Immune reconstitution in HIV-1-infected children on antiretroviral therapy: role of thymic output and viral fitness

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Objective To investigate the role of thymic output and viral fitness in immune reconstitution in HIV-1-infected children on antiretroviral therapy.

Methods Thymic output was studied by measuring levels of T-cell receptor rearrangement excision circles (TREC) in peripheral blood lymphocytes, using a real-time quantitative PCR assay. Recombinant viruses containing pre-therapy or post-therapy HIV-1 protease domains were evaluated for viral infectivity in a quantitative single-cycle assay.

Results Eighteen HIV-1-infected children who showed a significant increase in CD4 T-cell count after therapy were studied; HIV-1 plasma viraemia was substantially suppressed in 12 children (virological responders), but not in the other six (virological non-responders). TREC were quantified at baseline, and sequentially during the first 12 months of therapy. Both virological responders and non-responders showed an increase in TREC levels that was inversely correlated with baseline TREC and CD4 T cell counts. Changes in TREC positively correlated with CD4 T-cell count increases in virological responders, but not in non-responders; moreover, the ratios between TREC and CD4 T-cell count increases were higher in non-responders than in responders, suggesting a persistence of peripheral CD4 T-cell loss in the former. Drug-resistant viruses with reduced replicative capacity were documented in three out of six non-responders.

Conclusions These findings indicate that recovery of thymic function is a pivotal event in immune reconstitution, and suggest that CD4 T-cell increase despite persistent viraemia is sustained by a continuous thymic output that compensates peripheral CD4 T-cell depletion which might be slowed down by emerging viruses with reduced fitness.

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Introduction

HIV-1 infection leads to a progressive decrease in CD4 T cells. Several mechanisms contribute to the CD4 T-cell decline, including a shortened cell half-life, cell death at the periphery, and an inefficient cell renewal capacity [1–3]. Highly active antiretroviral therapy (HAART), a combination treatment that includes both protease and reverse transcriptase inhibitors, efficiently reduces the plasma HIV-1 RNA load often to unde-

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tectable levels, and increases the number of circulating CD4 T cells [4,5]. Although HAART does not eradicate HIV-1, due to a persistent low level of viral replication, and the longevity of latently infected cells [6–8], it does allow a substantial recovery of immune system functions [4,5].

Peripheral CD4 T-cell repopulation in HIV-1 infected adults on HAART is a biphasic process with an initial rapid increase in circulating CD4 lymphocytes, mainly memory cells, which is probably due to redistribution from lymph nodes where circulating lymphocytes had been trapped by the inflammatory status characteristic of HIV-1 replication. This first phase is followed by a slower increase in circulating CD4 lymphocytes, mainly naive cells, reflecting thymic production or extrathymic cellular proliferation, no longer counterbalanced by HIV-1-induced cellular sequestration and destruction [9–11]. In contrast, several studies have shown that in children a high increase in naive cells occurs after HAART initiation [12–15]. Given the involution of the thymus with age [16,17], it has been proposed that the differences in the immune reconstitution pattern between children and adults might be explained by the greater regenerative capacity of the thymus in the former [15,18].

Several studies have addressed the influence of HIV-1 infection on thymic function, but the methods used to estimate thymopoietic capacity caused concern. A new method that allows a more direct evaluation of thymic cell production has been described [19]. 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). Because TREC do not replicate with mitosis, they are diluted by cellular division or lost with cell death [20]; their detection in peripheral blood T cells could thus serve as a marker for recent thymic emigrant cells [19,21], and hence their quantification could constitute a tool to estimate thymic output. Using TREC measurements, it was found that although thymic function declines with age, limited thymic output was still measurable during old age [19,21]. TREC levels were lower in HIV-1-infected individuals than in age-matched normal controls [21,22]. An increase in TREC along with the HIV-1 decline was found in HIV-1-infected adults and children treated with antiretroviral therapy [19,22,23].

