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Relationship between changes in thymic emigrants and cell-associated HIV-1 DNA in HIV-1-infected children initiating antiretroviral therapy

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Objectives and methods: To investigate the relationship between cell-associated HIV-1 dynamics and recent thymic T-cell emigrants, HIV-1 DNA and T-cell receptor rearrangement excision circles (TREC, a marker of recent thymic emigrants) were measured in peripheral blood mononuclear cells in 181 samples from 33 HIV-1-infected children followed for 96 weeks after antiretroviral therapy (ART) initiation.

Results: At baseline, HIV-1 DNA was higher in children with higher TREC (P=0.02) and was not related to age, CD4 or HIV-1 RNA in multivariate analyses (P>0.3). Overall, TREC increased and HIV-1 DNA decreased significantly after ART initiation, with faster HIV-1 DNA declines in children with higher baseline TREC (P=0.009). The greatest decreases in HIV-1 DNA occurred in children with the smallest increases in TREC levels during ART (P=0.002). However, this inverse relationship between changes in HIV-1 DNA and TREC tended to vary according to the phase of HIV-1 RNA decline (P=0.13); for the same increase in TREC, HIV-1 DNA decline was much smaller during persistent or transient viraemia compared with stable HIV-1 RNA suppression.

Conclusions: Overall, these findings indicate that TREC levels predict HIV-1 DNA response to ART and suggest that immune repopulation by thymic emigrants adversely affects HIV-1 DNA decline in the absence of persistent viral suppression, possibly by providing a cellular source for viral infection and replication.

Introduction

Treatment with antiretroviral therapy (ART) has resulted in reductions in mortality and progression of HIV-1 disease in both adults and children [1–3]. Although response to ART, in terms of viral suppression, is broadly similar in adults and children, several studies have outlined differences in the pattern of peripheral immune reconstitution, notably that repopulation in children occurs mainly with naive T cells with only a small rise in memory T cells [4–6].

The main source of naive T cells in children is the thymus. During intrathymic T-cell differentiation, progenitor cells undergo rearrangement of the T-cell receptor, resulting in the formation of episomal DNA by-products [termed T-cell receptor rearrangement excision circles (TREC)]. Thus detection of TREC in peripheral blood T cells could serve as a marker of recent thymic emigrants [7]. An increasing level of TREC-bearing cells along with an increasing volume of the thymus in patients initiating ART was reported [8,9]. Besides thymic output, TREC levels in the peripheral blood may be influenced by other factors including the longevity of naive TREC-positive cells and their sequestration in lymphoid tissues [10,11]. Furthermore, as episomal DNA circles do not replicate with mitosis, TREC are diluted by cellular division; increased cellular division due to HIV-1 immune stimulation may thus dilute the content of TREC-bearing cells [12]. However, detection of TREC in peripheral blood T cells does provide evidence of their thymic origin and TREC quantification allows an estimate of the circulating reserve of naive T lymphocytes.

We have shown that previously untreated children initiating ART in a randomized trial [the Paediatric European Network for Treatment of AIDS (PENTA) 5] [13] with combinations of zidovudine, lamivudine, abacavir and nelfinavir had significant increases in TREC levels [14]. These increases were greatest in children with lowest baseline CD4% and in those with

greatest increases in CD4% following ART. There was also a trend towards greater TREC increases in children with the lowest baseline TREC levels. Moreover, TREC increases were strongly associated with increases in naive CD4 CD45RA cells, but not memory CD4 CD45RO cells. Furthermore, we also showed that children with HIV-1 RNA <50 copies/ml of plasma had smaller increases in TREC than those without viral suppression, even after adjusting for changes in CD4% [14].

It is well established that despite strong inhibition of viral replication in patients on ART, HIV-1 may persist in peripheral blood cells and lymphoid tissues. Importantly, whilst HIV-1 DNA decline is dramatic, particularly in the first month after ART initiation during which period plasma HIV-1 RNA suppression is also substantial, children with the greatest declines in HIV-1 RNA are not more likely to have the greatest declines in HIV-1 DNA [15]. This finding may be explained by persisting HIV-1 DNA both within cells from the memory compartment, which can have very long half-lives, and within newly infected cells. Therefore it is clear that true HIV-1 suppression within the human host depends on a dynamic relationship that exists between HIV-1 replication within infected cells and infection of hitherto uninfected lymphocytes.

