Reduced markers of HIV persistence and restricted HIV-specific immune responses after early antiretroviral therapy in children

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Objective: Understanding the extent to which early antiretroviral therapy (ART) can limit the establishment and persistence of the HIV reservoir is an important step to designing interventions aimed at achieving HIV cure. We measured the markers of HIV persistence and HIV-specific immunity in early treated children.

Design: This is a cross-sectional study that enrolled 15 children older than 2 years of age who initiated ART before 6 months of age and had sustained viral suppression. Total and integrated HIV DNA, and 2-LTR circles in $CD4^+$ T cells, HIV antibody response by fourth generation HIV enzyme immunoassay, and $CD4^+$ and $CD8^+$ T-cell responses to gag/env peptides by intracellular cytokine staining of $CD4^+$ and $CD8^+$ T cells were measured.

Results: The median current age was 6.3 years and age at ART initiation was 17 weeks. The median duration of viral suppression was 6 years, and all had HIV RNA less than 50 copies/ml. The median $CD4^+$ T cells was 44%. The median total HIV DNA was 132 copies/10^6 $CD4^+$ T cells (range 11–1804) and integrated HIV DNA was 17 copies/10^6 $CD4^+$ T cells (range 0–516), and no one had detectable 2-LTR circles. Nine of the 15 children (60%) had undetectable or extremely low integrated HIV DNA (<20 copies/10^6 $CD4^+$ T cells). All except one (93%) had undetectable HIV-specific $CD4^+$/$CD8^+$ cell responses and seven (47%) had nonreactive enzyme immunoassay.

Conclusion: Early ART resulted in very low levels of markers of HIV persistence and undetectable HIV-specific immune responses in the majority of HIV-infected children who started ART before 6 months of age.

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Introduction

Worldwide, HIV currently afflicts 34 million people, including 3.3 million children [1]. With increased availability of antiretroviral therapy (ART), child mortality rates have decreased, but children continue to cope with HIV as a chronic, highly stigmatized illness [2]. Evaluating factors that may enhance the chance for cure will be of paramount benefit.

Seeding of the HIV reservoir occurs early during acute HIV infection and sets the stage for establishment of stable reservoirs in long-lived cells [3,4]. Intervening with ART early in the course of infection can limit the size of the HIV reservoir in adults and children [5–7]. The frequency of latently infected cells in early treated children also declined at a more rapid rate than in chronic HIV-infected adults on suppressive ART [6]. Some early treated infants do not display HIV-specific antibody or cellular responses, indicative of restricted HIV reservoir size [8–10]. The low reservoir burden driven by early ART may be critical to achieve natural control of viral replication upon ART withdrawal, a working definition of HIV functional cure. Indeed, such control observed in the 'Mississippi baby' was likely because of the early ART initiated within days of birth and presumably onset of infection [11].

Perinatally HIV-infected children are unique because the onset of HIV infection is known to occur mostly at the time of delivery, and immediate ART in infancy is the standard of care [12]. However, knowledge on the effect of early treatment on the latent reservoir in older HIV-infected children who have had continuous viral suppression is limited. Understanding the extent for which early ART can affect the degree of HIV persistence is an important step to designing interventions aimed at achieving HIV cure in this population.

Methods

This cross-sectional study was conducted at four sites in Bangkok, Thailand: the HIV Netherlands Australia Thailand Research Collaboration, King Chulalongkorn Memorial Hospital, Siriraj Hospital and Queen Sirikit National Institute of Child Health. Children were eligible if they were older than 2 years of age, had positive HIV DNA PCR, initiated ART during the first 6 months of life, and had sustained HIV RNA less than 400 copies/ml. Children with ART failure at any time or who had periods in which ART was interrupted for more than 1 month were excluded. Parents gave their consent, and the study was approved by all ethics committees.

Medical history and information relevant to HIV infection were obtained from medical records. HIV enzyme immunoassay (EIA) was performed using the fourth generation EIA that detects HIV antigen and HIV immunoglobulin M (Architect; Abbott Laboratories, Wiesbaden, Germany) [13]. HIV-specific $CD4^+$ and $CD8^+$ cell responses were measured by intracellular cytokine staining after stimulating with HIV-specific gag and env pooled peptides [14]. Two criteria were used to determine a positive response: more than twice the background response [tissue culture medium alone with 0.45% dimethyl sulfoxide (DMSO)]; and the response, without subtracting the background, is more than or equal to a predetermined cut-off of 0.3 and 0.65% for $CD4^+$ and $CD8^+$ T cells, respectively.