Although many patients experience both immunological and virological responses to HAART in terms of increased peripheral blood CD4 T cell counts and reduced plasma HIV-1 RNA levels, respectively, several studies have described patients who have a paradoxical response to HAART in that they showed a significant increase in CD4 T-cell counts despite persistent detectable viraemia [24–28]. Although the mechanisms that might explain this discordant response to HAART have not been fully elucidated, it was recently suggested that thymic output [29], and an impaired replicative capacity of drug-resistant viruses [30] might be involved.

The objective of this study was to investigate the role of thymic output in the immune restoration process occurring in virological responder and virological non-responder HIV-1-infected children treated with HAART, and to evaluate the contribution of viral fitness to the increase in CD4 T cells despite lack of viral suppression.

Materials and methods

Patients

We studied 18 HIV-1 infected children, all born to HIV-1-seropositive mothers and followed by the Paediatric Department of Padova University since birth. All children were naive for prior protease inhibitor treatment. The median age at the time of HAART initiation was 8.5 years (range, 2.4–14.4 years). The HAART regimen consisted of a triple-drug combination, including two reverse transcriptase inhibitors (zidovudine, didanosine, stavudine or nevirapine) and one protease inhibitor (nelfinavir or ritonavir). The median follow-up time after HAART initiation was 20.5 months (range, 12–24 months). The inclusion criterion for this study was $\geq$ 10% increase in CD4 T-cell percentage at 12 months of therapy compared with baseline values (immunological response). Patients' CD4 and CD8 T-cell counts, and plasma HIV-1 RNA levels were followed over time. Levels of TREC and proviral HIV-1 DNA were evaluated in cryopreserved samples collected at HAART entry, and during follow-up. Virological responders were defined as children in whom the HIV-1 RNA load decreased and remained at $<$ 400 HIV-1 RNA copies/ml plasma; children who did not meet this criterion were considered virological non-responders.

Isolation of peripheral blood mononuclear cells (PBMC) and CD4 T-cell purification

PBMC were isolated from peripheral blood by centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient, as described previously [31,32]. CD4 T cells were isolated from PBMC using magnetic beads coated with monoclonal antibodies (mAb) against human CD4 (Dynabeads M-450 CD4; Dynal, Oslo, Norway) according to the manufacturer's instructions.

Immunophenotyping

PBMC were stained for two-colour flow cytometry with the following combinations of mAb: fluoresceine

isothiocyanate (FITC)-conjugated anti-CD3, and phycoerythrin (PE)-conjugated anti-CD4 or anti-CD8 mAb (Becton Dickinson, San Jose, California, USA), FITC-conjugated anti-CD4 or anti-CD8 mAb (Becton Dickinson) with PE-conjugated anti-CD45RA or anti-CD45RO mAb (Caltag, San Francisco, California, USA). Aliquots (50 μl) of whole blood samples were incubated with different mAb combinations for 20 min on ice, followed by red cell lysis and fixation, using the ImmunoPrep system (Coulter Corporation, Hialeah, Florida, USA). Cells were stored at 4°C before analysis by flow cytometry (EPICS ELITE, Coulter). A total of 5000 cells was analysed in a manually set lymphocyte gate. Positive cut-off for fluorescence was set to include $< 2\%$ of negative control mouse IgG conjugates.