Since thymic emigrants are an important source of peripheral immune repopulation in children, we consider here the dynamics of cell-associated HIV-1 DNA and thymic emigrants in HIV-1-infected children after initiation of ART.

Materials and methods

Subjects and samples

As reported elsewhere [14,15], in 33 of the 128 previously untreated children in the PENTA 5 trial [13] (18 in Italy, 15 in the UK), sequential cellular samples were available for quantification of TREC and cell-associated DNA in peripheral blood mononuclear cells (PBMCs) at baseline and then at scheduled visits 4 (n=24), 12 (n=33), 24 (n=34), 48 (n=38) and 96 (n=19) weeks after ART initiation (181 samples in total). Six children had two samples at different time points corresponding to the same scheduled visit week. Missing 96-week samples were primarily due to the trial ending when the last child reached 48 weeks of follow-up. Cell-associated DNA and TREC levels were quantified by real-time quantitative PCR assay as previously described [14–16]. HIV-1 RNA and CD4 cell subsets were measured at ART initiation, at 2, 4, 8, 16, 20 and 24 weeks and then every 8 weeks subsequently. The protocol for the PENTA 5 trial (and for samples to be taken for immunological testing) was approved by the ethics committee for each participating centre. All primary caregivers gave written consent to participate and additional written consent was obtained from children, where appropriate, according to their age and knowledge of HIV-1 status.

Statistical methods

Results were expressed as copies per 10^6 PBMC (2×10^6 β-actin copies), having previously demonstrated good calibration between changes in TREC from baseline (initiation of ART) in PBMCs compared with separated CD4 cells in a subset of samples from these children [14]. In addition, as children in PENTA 5 experienced substantial CD4 increases [13], both HIV-1 DNA and TREC were also transformed to measurements per ml of blood according to the total number of lymphocytes per ml. Normal linear regression models were used to determine the multivariate relationship between $\log_{10}$ HIV-1 DNA, $\log_{10}$ TREC, CD4 [percentage or absolute level (square root transformed for stability)], $\log_{10}$ HIV-1 RNA and age at baseline. Longitudinal mixed normal models [17] were then used to assess the effects of baseline levels and subsequent changes in these variables on absolute $\log_{10}$ HIV-1 DNA after initiation of ART, in order to avoid problems of regression to the mean due to errors in the measurement of the baseline value [18]. These methods adjust for the fact that the same children were observed repeatedly. A change in underlying HIV-1 DNA slope was allowed at 4 weeks to capture the biphasic decline previously reported [15], with random effects allowing both the baseline HIV-1 DNA and initial decline to vary across children. Of the HIV-1 DNA values, 25% were below the level of detectability (10 copies/10^6 PBMC) and these were replaced with half the cut-off value [19]. Lack of sensitivity to this approximation was confirmed for main results using maximum likelihood methods [20].

Firstly TREC, CD4, CD8, HIV-1 RNA, age and disease stage (AIDS/not AIDS) at ART initiation (baseline) were investigated as predictors of changes in $\log_{10}$ HIV-1 DNA in these mixed models using backwards elimination. Any additional effect of changes in these variables after ART were then explored. In addition to predefined HIV-1 RNA categorization below 50 copies/ml, we also considered the phase of HIV-1 RNA decline. Specifically, each plasma HIV-1 RNA measurement for each child was categorized as belonging to i) the initial decline in HIV-1 RNA to its lowest level ('initial decline'); ii) after this initial response, to time periods with stable HIV-1 RNA <50 copies/ml, requiring both two previous HIV-1 RNA measurements to be <50 copies/ml ('stable response'); iii) time periods with HIV-1 RNA ≥50 copies/ml with at least one of the two previous

HIV-1 RNA being <50 copies/ml ('transient viraemia') (subsequent time points <50 copies/ml included as transient viraemia until satisfying 'stable' criteria); or iv) any other time period with HIV-1 RNA ≥50 copies/ml ('non-response'). Thus each HIV-1 RNA measurement from each child could be categorized as being during periods of initial decline, stable response, transient viraemia or non-response in terms of the overall pattern of HIV-1 RNA decline, and each child can contribute data from two or more phases of HIV-1 RNA decline as all children experience an initial decline in HIV-1 RNA.

