeks of cART, and virologic suppression maintained through 96 weeks of study. Intermittent “blips” in pVL were permitted and defined as transient viremia after achieving virologic suppression to<400 copies/mL. The dynamics and size of the replication-competent, resting CD4+ T cell latent reservoir (infectious units per million resting CD4+ T cells (IUPM)) was previously reported in 14 of the 19 infants [12].

One infant was excluded from the present study after testing negative for HIV-1 DNA in peripheral blood mononuclear cells (PBMCs) due to primer mismatch with subtype A/G recombinant HIV-1 (Figure 1). Not all infants had samples available for both HIV-1 DNA and cellular RNA at all-time points (Figure 1).

Quantification of HIV-1 DNA and 2-LTR Circles

Genomic DNA was isolated from PBMCs using Qiagen Blood Midi kit (Qiagen, Valencia, California). HIV-1 DNA levels were quantified using modified methods for droplet digital polymerase chain reaction (ddPCR) published by Strain et al [15]. The median limit of detection (LOD) for both HIV-1 DNA and 2-long terminal repeat (2-LTR) was 0.41 log10 cpm. For samples below the LOD, values were adjusted to their respective LOD.

Quantification of Multiply Spliced- and Unspliced- HIV-1 RNA

Total RNA was isolated from cryopreserved PBMCs using Trizol (Life Technologies, Grand Island, New York) and DNase-treated (Life Technologies, Grand Island, New York). cDNA synthesis was performed from 500 ng of cellular RNA using iScript Advanced cDNA Synthesis Kit (Bio Rad, Hercules, California). Cellular HIV-1 RNA species were then quantified using a published primer set for HIV-1 msRNA targeting Tat/Rev and HIV-1 usRNA targeting Gag [16, 17]. For each sample, residual DNA was assessed in parallel with a no reverse transcriptase (RT) control reaction. Copy numbers of msRNA and usRNA species were normalized to total RNA concentration and reported as copies per 1 µg of total input cellular-derived RNA. The lower limit of detection for msRNA and usRNA was −0.74 log10 and −0.46 log10 copies per µg total input RNA from PBMC respectively. For samples below LOD, values were adjusted to their respective LOD.

Statistical Analyses

HIV-1 DNA half-life was calculated using a pharmacokinetic analysis for the entire cohort and also after excluding 4 patients who only had available blood samples for measurements at 2 time points. HIV-1 DNA was modeled over the follow-up period using linear mixed effects (LME) model with a random intercept for each infant. Nonlinear HIV-1 DNA trajectories were represented with 3 linear splines with knots at 24 and 48 weeks. The model also included concurrent CD4+ T-cell percent and age. Random slopes for each infant were tested to account for heterogeneity in HIV-1 DNA trajectories but did not improve model fit and, therefore, were not included in the final model. LME was also used to calculate differences in cellular usRNA concentrations during cART. Undetectable concentrations of ms RNA by 48 weeks post-cART precluded similar analyses of this marker.

Correlations between HIV-1 DNA, msRNA, usRNA, IUPM, and VL were calculated at each study visit using either Pearson (r) (for log-transformed variables) or Spearman correlation (ρ) coefficients. Time to undetectable viral load following cART initiation was modeled using a discrete-time proportional odds model.

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RESULTS

Patient Characteristics
Among the 18 infants who achieved virologic suppression, the median age at cART was 2.0 months (Table 1). Five infants were <6 weeks of age (median: 5.7 weeks), and 13 infants were ≥6 weeks of age (median: 11.1 weeks) at cART initiation. At the start of cART, the median pVL was 5.8 log10 copies/mL, and the median CD4+ T-cell percentage was 35% (Table 1). The median time to undetectable pVL to <400 copies/mL was 12 weeks (range: 4 to 48 weeks). Ten of 18 (56%) participants experienced intermittent episodes of viremia above 400 copies/mL (range: 410 to 93,800) through 96 weeks of cART.

The 6 infants who did not achieve effective virologic control did not differ from suppressors in patient demographics age at cART (P-value: .71), pre-cART HIV DNA load (P-value: .94), pVL (P-value: .28) or CD4+ T-cell percentages (P-value: .78).