TREC quantification

TREC levels were analysed by real-time quantitative PCR. Cells were lysed as reported previously [33]. Cell lysate equivalent to 80 000 cells (10 μl) was used as template for PCR amplification. The reaction volume was 50 μl containing, in addition to the template, 1.0 × TaqMan Universal PCR Master Mix (PE Applied Biosystems, Foster City, California, USA), 300 nM each primer (forward, 5′-CACATCCCTTT CAACCATGCT-3′; reverse, 5′-GCCAGCTGCAGG GTTTAGG-3′) and 100 nM of the fluorogenic probe (5′-ACACCTCTGGTTTTTGTAAAGGTGCCCAC T-3′) conjugated with the fluorophores FAM (6-carboxyfluorescein) at the 5′-end, and TAMRA (6-carboxytetramethilrhodamine) at the 3′-end. The PCR primers and the fluorogenic probe were specifically designed for the detection of human TREC. The thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, and 45 cycles each of 95°C for 15 sec and 60°C for 1 min. The reaction was performed in a spectrofluorometric thermal cycler (ABI PRISM 7700 Sequence Detector, PE Applied Biosystems). For each run, a standard curve was generated from duplicate samples of five-fold serially diluted known copies of plasmid DNA obtained by inserting a human signal joint TREC fragment in the pCR-Blunt Vector. Under our experimental conditions, the assay was sensitive enough to detect 2.5 copies of TREC, and showed a dynamic range of at least 5 $\log_{10}$ (from 2.5 copies to 2×10^5 copies). Each sample was run in duplicate. To normalize for cell equivalents, the β-actin gene was quantified under real-time PCR conditions similar to those used for TREC quantification; the primer and probe concentrations were as follows: 300 nM of the forward primer (5′-TCACCCA CACTGTGCCCATCTACGA-3′), 600 nM of the reverse primer (5′-CAGCGGAACCGCTCATTGC CAATGG-3′) and 200 nM of the fluorogenic probe (5′-ATGCCCTCCCCCATGCCATCCTGCGT-3′) conjugated with the fluorophore VIC (Applied Biosytems, Foster City, CA, USA) at the 5′-end, and TAMRA at the 3′-end. The β-actin standard curve was obtained from fivefold serial dilutions of DNA extracted from the 8E51 cell line.

HIV-1 DNA quantification

HIV-1 DNA levels were determined by real-time quantitative PCR assay. The thermal cycling, reaction conditions, and the methodological approach used for quantification were similar to those described for TREC quantitative analysis. The PCR primers (forward, 5′-TTAAGTGTTTCAATTGTGGCAAAGA-3′; reverse, 5′-AAAAAATTA GCCTGTCTCTCAG TACAATCT-3′) and the fluorogenic probe (5′-FAM-CCCCTAGGAAAAAGGGCTGTTGGAAATG-TA MRA-3′) were designed to detect a conserved region of the HIV-1 *gag* gene. The primer and probe concentrations used were 600 nM and 100 nM, respectively. The standard curve was obtained by fivefold serial dilutions of DNA extracted from 8E51 cells, which contain one proviral genome per cell. The assay was sensitive enough to detect 1.2 copies of HIV-1 DNA, and showed a dynamic range of at least 5 $\log_{10}$ (from 1.2 to 5×10^5 copies). The HIV-1 copy number was normalized against the β-actin copy number, as described above.

HIV-1 RNA quantification

Plasma HIV-1 RNA levels were determined using the Amplicor HIV-1 Monitor Test (Roche Diagnostic Systems, Branchburg, New Jersey, USA). The lower limits of detection were 400 HIV-1 RNA copies/ml of plasma using the Standard protocol, and 50 HIV-1 RNA copies/ml of plasma using the Ultrasensitive protocol.

Viral infectivity assay

Recombinant viruses, containing the protease sequence of the patients' pre- or post-therapy plasma viruses, were produced as described by Race *et al.* [34]. This technique relies on the complementation of a viral vector, devoid of protease coding sequence, by a PCR product obtained from plasma virus. Complementation is achieved by homologous recombination during 293-T cell transfection. The single-cycle titre of the recombinant viruses was measured on indicator P4 cells (HeLa-CD4,LTR-*lacZ*) as described previously [35]. Briefly, triplicate subconfluent P4 cells in 96-well plates were infected with the equivalent of 0.25, 1.5, 1 and 2 ng HIV-1 p24 of the different recombinant viruses obtained by transfection, in the presence of 20 μg/ml DEAE-dextran. The infection titre was measured by quantification of the β-galactosidase activity in P4 lysates, using a colorimetric assay (termed the CPRG assay) based on the cleavage of chlorophenol red-β-D-galactopyranoside by β-galactosidase; post-therapy viral isolate infectivity was expressed as a percentage of wild-type isolate infectivity. The protease region sequences were determined from bulk PCR products amplified from plasma as described previously [36].