Results

Population

As previously described [14], the median age of the 33 ART-naive children was 7.1 years at baseline (Table 1) and the mean plasma HIV-1 RNA was 5.01 $\log_{10}$ copies/ml (SD=0.76). Children received dual nucleoside backbones containing zidovudine, lamivudine and abacavir, with (*n*=26) or without (*n*=7) nelfinavir. None of the seven children on double NRTI only changed from dual NRTI therapy and all but one suppressed HIV-1 RNA below 400 copies/ml. At 48 weeks after ART initiation and at the latest HIV-1 DNA/TREC measurement, 13 (39%) and 12 (36%) children had HIV-1 RNA <50 copies/ml, respectively (Table 1); however, only six children consistently maintained HIV-1 RNA <50 copies/ml after initial HIV-1 RNA decline.

Table 1. Characteristics

At initiation of ART (previously untreated)	*n*=33
Girls, *n* (%)	11 (33%)
Age, median [IQR] (range)	7.1 [4.6–9.6] (0.3–15.5)
AIDS, *n* (%)	4 (12%)
CD4 percent, median [IQR] (range)	17% [9–24%] (1–45)
CD4 absolute, median [IQR] (range)	408 [265–729] (2–2580)
HIV-1 RNA $\log_{10}$ copies/ml, mean (SD) (range)	5.01 (0.76) (3.2–6.6)
First ART regimen, *n* (%)	
Two NRTIs*	7 (21%)
Two NRTIs* + nelfinavir	26 (79%)
At 48 weeks	***n*=33**
Increase in CD4 percent, mean (SD)	11.0% (8.0%)
Increase in CD4 absolute, mean (SD)	348 (447)
HIV-1 RNA <50 copies/ml, *n* (%)	13 (39%)
At last HIV-1 DNA/TREC measurement	***n*=33**
Week of last measurement, median [IQR] (range)	63 [42–93] (24–120)
last measurement before week 36, *n* (%)	1 (3%)
Increase in CD4 percent, mean (SD)	12.3% (8.4%)
Increase in CD4 absolute, mean (SD)	325 (326)
HIV-1 RNA <50 copies/ml, *n* (%)	12 (36%)

*Two of zidovudine, lamivudine or abacavir [13]. ART, antiretroviral therapy; IQR, interquartile range; NRTI, nucleoside reverse transcriptase inhibitor; TREC, T-cell receptor rearrangement excision circle.

In ART-naive children, HIV-1 DNA is associated with TREC

At ART initiation (baseline), median TREC level was 37 090 per 10^6 PBMC (range 1260–167 910) corresponding to 83 143 per ml of blood (range 252–592 483). Median HIV-1 DNA level was 440 copies per 10^6 PBMC (range <10–17 690) corresponding to 554 per ml of blood (range <2 to 33 485). Children with higher HIV-1 DNA at ART initiation had higher TREC levels (*P*=0.002) (Table 2). However, whilst HIV-1 DNA appeared to depend on CD4 in univariate analyses (*P*=0.02, Table 2), this was due to the strong relationship between CD4 and TREC; in multivariate analyses HIV-1 DNA did not depend on age, CD4 or HIV-1 RNA after adjusting for TREC (*P*>0.3). In contrast, higher TREC levels at baseline were independently associated with higher CD4, higher HIV-1 DNA, lower HIV-1 RNA and younger age (*P*<0.06; Table 2). AIDS status at baseline did not add independent information to any relationship (*P*>0.14). Relationships between CD4, HIV-1 RNA and age appeared to be mediated through TREC and did not persist once TREC values were taken into account.