Cellular HIV-1 DNA
The median HIV-1 DNA concentration prior to cART was 3.2 log10 copies per million (cpm) PBMCs and was lower at all subsequent time points with median levels of 2.0 log10 (interquartile range [IQR]: 1.7, 3.0), 2.1 log10 and 2.1 log10 cpm PBMCs at 24, 48, and 96 weeks of cART, respectively (Table 2). One infant reached an undetectable HIV-1 DNA by 48 weeks of cART but was subsequently lost to follow-up, precluding additional

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Figure 1. Study population: IMPAACT P1030. Abbreviations: 2-LTR, 2-long terminal repeat; cART, combination antiretroviral therapy; EVR, effective virologic response; HIV-1, human immunodeficiency virus type 1; IMPAACT, International Maternal Pediatric Adolescent AIDS Clinical Trials Group; ms, multiply spliced; us, unspliced.
longitudinal analyses. The median half-life of HIV-1 DNA among the 18 infants was estimated at 26.8 weeks. After excluding 4 patients with only 2 measurements, the median half-life was 32.6 weeks.

The results of the linear mixed effects model indicated a statistically significant change in patient-specific slopes at 24 weeks (P-value < .0001) and at 48 weeks (P-value = .003) of cART. HIV-1 DNA levels decayed rapidly at an estimated rate of $-0.040 \log_{10} \text{cpm PBMCs per week}$ (95% CI, $-0.05, -0.03$; P-value < 0.0001) from 0 to 24 weeks of cART, followed by a slowed estimated decay rate of $-0.017 \log_{10} \text{cpm PBMCs per week}$ (95% CI, $-0.024, -0.01$; P-value < 0.001) from 24 to 48 weeks and no further statistically significant decay at an estimated rate of $-0.0021 \log_{10} \text{cpm PBMCs per week}$ (95% CI, $-0.006, .002$; P-value = .295) from 48 to 96 weeks of cART (Figure 2A). The estimated decay rates did not substantially change after adjusting for age at cART and concurrent CD4$^+$ T-cell percentages. Among the nonsuppressors, HIV-1 DNA levels decayed at an estimated rate of $-0.029 \log_{10} \text{cpm PBMCs per week}$ (95% CI, $-0.045, -0.014$; P-value < 0.0001) from 0 to 24 weeks of cART, followed by a slowed estimated decay rate of $-0.008 \log_{10} \text{cpm PBMCs per week}$ (95% CI, $-0.024, .007$; P-value = .296) from 24 to 48 weeks. The small number of nonsuppressors limited the power of these analyses and additional data on nonsuppressors is required to test for differences in slopes.

We examined differences in HIV-1 DNA concentration as a function of age at cART following the IMPAACT P1030 trial’s original design of stratification at 6 weeks of age. The median HIV-1 DNA concentration before cART initiation in infants treated at <6 weeks of age (n = 5) was not statistically significantly different from those treated at $\geq$6 weeks of age (n = 13), P-value = .961, with medians of 3.1 (IQR: 2.9, 3.2) and 3.6 cpm PBMCs (IQR: 2.6, 3.9), respectively. HIV-1 DNA was lower but not statistically significantly different in the <6 week age group compared to $\geq$6 weeks age group at weeks 24, 48, and 96 weeks of cART (data not shown).

Fifteen of 18 (83%) infants had 2-LTR circles detected before cART initiation with a median concentration of 2.0 log$_{10}$ cpm (Table 2). 2-LTR circles decayed in parallel with total HIV-1 DNA with increased duration of cART (Figure 2B) and remained detectable but at substantially lower concentrations.
in 11/16 (69%), 10/14 (71%), and 11/12 (92%) infants at 24, 48, and 96 weeks of cART (Table 2).

We examined the ratio of HIV-1 DNA concentration to replication-competent HIV-1, as indicated by IUPM previously reported for these infants [12]. HIV-1 DNA concentrations in total PBMC were correlated with IUPM in resting CD4+ T cell at 24 weeks but not at 48 or 96 weeks (Table 3). However, HIV-1 DNA concentrations exceeded replication-competent viral genomes by 66.0-, 170-, and 148-fold at 24, 48, and 96 weeks post-cART, respectively.