Statistical analysis

TREC and CD4 T-cell count data obtained for each patient at baseline and at different time points during the first 12 months of therapy were analysed by linear regression analysis, and the slopes of TREC/1×10^5 cells per day and CD4 T cells/l per day were calculated individually. Correlations between different parameters were analysed using the non-parametric Spearman's test. Comparisons between groups were performed by using the non-parametric Mann–Whitney test. Analyses were performed with SAS institute statistical software (SAS version 6.12).

Results

Virological and immunological characteristics of patients

We studied 18 HIV-1-infected children whose CD4 T-cell percentages showed a $\geq 10\%$ increase over baseline values after 12 months on HAART (i.e., immunological responders); of these, 12 were classified as virological responders and six as virological non-responders, according to the decline in HIV-1 plasma viraemia during therapy. The virological and immunological characteristics of the two groups of children at baseline and during follow-up are summarized in Fig. 1. In virological responders, the mean baseline value of HIV-1 RNA was 4.6 (SD, 0.7) $\log_{10}$ copies/ml and decreased by 1.6 (SD, 1.0), 2.4 (SD, 1.0), and 2.7 (SD, 0.6) $\log_{10}$copies/ml at 1, 3 and 6 months of therapy, respectively (Fig. 1a). Plasma HIV-1 RNA levels dropped to < 50 copies/ml in 10 children, and remained undetectable for the entire follow-up period in all but one, in which spikes from 50 to < 400 copies/ml were observed; in two other children, plasma HIV-1 RNA fell to < 400 copies/ml, and remained stable at levels between 50 and 400 copies/ml for the entire follow-up period. The mean baseline HIV-1 DNA level was 1.8 (SD, 0.7) $\log_{10}$ copies/1×10^5 PBMC, and decreased by 0.6 (SD, 0.3) $\log_{10}$ copies/1×10^5 PBMC at 6 months of therapy (Fig. 1a), but remained detectable for the entire follow-up period in all children but one; after 24 months of therapy the mean value was 0.5 (SD, 0.4) $\log_{10}$ copies/1×10^5 PBMC.

In the virological non-responders, the mean baseline HIV-1 RNA load was 4.7 (SD, 1.3) $\log_{10}$ copies/ml and decreased by 1.1 (SD, 0.8), and 0.8 (SD, 0.6) $\log_{10}$ copies/ml at 1, and 3 months of therapy, respectively; thereafter, HIV-1 RNA plasma viraemia rebounded to pre-therapy levels, and at 12 months of therapy was 4.6