The only baseline factors influencing HIV-1 DNA response to ART are TREC and HIV-1 DNA

HIV-1 DNA declined by an average of 0.25 $\log_{10}$ per ml at week 4, with slower subsequent decline to a total of around 0.55 $\log_{10}$ per ml at 96 weeks. In contrast, TREC levels remained at baseline levels to week 12, then increased by an average of 0.4 $\log_{10}$ per ml at week 24, remaining stable thereafter. When we considered pre-ART values as predictors of subsequent declines in HIV-1 DNA, baseline TREC was the most important predictor of HIV-1 DNA response to ART: children with higher TREC at baseline had faster initial declines in HIV-1 DNA to week 4 (on average 0.08 faster decline in $\log_{10}$ HIV-1 DNA per ml per week for each $\log_{10}$ higher baseline TREC per ml, 95% CI 0.02–0.15, *P*=0.009). There was also a trend towards children with higher baseline cell-associated HIV-1 DNA having faster initial HIV-1 DNA decline (*P*=0.10). After adjusting for baseline TREC, none of the other baseline factors (age, CD4, HIV-1 RNA and disease stage) were associated with decreases in HIV-1 DNA per ml after initiation of ART, either during the

Table 2. Relationship between TREC, HIV-1 DNA, HIV-1 RNA, CD4 cell count and age at initiation of antiretroviral therapy

	Univariate model			Multivariate model*		
Baseline factor and effect of predictors	Change	(95% CI)	*P*	Change	(95% CI)	$P^{\dagger}$
HIV-1 DNA (units $\log_{10}$/ml)						
TREC (per 1 $\log_{10}$/ml higher)	+0.82	(0.32,1.32)	0.002	+0.82	(0.32,1.32)	0.002
HIV-1 RNA (per 1 $\log_{10}$/ml higher)	–0.22	(–0.76,0.32)	0.41			*0.64*
Absolute CD4 (per 5 $CD4^{1/2}$ higher)	+0.21	(0.04,0.39)	0.02			*0.82*
Age (per 2 years older)	–0.09	(–0.30,0.13)	0.40			*0.39*
TREC (units $\log_{10}$/l)						
HIV-1 DNA (per 1 $\log_{10}$/ml higher)	+0.33	(0.13,0.52)	0.002	+0.12	(0.00,0.25)	0.06
HIV-1 RNA (per 1 $\log_{10}$/ml higher)	–0.14	(–0.48,0.20)	0.42	–0.26	(–0.46,–0.06)	0.01
Absolute CD4 (per 5 $CD4^{1/2}$ higher)	+0.27	(0.20,0.34)	<0.001	+0.19	(0.12,0.27)	<0.001
Age (per 2 years older)	–0.19	(–0.31,–0.07)	0.002	–0.13	(–0.22,–0.04)	0.007
Absolute $CD4x10^6$/l (units 5 $CD4^{1/2}$)						
TREC (per 1 $\log_{10}$/ml higher)	+2.45	(1.82,3.08)	<0.001	+2.45	(1.82,3.08)	<0.001
HIV-1 DNA (per 1 $\log_{10}$/ml higher)	+0.76	(0.13,1.39)	0.02			*0.82*
HIV-1 RNA (per 1 $\log_{10}$/ml higher)	–0.01	(–1.04,1.02)	0.99			*0.26*
Age (per 2 years older)	–0.53	(–0.89,–0.17)	0.005			*0.53*
HIV-1 RNA (units $\log_{10}$/ml)						
TREC (per 1 $\log_{10}$/ml higher)	–0.15	(–0.54,0.23)	0.42	–0.56	(–0.91,–0.21)	0.002
HIV-1 DNA (per 1 $\log_{10}$/ml higher)	–0.10	(–0.34,0.14)	0.41			*0.91*
Absolute CD4 (per 5 $CD4^{1/2}$ higher)	0.00	(–0.13,0.13)	0.98			*0.36*
Age (per 2 years older)	–0.18	(–0.31,–0.06)	0.005	–0.30	(–0.43,–0.17)	<0.001