Cellular HIV-1 RNA
Before cART initiation, msRNA, a general marker of productively infected cells, was detected in 100% of 15 infants tested and at a median concentration of 2.7 log_{10} copies/µg total RNA (Table 4). Within 24 weeks of cART, msRNA expression decreased to −0.07 log_{10} copies/µg total RNA; remained detectable in 5/10 (50%) infants (Figure 3A) and was less frequently detected by 48 weeks in 0/6 infants and 96 weeks in 1/8 (12.5%) infants (Table 4).

Before cART initiation, usRNA was detected in 14/15 (93%) infants at a median concentration was 3.4 log_{10} copies/µg total RNA (Table 4). Within 24 weeks of cART, median concentrations of usRNA decreased significantly to 1.2 log_{10} copies/µg total RNA but remained detectable in 5/6 (83%) and 6/8 (75%) infants, respectively (Table 4). The median ratio of HIV-1 usRNA/total DNA before cART initiation was 18.2, which declined to 2.3, 0.67, and 2.5 by 24, 48, and 96 weeks of cART respectively (Table 5).

Correlations Between Virologic and Immune Markers of HIV-1 Infection Following cART
HIV-1 DNA concentrations prior to cART was positively correlated with time to first virologic suppression: a 1 log_{10} increase in HIV-1 DNA before cART was associated with a 76% decreased odds (95% CI, 32%, 91%; P-value = .007) of reaching an undetectable VL at any week assuming a constant effect of HIV-1 DNA over the entire follow-up time. Higher baseline pVL was also associated with lower probability of achieving undetectable pVL: a 1 log_{10} increase in pVL was associated with a 78% decreased odds (95% CI, 13%, 94%; P-value = .031) of reaching an undetectable pVL at any week. However, no association was found between age at cART and baseline CD4+ T-cell percentage with time to virologic suppression. Pre-cART

Table 3. Summary of Relationship Between Human Immunodeficiency Virus Type 1 (HIV-1) DNA Concentrations in Peripheral Blood Mononuclear Cells and Replication Competent HIV-1 in Resting CD4+ T Cells Among Infants With Detectable Levels by Both Measurements

<table>
<thead>
<tr>
<th>Time on Combination Antiretroviral Therapy (cART)</th>
<th>24 Wks</th>
<th>48 Wks</th>
<th>96 Wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>11</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Spearman’s ((\rho)) of HIV-1 DNA and Replication competent DNA</td>
<td>.66</td>
<td>.39</td>
<td>.44</td>
</tr>
<tr>
<td>P-value ((P))</td>
<td>.028</td>
<td>.34</td>
<td>.38</td>
</tr>
<tr>
<td>Min-Max</td>
<td>7.1, 642</td>
<td>8.7, 752</td>
<td>53.6, 933</td>
</tr>
</tbody>
</table>

Abbreviations: HIV-1, human immunodeficiency virus type 1; IQR, interquartile range.
HIV-1 DNA concentration was highly correlated with post-cART HIV-1 DNA concentration at all study visits (Figure 4) and was not significantly correlated with age \( r = 0.30; P\text{-value} = .23 \), plasma viral load \( r = 0.43; P\text{-value} = .08 \), or CD4\(^+\) T-cell percentage \( r = -0.03; P\text{-value} = .91 \) at cART initiation.

Prior to cART initiation, msRNA was significantly correlated with HIV-1 DNA concentration \( \rho = 0.73; P\text{-value} = .002 \) but not pVL \( \rho = 0.27; P\text{-value} = .334 \), whereas usRNA was correlated with both pVL \( \rho = 0.59; P\text{-value} = .02 \) and HIV-1 DNA concentration \( \rho = 0.68; P\text{-value} = .005 \). Analyses of correlations between HIV-1 RNA species and HIV-1 DNA concentrations were limited due to undetectable levels of msRNA in 50% of study participants by week 24 and limited sample availability for ms- and usRNA analysis at later time points.