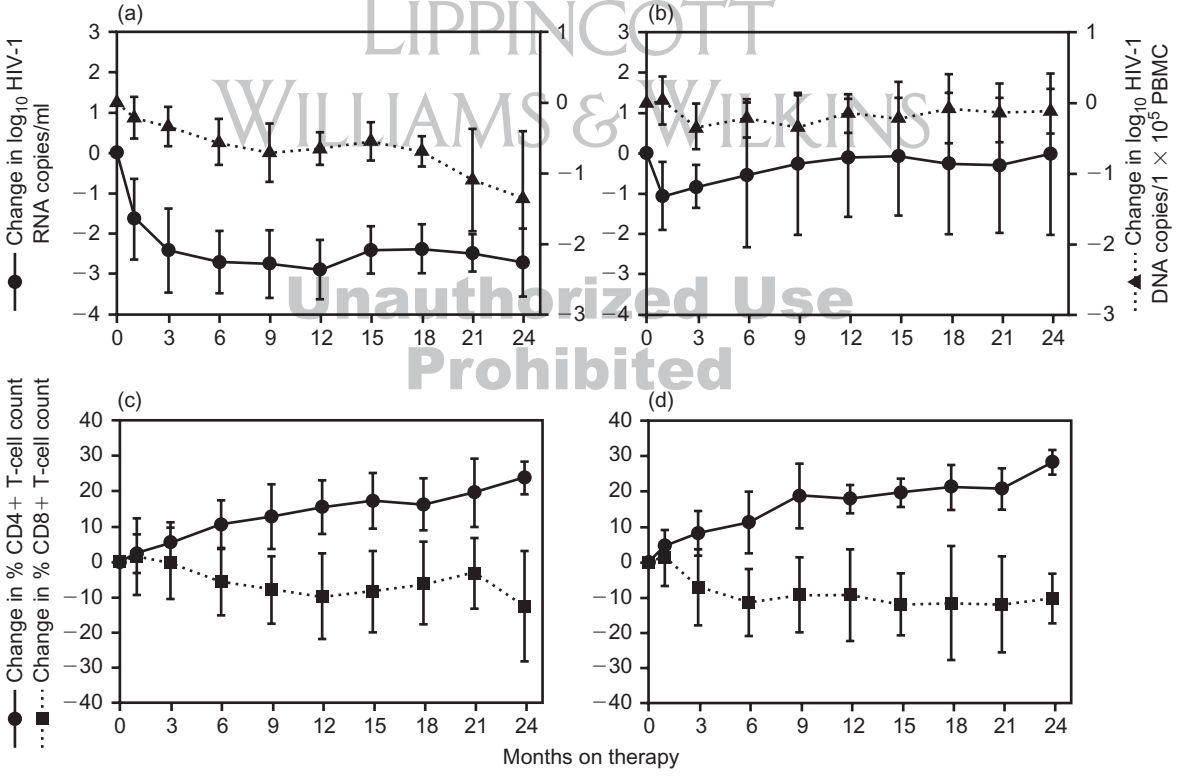

Fig. 1. Viral dynamics and T-cell count kinetics in virological responders (a and c), and non-responders (b and d). Analyses were performed at the start of therapy, at 1 month of therapy and subsequently every 3 months. Mean values and SD (vertical bars) are shown.

(SD, 1.0) $\log_{10}$ copies/ml (Fig. 1b). Mean baseline HIV-1 DNA burden was 1.9 (SD, 1.1) $\log_{10}$ copies/1×10^5 PBMC, and remained fairly stable for the entire follow-up period (Fig. 1b).

In virological responders, the mean baseline CD4 T-cell percentage was 15% (range, 1%–30%), and increased to 30% (range, 17%–44%) at 12 months of therapy (Fig. 1c). A similar increase was observed in the virological non-responders, despite the persistence of HIV-1 plasma viraemia; the mean baseline CD4 T-cell percentage was 10% (range, 2%–24%), and increased to 28% (range, 21%–40%) at 12 months of therapy (Fig. 1d). CD8 T-cell percentages decreased in both groups of children (Fig. 1c and d).

Quantification of TREC levels in PBMC and CD4 T cells

To investigate the contribution of thymic output to peripheral T-cell repopulation in response to antiretroviral treatment, we quantified TREC levels in PBMC samples and, when available, in CD4 T-cell fractions at therapy initiation and sequentially during the first 12 months of treatment (Fig. 2). The median baseline value of TREC/1×10^5 PBMC in virological responders and non-responders collectively was 943 (range,