*Multivariate models constructed using backwards elimination. †*P* values in italics show additional effect of non-significant predictors when added into multivariate model. Results for CD4 percentage were similar to those for absolute CD4. Similar results were also obtained expressing TREC and HIV-1 DNA per 10^6 PBMCs. CD8 (absolute or percentage) was not an additional significant predictor for any other baseline variable. TREC, T-cell receptor rearrangement excision circles.

first 4 weeks or subsequently (all $P>0.25$, data not shown).

Changes in TREC after ART are the strongest overall predictor of HIV-1 DNA response to ART

When we also considered how changes in other measurements following ART initiation might be related to declines in HIV-1 DNA, we found that, overall, children with the greatest increases in TREC after starting ART had the smallest decreases in HIV-1 DNA ($P=0.002$; Table 3), and TREC changes were by far the strongest predictor of HIV-1 DNA response to ART. Changes from baseline and actual levels of percentage/absolute CD4 and HIV-1 RNA per ml were not associated with changes in HIV-1 DNA after ART initiation (all $P>0.25$, Table 3). However, only six children consistently maintained full virological response (that is, plasma HIV-1 RNA suppression <50 copies/ml at every visit after first response) and several children experienced transient plasma viraemia above 50 copies/ml with or without subsequent persistent rebound above 50 copies/ml (Table 1). Taking these periods of transient viraemia into account, we found that the phase of HIV-1 RNA decline was associated with change in HIV-1 DNA ($P=0.04$), with decline in HIV-1 DNA being greater during stable response and less during transient viraemia (Table 3). In particular, HIV-1 DNA decline was 0.19 [=(–0.26) minus (–0.07)] and 0.42 [=(–0.26) minus (+0.17)] $\log_{10}$ per ml greater during stable virological response than during non-response and transient viraemia, respectively.

The relationship between change in TREC and HIV-1 DNA response to ART appears to vary according to phase of HIV-1 RNA decline following ART initiation

Table 3 assumes that any increase in TREC is associated with the same change in HIV-1 DNA in each phase of HIV-1 RNA decline. However, there was a suggestion from an interaction model that HIV-1 DNA decline was in fact affected least by TREC increases occurring during stable virological response and most by TREC increases occurring during transient viraemia ($P=0.13$) (Table 4). For each 0.5 $\log_{10}$ increase in TREC, decline in HIV-1 DNA during stable virological response appeared to be greater by 0.10 (=0.10 minus 0.20) and 0.26 (=0.10 minus 0.36) $\log_{10}$ per ml compared with virological non-response and transient viraemia, respectively. Adjusting for this inter-relationship, in the absence of any changes in TREC, overall HIV-1 DNA decline during stable virological response was greater by 0.10 and 0.27 $\log_{10}$ per ml on average than during non-response and transient viraemia respectively (Figure 1).

Table 3. Predictors of decline in HIV-1 DNA after initiation of antiretroviral therapy

	Univariate model			Multivariate model		
Factor	Difference*	(95% CI)	*P*	Difference*	(95% CI)	*P*†
TREC						
no change from baseline	0			0		
per 0.5 $\log_{10}$ per ml increase	+0.15	(+0.06,+0.25)	0.002	+0.16	(+0.07,+0.26)	0.0008
%CD4						
no change from baseline	0					
per 5% increase	+0.01	(–0.05,+0.08)	0.67			*0.75*
CD4						
no change from baseline	0					
per 5 $CD4^{1/2}$ increase	+0.04	(–0.03,+0.11)	0.26			*0.50*
%CD8						
no change from baseline	0					
per 5% increase	+0.04	(–0.02,+0.09)	0.19			*0.12*
CD8						
no change from baseline	0					
per 5 $CD8^{1/2}$ increase	+0.04	(–0.01,+0.09)	0.13			*0.64*
HIV-1 RNA						
no change from baseline	0					
per 2 $\log_{10}$ decrease	+0.03	(–0.15,+0.20)	0.78			*0.58*
HIV-1 RNA						
≥50 copies/ml	0					
<50 copies/ml	–0.08	(–0.27,+0.11)	0.39			*0.91*
HIV-1 RNA phase						
initial decline	0		0.08	0		0.04
non-response	–0.01	(–0.23,+0.22)		–0.07	(–0.29,+0.15)	
stable response	–0.22	(–0.48,+0.05)		–0.26	(–0.51,+0.00)	
transient viraemia	+0.20	(–0.07,+0.46)		+0.17	(–0.09,+0.42)	