**DISCUSSION**

To our knowledge, this is the first study to assess the effect of early cART on HIV-1 DNA and cellular RNA dynamics, along with estimates of the ratio of total HIV-1 DNA to replication-competent genomes persisting during the first 2 years of life in infected infants on effective cART. We identified early generation of high concentrations of HIV-1 infected cells in peripheral blood mononuclear cells within 2 months of birth. Pre-cART concentrations of HIV-1-infected cells were highly correlated with the concentrations of HIV-1-infected cells persisting under cART through two years of age and time to virologic suppression. Time to virologic suppression was also associated with pre-treatment plasma viral load. HIV-1 DNA concentrations decreased in a bi-phasic manner over the first 48 weeks of cART, before becoming stable between 48 and 96 weeks of cART. In the nonsuppressors, HIV-1 decayed significantly in the first 24 weeks but did not decay significantly between 24 and 48 weeks of nonsuppressive cART. Total HIV-1 DNA

<table>
<thead>
<tr>
<th>Time on Combination Antiretroviral Therapy (cART)</th>
<th>0 Wks</th>
<th>24 Wks</th>
<th>48 Wks</th>
<th>96 Wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \log_{10} (\text{msRNA c/µg total RNA}) )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>15</td>
<td>10</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>2.7 [2.3, 3.3]</td>
<td>-0.07 [-0.74, 0.75]</td>
<td>-0.74 [-0.74, -0.74]</td>
<td>-0.74 [-0.74, -0.74]</td>
</tr>
<tr>
<td>Min-Max</td>
<td>0.90, 4.0</td>
<td>-0.74, 1.2</td>
<td>-0.74, -0.74</td>
<td>-0.74, 0.38</td>
</tr>
<tr>
<td>Detectable [%]</td>
<td>15/15 [100%]</td>
<td>5/10 [50%]</td>
<td>0/6 [0%]</td>
<td>1/8 [12.5%]</td>
</tr>
<tr>
<td>( \log_{10} (\text{usRNA c/µg total RNA}) )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>15</td>
<td>10</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>3.4 [3.1, 4.2]</td>
<td>1.2 [0.9, 2.2]</td>
<td>0.83 [0.41, 1.6]</td>
<td>0.94 [0.2, 1.5]</td>
</tr>
<tr>
<td>Min-Max</td>
<td>-0.46, 4.5</td>
<td>-0.46, 2.5</td>
<td>-0.46, 1.7</td>
<td>-0.46, 2.2</td>
</tr>
<tr>
<td>Detectable [%]</td>
<td>14/15 [93%]</td>
<td>8/10 [80%]</td>
<td>5/6 [83%]</td>
<td>6/8 [75%]</td>
</tr>
</tbody>
</table>

Abbreviations: c/µg, copies per microgram; IQR, interquartile range; ms, multiply spliced; us, unspliced.

**Figure 3.** Longitudinal decay of cellular HIV-1 RNA species following cART. A, Decay of msRNA (circles). B, Decay of usRNA (squares). ***\( P \leq .0001 \); *\( P \leq .05 \) statistical significance. Dotted lines indicate limit of detection for the RNA assay. Open symbols indicate RNA measurements below limit of detection. Abbreviations: c/µg, copies per microgram; cART, combination antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; ms, multiply spliced; us, unspliced.
concentrations in PBMC was in large excess to replication-competent HIV-1 at an estimated 148-fold at 96 weeks of cART, emphasizing early and large contributions of defective genomes in the pathogenesis of HIV-1 infection [18, 19]. A similar correlation between pre- and post-cART HIV-1-infected cell concentrations was also reported in children initiating cART during chronic infection [20] and in infected adults [21]. The association between pre-cART HIV-1 DNA concentrations and time to virologic suppression is consistent with ongoing viremia from long-lived cells generated pre-cART and supported by data in adults on its correlation with residual viremia despite years of effective treatment [22]. Together these findings support the early establishment of long-lived cells in perinatal infection.