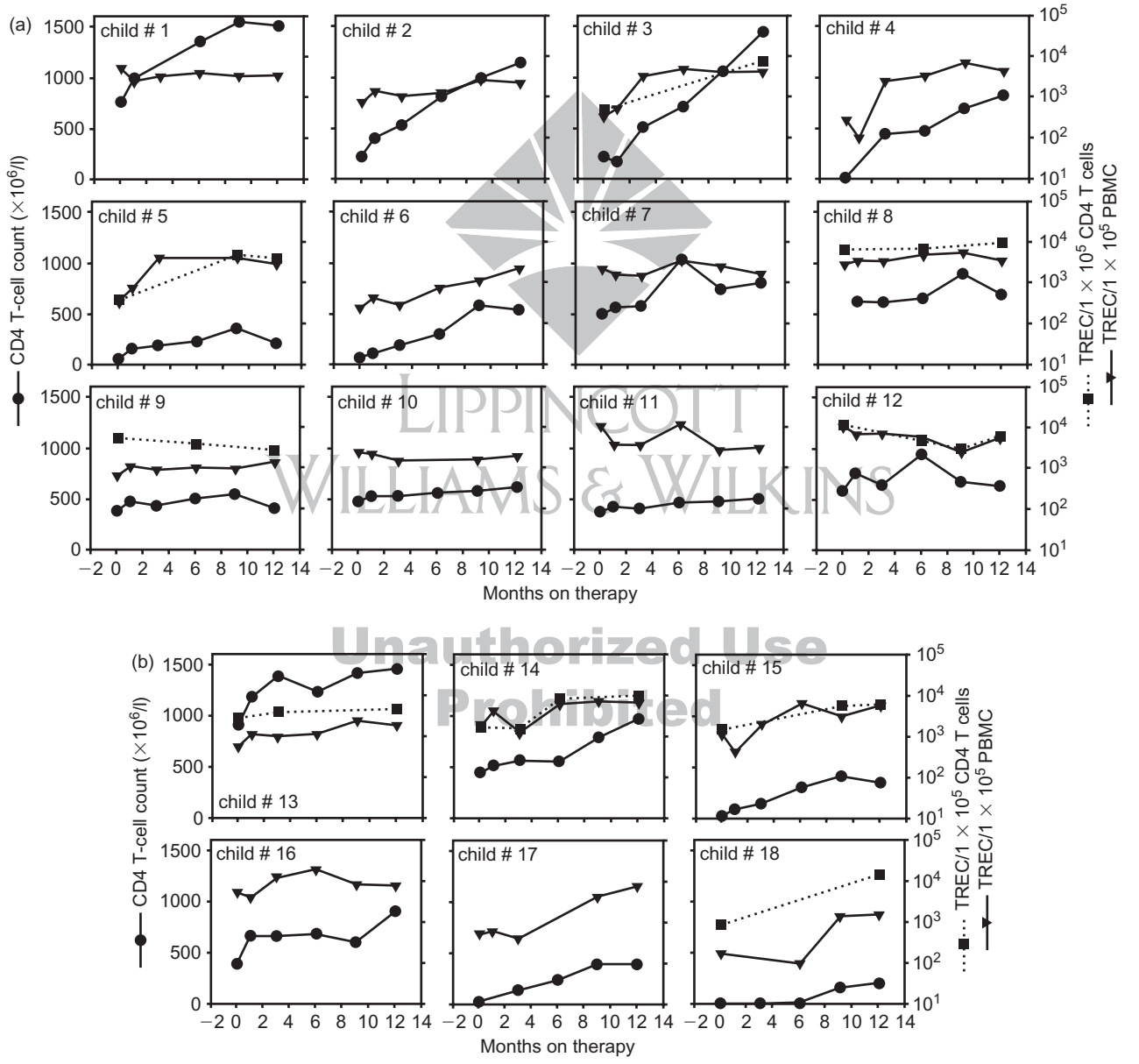

Fig. 2. Kinetics of TREC levels and CD4 T-cell counts in virological responders (a) and virological non-responders (b). TREC were estimated by real-time PCR in PBMC and in purified CD4 T cells at the start of therapy, and subsequently during the first 12 months of treatment.