*Positive values mean HIV-1 DNA decline smaller by this amount ($\log_{10}$ per ml) and negative values mean HIV-1 DNA decline greater by this amount ($\log_{10}$ per ml).
†*P* values in italics show additional effect of non-significant predictors when added into the multivariate model. All multilevel models adjust for time since initiation of antiretroviral therapy and the effect of baseline TREC on HIV-1 DNA decline (not statistically significant if change in TREC from baseline is included in the model). The effect of continuous factors are presented for approximate mean change from baseline to 24 weeks. TREC, T-cell receptor rearrangement excision circles.

Conclusions

In this study we have sought to gain an insight into the complex viral and T-cell dynamics that occur in HIV-1-infected children initiating ART. This analysis follows our previous report that the increase in CD4 cells following ART in children was predominantly due to naive CD45RA cells, and that these naive cells are likely to be thymically derived [4,14]. However, the influence that such T-cell changes may have on HIV-1 infection of T cells have as yet to be fully determined.

We started by considering what factors affected absolute levels of cell-associated HIV-1 DNA in naive children at baseline (pre-therapy). Potential factors such as age, absolute and percentage CD4, HIV-1 RNA and TREC are all highly inter-dependent and thus it was impossible to assess the independent effects of these variables using univariate analyses. By multivariate analyses, the only independent association we found at baseline was that levels of cell-associated HIV-1 DNA were positively associated with TREC. This is intriguing and could be caused by a number of mechanisms, which are not mutually exclusive. The simplest explanation is that TREC-bearing cells are preferentially infected by HIV-1 [21–23]. However, this is unlikely to be the sole explanation as TREC-bearing cells are also more likely to be naive, whereas it is the memory CD4 population that appears to be preferentially infected [21–23]. However, thymic emigrants may differentiate into memory cells thereby providing a greater source of cells susceptible to infection [23,24]. Another possibility is that the relationship between TREC and HIV-1 DNA reflects homeostasis between peripheral CD4 cells and thymic output, that is, the higher the HIV-1 DNA levels, the greater the peripheral CD4 depletion and, in turn, the greater the thymic output. Future studies are required to address this fully.

Baseline TREC level was also a predictor of HIV-1 DNA response to ART, with children with higher baseline TREC levels having faster initial HIV-1 DNA

Table 4. Variation in the relationship between decline in HIV-1 DNA and increase in TREC according to phase of HIV-1 RNA decline after initiation of antiretroviral therapy

Factor	Difference in HIV-1 DNA*	(95% CI)
HIV-1 RNA phase		
initial decline	0	
non-response	−0.11	(−0.33,+0.12)
stable response	−0.21	(−0.47,+0.06)
transient viraemia	+0.07	(−0.22,+0.34)
TREC		
no change from baseline	0	
per 0.5 $\log_{10}$ per ml increase during:		
non-response	+0.20	(+0.08,+0.33)
stable response	+0.10	(−0.03,+0.22)
transient viraemia	+0.36	(+0.08,+0.64)
test for interaction between TREC		
and phase of HIV-1 RNA decline	P=0.13	

*Positive values mean HIV-1 DNA decline smaller by this amount ($\log_{10}$ per ml) and negative values mean HIV-1 DNA decline greater by this amount ($\log_{10}$ per ml). Multivariate multilevel model, also adjusted for time since initiation of antiretroviral therapy, the effect of baseline TREC on HIV-1 DNA decline and interactions between phase of HIV-1 RNA decline and TREC. TREC, T-cell receptor rearrangement excision circles.

declines after ART initiation. We have previously demonstrated that TREC and CD4 increases after ART initiation are smaller in children with higher baseline TREC and CD4 [14,16]; thus a plausible explanation would be that HIV-1 DNA decline is faster when there is a smaller increase in the number of circulating cells that can potentially become infected. It is also possible that children with high TREC levels have a more rapid turnover of infected cells, and so the reduction in HIV-1 RNA following the addition of ART could, in this context, lead to a reduction in newly infected cells.