The increased time to undetectable plasma viral load and the occurrence of “blips” during cART may represent ongoing low-level replication or virus production from long-lived cells [23]. 56% of the infants experienced “blips” during the 96 weeks of cART. Analyses of 2-LTR circles, which are frequently cited as a measure of ongoing virus replication [23], failed to distinguish between those with and without blips (data not shown). Indeed, 2-LTR circles decayed in parallel with HIV-1 DNA, and persisted in 11/12 (92%) infants through 96 weeks of cART, suggesting stability of 2-LTR circles in effectively treated perinatal infection. Long-term detection of 2-LTR circles was observed in HIV-1-infected adults with up to 10 years of cART [21], supporting that this byproduct of abortive integration event may also be long-lived. However, in support for an overall lack of ongoing virus replication is the finding that the decay of HIV-1 DNA was consistent across all of the infants despite heterogeneity in time to virologic suppression.

Before cART, levels of HIV-1 transcripts were high and correlated with HIV-1 DNA and/or plasma viral load confirming the transcriptionally active state of the infected cell pool. The detection of msRNA transcripts, Tat and Rev, generally indicates presence of productively infected cells [24–26], whereas usRNA transcripts represents transcriptionally active genomes that may not necessarily lead to productive infection. The inability to detect msRNA by 48 weeks is consistent with clearance of productively infected cells with cART [26]. The clinical relevance of persistent detection of usRNA transcripts through 96 weeks of cART is unclear. Studies in perinatally infected children treated in later childhood reported persistence of unspliced transcripts through 48 months of cART [27], and even with up to 7–8 years of early cART [28]. However, the extent to which these unspliced transcripts represent readthrough transcripts or LTR-derived HIV-1 transcripts, is unclear [29]. Whether these transcripts are defective or capable of virus production will require further investigation as this will be important for immune-based strategies aimed at reducing HIV-1 reservoirs [30].

Our study was limited by small sample size and the inability to measure HIV-1 DNA and RNA transcripts among all infants at all study visits, in addition to knowledge of the timing of infection, which may have influenced their decay dynamics. Blood volume limitations also precluded quantitative analyses of HIV-1 infection within the various CD4+ T-cell subsets. However, we observed

### Table 5. Summary Ratio of Human Immunodeficiency Virus Type 1 Unspliced RNA and Total DNA at pre-Combination Antiretroviral Therapy (cART) and 24, 48, and 96 Weeks after cART Among Infants With Detectable Levels

<table>
<thead>
<tr>
<th>Time on Combination Antiretroviral Therapy (cART)</th>
<th>HIV-1 usRNA/total DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Wks 24 Wks 48 Wks 96 Wks</td>
<td>N 14 8 5 6</td>
</tr>
<tr>
<td>Median [IQR]</td>
<td>18.2 [6.3, 37] 2.3 [0.56, 3.3] 0.67 [0.26, 0.84] 2.5 [0.77, 3.4]</td>
</tr>
<tr>
<td>Min-Max</td>
<td>0.05, 70 0.06, 15 0.03, 1.3 0.59, 8.1</td>
</tr>
</tbody>
</table>

Abbreviations: HIV-1, human immunodeficiency virus type 1; IQR, interquartile range; us, unspliced.

Figure 4. Correlations between pre- and post-cART HIV-1 DNA concentrations at 24, 48, and 96 weeks. Abbreviations: cART, combination antiretroviral therapy; cpm, copies per million; HIV-1, human immunodeficiency virus type 1; PBMC, peripheral blood mononuclear cells.
strikingly similar patterns of decay over time between individuals. The ratio of HIV-1 DNA in PBMCs to replication-competent HIV-1 from resting CD4+ T cells is likely an underestimate of the true relationship. As the major contributing cell type to the circulating latent HIV-1 reservoir is resting CD4+ T cells [31], it is expected that the concentration of total DNA would be higher among samples containing purified resting CD4+ T cells.

Large stable pools of HIV-1-infected cells were established within 2 months of age in perinatally infected infants that influenced the concentrations of infected cells persisting through 96 weeks of cART and time to virologic suppression, although a substantial proportion were replication defective but maintained the capacity to produce unspliced HIV-1 transcripts. These findings support early establishment of long-lived HIV-1-infected cells in perinatal infection that creates an early barrier to HIV-1 eradication. Dramatically limiting the size of replication-competent HIV-1 reservoirs will therefore require substantially earlier timing of cART as was observed in the case of the Mississippi child [6].

Notes

Disclaimer. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health (NIH).

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Potential conflicts of interest. All authors: No potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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