174–10498), with no significant difference between the two groups (median 1503; range, 247–10498 versus median, 837; range, 174–5448; $P = 0.60$). A positive correlation between baseline levels of TREC/1×10^5 PBMC and baseline levels of CD4 T cells/l was observed (r_s, 0.66; $P = 0.0031$) (Fig. 3a). The kinetics of TREC levels during therapy varied widely from child to child; of note, a prompt increase was observed in infants who had low levels of TREC and low CD4 T-cell counts at baseline (i.e., children 3, 4 and 5; Fig. 2a), whereas the increase in TREC levels occurred later in others (i.e., child 6, Fig. 2a and children 14, 15, 17 and 18; Fig. 2b); no increase was observed in children who had baseline TREC values $\geqslant 1000/1 \times 10^5$ PBMC (i.e., children 1, 10, 11 and 12; Fig. 2a), comparable to values reported in HIV-1 uninfected age-matched normal controls ([22] and unpublished observation). Overall, the slopes of the TREC increases over 12 months of therapy were inversely correlated with the baseline levels of both

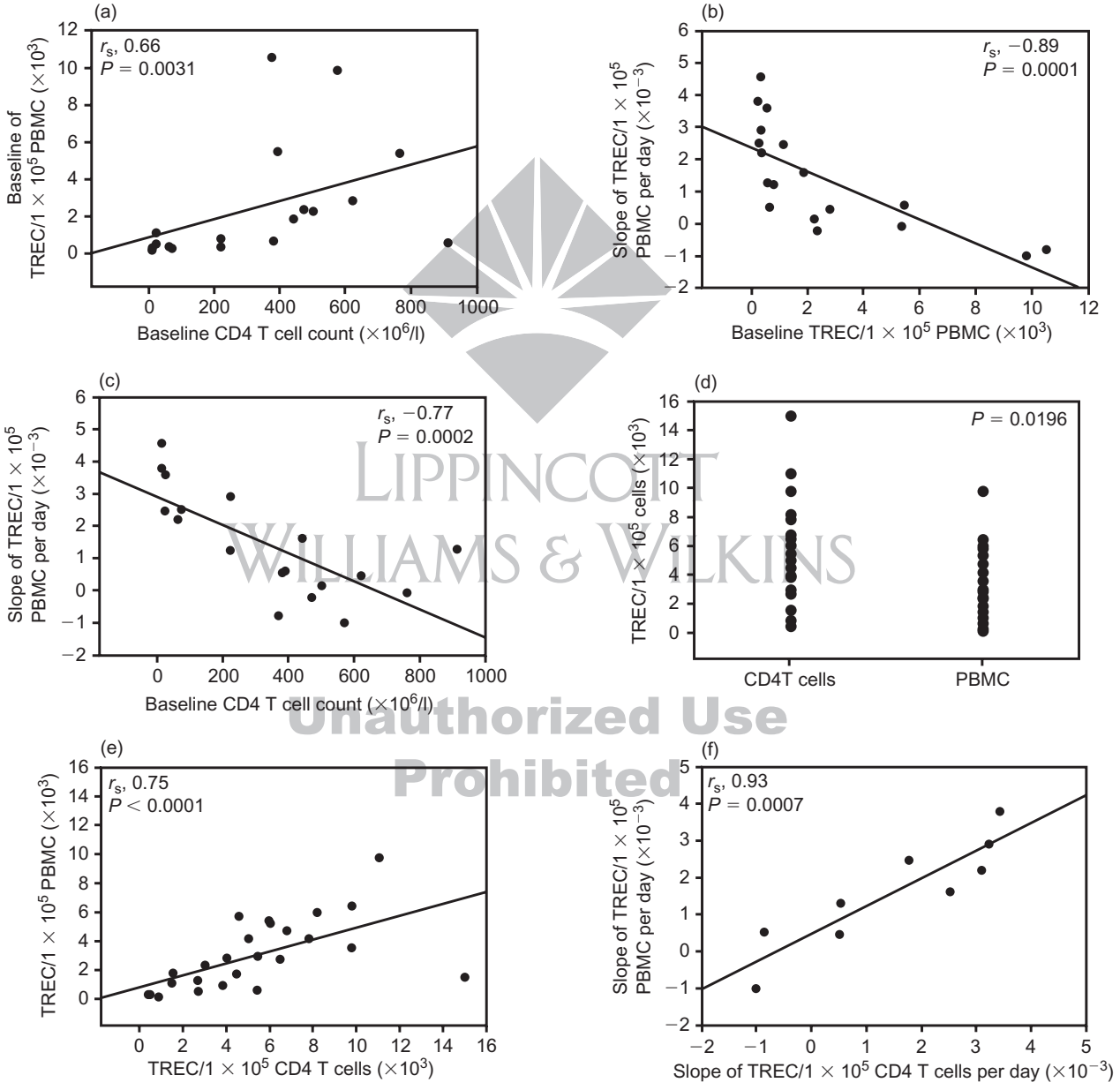

Fig. 3. Correlation between TREC levels and different parameters at the start of therapy and during the first 12 months of therapy in the 18 children studied. Correlation between (a) baseline levels of TREC in PBMC and baseline CD4 T-cell counts, (b) slopes of TREC in PBMC and baseline levels of TREC, and (c) between slopes of TREC in PBMC and baseline CD4 T-cell counts. (d) TREC levels in PBMC and in the corresponding purified CD4 T-cell fraction. Correlation between (e) TREC levels in PBMC and in purified CD4 T cells and (f) slopes of TREC in PBMC and in purified CD4 T cells. r_s, Spearman's correlation coefficient.

TREC (r_s, −0.89; $P = 0.0001$; Fig. 3b) and CD4 T-cell counts (r_s, −0.77; $P = 0.0002$; Fig. 3c). In this group of children, no relationship emerged between age and baseline TREC levels (r_s, 0.084; $P = 0.74$) nor between age and changes in TREC levels during therapy (r_s, −0.157; $P = 0.534$).