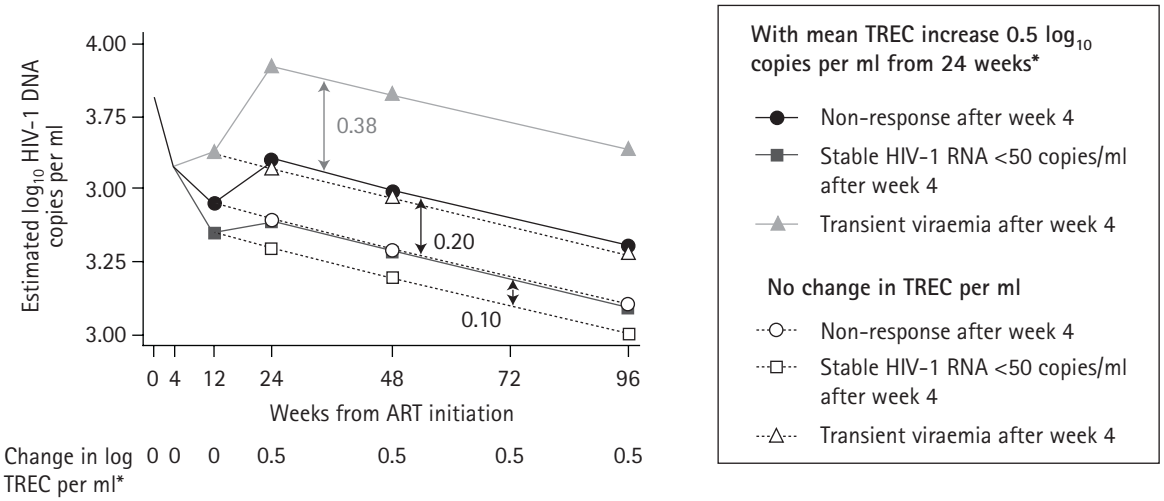
Following the introduction of ART, we found that greater increases in TREC were associated with smaller long-term HIV-1 DNA declines. As naive CD4 cells only become infected at low rates [23], it seems unlikely that infection of developing thymic emigrants themselves leads to a substantial pool of cell-associated HIV-1 DNA. Alternatively, it is possible that thymic emigrants, in the context of ongoing viraemia, will be activated from circulating viral antigen [25], proliferate [26] and differentiate into memory cell subsets, which are more vulnerable to HIV-1 infection. This concept is supported by our exploratory findings that the inverse relationship between decline in HIV-1 DNA and increase in TREC during ART varied according to the phase of HIV-1 RNA decline. We found that for the same increase in TREC, HIV-1 DNA decline appeared to be only slightly affected during stable HIV-1 RNA suppression but was strongly reduced during ongoing viraemic phases. It is pertinent that we have previously shown that TREC increases during ART were higher in viraemic children than in children with stable viral suppression [14,16].

Of particular interest was our finding that the inverse relationship between TREC increase and HIV-1 DNA decline was strongest during transient viraemia. Slower HIV-1 DNA declines in children with transient viraemia could be partially explained by larger prior CD4 increases providing more target cells for infection than in non-responders. In addition, transient viraemia could be associated with increased survival of infected cells in the periphery compared with non-response. These findings may imply that the use of short cycles of pulse ART in children could be detrimental to the long-term reduction in cell-associated HIV-1. They also highlight the importance of maintaining maximal adherence to ART regimens to promote and maintain stable, complete HIV-1 RNA suppression.