We assessed HIV persistence by measuring the frequency of cells harboring total and integrated HIV DNA and 2-LTR circles in $CD4^+$ T cells (purity >95%) isolated by negative magnetic selection (Stemcell, Vancouver, British Columbia, Canada) and using a validated assay optimized for all HIV clades, including the common CRF01_AE clade in Thailand (limit of detection 1 copy/reaction tube for HIV DNA and 2-LTR circles) [5,15].

The frequencies of memory $CD4^+$ T-cell subsets and activated $CD4^+$ and $CD8^+$ T cells were determined by flow cytometry. We used the following 10 colors antibody panel: CD3-A700, CD4-Qdot605, CD8-Pacific Blue, CD45RA-APCH7, CD27-BV650, HLA-DR-APC, CCR7-PE-Cy7, HLA-DR-PerCP, CD38-PE, and PD-1-Alexa647. All antibodies were from BD Biosciences (La Jolla, California, USA) except CD4-QDot605 (Invitrogen, Carlsbad, California, USA) and CD27-BV650 (Biolegend, San Diego, California, USA). Dead cells were excluded with the LIVE/DEAD Aqua marker (Invitrogen).

Frequencies of naive ($CD45RA^+CCR7^+CD27^+$), central memory ($CD45RA^-CCR7^+CD27^+$), transitional memory ($CD45RA^-CCR7^-CD27^+$), and effector memory ($CD45RA^-CCR7^-CD27^-$) $CD4^+$ T cells were determined by flow cytometry by gating on total $CD4^+$ T cells ($CD3^+CD4^+$). Frequencies of activated ($CD38^+$ $HLA\text{-}DR^+$) cells were measured by gating on total $CD4^+$ and total $CD8^+$ T cells. Cells were acquired on a LSRII flow cytometer using the FACSDiva software (Becton Dickinson, Rutherford, New Jersey, USA) and analyzed using FlowJo (Tree Star, Ashland, Oregon, USA).

Demographic and HIV-disease and treatment-related characteristics were described as median (range) or frequencies. Categorical descriptions of detectable vs. undetectable were made for HIV DNA and HIV-specific

immune responses. Formal comparisons were made using a Mann–Whitney *U*-test.

Results

From June to July 2013, 15 children were enrolled. The median (range) age at HIV diagnosis was 12 weeks (birth to 25 weeks) old, and three had positive HIV DNA PCR at birth. Six mothers and three neonates had no antiretroviral prophylaxis for mother-to-child transmission, and the information was unknown in three mothers and two neonates. The remaining six mothers received either zidovudine alone (four), single-dose nevirapine alone (one), or zidovudine with single-dose nevirapine (one). Three neonates received triple ART with zidovudine, lamivudine, and nevirapine, and the remaining had either zidovudine alone (three), zidovudine with single-dose nevirapine (three), or zidovudine, lamivudine along with single dose of nevirapine (one). These 15 children initiated ART at about 4 months of age and had been virally suppressed on ART for around 6 years (Table 1). All had HIV RNA less than 50 copies/ml. Every child was treated with optimal triple ART regimens, and no one switched ART because of treatment failure. First ART regimen contained lopinavir/ritonavir in six, and nevirapine in nine children, and at enrollment, four were on lopinavir/ritonavir, and five and six were on efavirenz and nevirapine, respectively.

The median total and integrated HIV DNA levels in $CD4^+$ T cells were low (132 and 17 copies per 10^6 $CD4^+$ T cells, respectively) and none had detectable 2-LTR circles. Nine (60%) had integrated HIV DNA below 20 copies/10^6 $CD4^+$ T cells, and in two, it was undetectable (Fig. 1). Seven (47%) had nonreactive fourth generation EIA, and all except one (93%) had undetectable HIV-specific $CD4^+$ and $CD8^+$ T-cell responses (Table 1). This child had the largest frequency of cells with integrated HIV DNA (516 copies/10^6 $CD4^+$ T cells). Comparing nine children with integrated HIV DNA less than 20 copies to six children with at least 20 copies/10^6 $CD4^+$ T cells, their median (range) age at HIV diagnosis [12 (0–25) vs. 12 (0–23) weeks, $P = 0.56$] and age at ART initiation [17 (0–25) vs. 17 (0–25) weeks, $P = 0.64$] were not different.

Table 1. Characteristics of early treated children.

Parameters	Value ($n = 15$)
Current age (years)	6.3 (4–12)
Age at ARV start (weeks)	17 (0–25)
Duration of viral suppression (years)	6 (3.5–11.9)
Total HIV DNA (copies/10^6 $CD4^+$ T cells)	132 (11–1804)
Integrated HIV DNA (copies/10^6 $CD4^+$ T cells)	17 (0–516)
2-LTR circles (copies/10^6 $CD4^+$ T cells)	0 (0–0)
Nonreactive fourth generation EIA	7 (47)
Undetected HIV-specific $CD4^+$ and $CD8^+$ T-cell responses	14 (93)
% Total $CD4^+$ T cells	44 (23–54)
% Naive $CD4^+$ T cells	60 (45–77)
% Central memory $CD4^+$ T cells	18 (10–24)
% Transitional memory $CD4^+$ T cells	8 (4–16)
% Effector memory $CD4^+$ T cells	4 (1–18)
% Activated $CD4^+$ T cells	2 (1–9)
% Total $CD8^+$ T cells	41 (34–57)
% Activated $CD8^+$ T cells	9 (3–27)

Values are shown as median (range) or number (%). Markers used were $CD3^+CD4^+$ (total $CD4^+$ T cells), $CD45RA^+CCR7^+CD27^+$ (naive $CD4^+$ T cells), $CD45RA^-CCR7^+CD27^+$ (central memory $CD4^+$ T cells), $CD45RA^-CCR7^-CD27^+$ (transitional memory $CD4^+$ T cells), $CD45RA^-CCR7^-CD27^-$ (effector memory $CD4^+$ T cells), $CD4^+CD38^+HLADR^+$ (activated $CD4^+$ T cells), $CD3^+CD8^+$ + (total $CD8^+$ T cells), and $CD8^+CD38^+HLADR^+$ (activated $CD8^+$ T cells). ARV, antiretroviral; EIA, enzyme immunoassay.

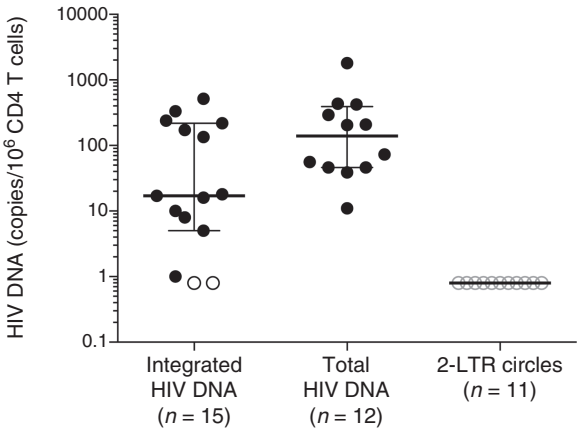

Fig. 1. Markers of HIV persistence in early treated children. The opened circles represent undetectable values (limit of detection 1 copy per PCR reaction tube).

Three children initiated triple ART with zidovudine, lamivudine, and nevirapine at birth for prophylaxis, and after HIV was diagnosed, the regimen was continued without interruption. They were boys, aged 5.8, 5.4, and 6.8 years. Their integrated HIV DNA levels were 0, 18, and 134 copies/10^6 $CD4^+$ T cells, respectively. The total HIV DNA levels were 11 and 46 copies/10^6 $CD4^+$ T cells in the first two children, and because of limited number of cells, it was not measured in the last child. All three had undetectable HIV-specific $CD4^+$ and $CD8^+$ T-cell responses. The fourth generation EIA was nonreactive in two (the first and last child) despite all three having positive HIV DNA PCR at birth.

Immunophenotyping showed that these children had median $CD4^+$ (44%) similar to referenced value of 35% in age-matched healthy Thai children (unpublished data). The median frequencies of activated $CD4^+$ (2%) and $CD8^+$ (9%) cells were lower than the referenced values in age-matched uninfected controls (3.9%, $P = 0.001$ and 14.2%, $P = 0.007$, respectively). Compared to acutely infected Thai adults treated successfully for 2 years [16], the frequency of naive $CD4^+$ T cells was higher in these children (29 vs. 60%, $P = 0.0001$), whereas the frequency

of memory $CD4^+$ T-cell subsets that have been shown to encompass the latent reservoir [15] was lower (36 vs. 18%, 16 vs. 8%, and 8 vs. 4% for central, transitional, and effector memory $CD4^+$ cells, respectively, $P<0.001$ for all).

Discussion

Although immediate ART initiation in infants diagnosed with HIV infection is the standard of care, there is little information regarding its effects on the size of the HIV reservoir and immune responses. Our data illustrate that early ART results in restricted HIV-specific immune responses. Sixty percentage of the children enrolled in this study had remarkably low frequency of cells harboring integrated HIV DNA and none had detectable 2-LTR circles, a marker of residual viral replication during ART [17]. There were high frequencies of $CD4^+$ T cells and low frequencies of cells expressing activation markers.