TREC were quantified in PBMCs rather than in purified CD4 T lymphocytes and this is a limitation of our study. However, we have previously shown good calibration between changes in TREC in PBMCs and separated CD4 cell subsets in samples from this same study [14]; thus appropriate inference should therefore be obtained from considering change in TREC in PBMCs rather than purified CD4 cells. We have also considered models based both on PBMCs (TREC content) and transformation to measurements per ml of blood (absolute TREC number) in order to account for the effects of the substantial increases in CD4 that occurred after ART initiation in these children. Furthermore, the advantage of measuring the absolute TREC number instead of the TREC content is that the absolute TREC number provides a more direct measurement of thymic output than TREC content, which is also affected by T cell division [27]. However, findings concerning the relationship between changes in HIV-1 DNA, TREC and phase of HIV-1 RNA decline were similar in both cases.

We have not been able to include information on CD4 phenotypes, which were only available from 17 children, nor were we able to assess markers of T cell proliferation or apoptosis as all studies were performed on stored samples and there was insufficient material remaining. However, despite these limitations, our analysis, derived from the most extensive longitudinal study to date examining the relationship between ART, TREC and HIV-1 DNA in previously untreated children, strongly suggests that HIV-1 DNA decline is affected by both changes in HIV-1 plasma viraemia and TREC. Both latently infected cells and *de novo* cell infection are likely to contribute to persistence of HIV-1 DNA [28,29]. Overall, our findings suggest that an increase in circulating thymic emigrants, while useful for peripheral immune reconstitution, might also serve

Figure 1. Fitted relationship between changes in TREC and HIV-1 DNA by stage of HIV-1 suppression



Estimated HIV-1 DNA trajectories corresponding to children with different increases in TREC occurring at different times and with different children in different phases of HIV-1 RNA decline (from the multivariate multilevel interaction model in Table 4). The impact of HIV-1 RNA decline and TREC increase is estimated under restricted scenarios: i) TREC increase: a child without any change in TREC compared with baseline versus a child with the average increase in TREC observed in this population (namely, no change at weeks 4 and 12 and then an increase of 0.5 $\log_{10}$ copies/ml at week 24 and subsequently) and ii) phase of HIV-1 RNA decline after week 4 (initial decline): non-response (always ≥50 copies/ml) versus stable response (always <50 copies/ml) versus transient viraemia. Thus to week 4 there is no difference between any scenario in terms of the model and all trajectories show an initial decline in HIV-1 DNA. From weeks 4 to 12, there is no difference between scenarios in terms of increase in TREC, so the trajectories show the impact of phase of HIV-1 RNA decline with the impact of TREC increases shown from week 24 onwards. *Mean change in TREC in this population [9]. TREC, T-cell receptor rearrangement excision circles.

as a source of CD4 cells for viral infection in the absence of persistent HIV-1 RNA suppression. Further studies addressing the HIV molecular profile, such as integrated versus unintegrated HIV-1 DNA and expression of HIV mRNAs in separate cell subsets, are required to confirm this finding.

A number of key questions remain unanswered in the treatment of HIV-1 infected children. In particular, the optimal timing of ART initiation has yet to be established. The findings from this study indicate that use of HIV-1 RNA and CD4 counts to guide initiation and modulation of ART in children with HIV-1 may be insufficient. We would advocate that further studies in children are required to refine our knowledge of the dynamic inter-relationship between HIV-1 and immune restoration in order to enable optimal treatment of paediatric HIV-1 infection.

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Appendix

PENTA committees and participants (alphabetical)

Executive Committee for PENTA 5: J-P Aboulker, A Babiker, A Compagnucci, J Darbyshire, M Debré, M Gersten (Agouron), C Giaquinto (chairperson), DM Gibb and A Jones (GlaxoSmithKline).

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PENTA Immunology/Virology Group: C Boucher, M Clerici, A de Rossi, N Klein, C Loveday, M Muñoz-Fernandez, D Pillay and C Rouzioux.

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