Persistence of HIV occurs because of a reversible nonproductive state of infection of individual cells, mostly $CD4^+$ T cells, and in particular, central memory $CD4^+$ cells [10,15,18]. In patients who initiate ART during chronic HIV infection, proviral DNA is almost always detected despite long-term viral suppression [15]. In contrast, ART during acute HIV infection in adults leads to a lower reservoir size [7], including undetectable integrated HIV DNA if treatment is commenced within the first month of infection [5]. Two of our children had undetectable integrated HIV DNA and 60% had very low levels. The lack of HIV-specific cellular responses in almost all children, and the absence of HIV antigen and HIV immunoglobulin M in 50% further supports the restricted reservoir size, although it could also be a result of therapy initiation prior to the ontogeny of these responses [19–21]. It is possible that if treatment was initiated sooner than the average age of 4 months, a lower frequency of cells harboring HIV DNA could be achieved. Two of three children who started triple ART at birth did have undetectable/low integrated HIV DNA despite both having in-utero infection. Studies have shown that the frequency of infected cells with replication-competent virus is significantly lower in infants treated before 6 weeks of age, and in those who achieve viral suppression by 24 weeks of life [6]. The half-life of latently infected cells is also shorter when ART is initiated early, and depletion of the latent reservoir continues with longer ART duration. Indeed, reports of treated children and adolescents with sustained viral suppression since infancy illustrate no detectable HIV DNA, replication competent virus, or HIV-specific humoral and cellular responses [8–10]. It is conceivable that little or no HIV is left in these older but early treated children; however, the test for HIV cure would require interrupting ART, the ethics of which requires careful consideration [22].

Massive immune destruction occurs alongside reservoir establishment early in infection and results in irreversible chronic HIV-associated immune deficiency and inflammation [23,24]. Our study corroborates findings in adults that early treatment can limit immune destruction and activation, as evidenced by the high frequencies of $CD4^+$ cells and low levels of cellular markers associated with immune activation [5,23]. In contrast, HIV-infected children who are ART-naive or virologically suppressed but started ART late have higher immune activation compared with their uninfected peers [25,26]. Undetectable 2-LTR circles in all children further support the lack of ongoing viral infection from the stable $CD4^+$ T-cell reservoir, although low-level viral replication was not directly measured in our patients. The high frequencies of naive $CD4^+$ cells and low frequencies of memory $CD4^+$ cells in these children may contribute to a lower frequency of reservoir cells as naive cells are rarely infected during ART while memory cells are the main reservoir site for HIV [15].

In addition to the small sample size, our study is limited by the lack of information on the frequency of cells that harbor replication competent virus. The quantitative viral outgrowth assay (Q-VOA), the gold standard for assessing reservoir size [27], was originally developed to measure this frequency, as it is known that most HIV DNA is defective and replication incompetent [28]. However, the Q-VOA requires a large blood volume, which is difficult to obtain in children [6,27]. The use of Q-VOA is further complicated by recent findings suggesting that this method may have underestimated the reservoir size by up to 60-fold. In that study, 11.7% of the previously defined 'replication incompetent' proviruses were found to be intact viral genomes capable of replicating [28]. Integrated HIV DNA is easier to measure, requires less blood, and is statistically the best correlate of the Q-VOA, although the predicted values can vary over a 10-fold range [27]. The intracellular cytokine staining method used in our study may not detect HIV-specific cellular responses in cases with low frequencies of responsive cells or with high background responses [14].

Despite conclusive evidence that early ART prevents deaths in young infants [29] and guidelines recommending immediate ART for infants [12], the uptake of early infant diagnosis and treatment has been poor [30]. The Mississippi baby's case has shifted the paradigm to the possibility that functional cure can be achieved with early ART alone [11], and this could drive the implementation of early infant diagnosis and treatment. As a first step toward achieving HIV cure in children, future research should focus on strategies to shorten the time to which infants are diagnosed with HIV and start on ART, and to provide appropriate ART and adequate adherence

support so that children can achieve sustained viral suppression. The transition from triple ART prophylaxis to treatment without interruption in infants may be key to achieving cure in children, and in fact, Thailand is implementing this strategy for infants at high risk for HIV, providing an opportunity to understand factors that may have contributed to the functional cure in the Mississippi baby. Finally, future research should address the unmet knowledge gap of the effect of early ART on the reservoir, HIV-specific immune responses, immune activation, and persistent viremia that underpins HIV cure interventions.

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J.A., T.P., P.S., K.C., S.J.K., and N.C. conceived and designed the study, and drafted the article. J.I. and A.M. participated in the study design, coordination, and data interpretation. R.F., W.B., A.M., and S.S. participated in the laboratory testing and its interpretation. All authors read, provided input in the article, and approved the final article.

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Conflicts of interest

All authors declare no conflict of interest.

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