Levels and changes of TREC in PBMC reflected those in purified CD4 T cells. By comparing samples in which analyses could be performed in both PBMC samples and corresponding CD4 T-cell fractions, in agreement with recent observations [22] we found that TREC levels were higher in CD4 T cells than in PBMC ($P = 0.0196$; Fig. 3d); however, TREC levels in PBMC and CD4 T cells were strongly correlated (r_s, 0.75; $P < 0.0001$; Fig. 3e); moreover, in nine children in whom analyses could be performed in both PBMC and in CD4 T cells at baseline and during therapy, slopes of TREC/1 × 10^5 CD4 T cells per day positively correlated with slopes of TREC/1 × 10^5 PBMC per day (r_s, 0.93; $P = 0.0007$; Fig. 3f), and inversely correlated with CD4 T-cell values at baseline (r_s, −0.64; $P = 0.04$).

Taken together, these findings suggest that advanced immunological impairment, while associated with a lower levels of TREC, might result in a more evident thymic output in response to therapy.

Relationship between TREC kinetics and CD4+ T cell increase in virological responder and non-responder children

To analyse better the relationship between TREC and CD4 T cells in virological responder and non-responder children, TREC and CD4 T-cell counts and slopes were compared. In virological responders, there was a good relationship between TREC levels and CD4 T-cell counts (r_s, 0.55; $P < 0.0001$; Fig. 4a); furthermore, the slopes of TREC in PBMC were positively correlated with the slopes of CD4 T-cell count increases (r_s, 0.60; $P = 0.04$; Fig. 4c). A similar result was obtained by comparing the slopes of TREC in CD4 T cells with the slopes of CD4 T cells (r_s, 0.80; $P = 0.08$). Conversely, in virological non-responders, there was no relationship between TREC levels and CD4 T-cell counts (r_s, 0.24; $P = 0.24$; Fig. 4a), nor between slopes of TREC in PBMC and slopes of CD4 T-cell increase (r_s, −0.3; $P = 0.56$; Fig. 4c); a similar lack of association was observed when slopes of TREC in CD4 T cells were compared with the slopes of CD4 T cells (r, −0.4; $P = 0.75$). Although caution is needed when considering these data due to the small number of virological non-responder children, these findings suggest that the interplay between thymic output and CD4 peripheral restoration was different in virological responders and non-responders. Furthermore, the ratios between the slopes of TREC and CD4 T-cell counts tended to be higher in virological non-responders than in responders [mean, 2.5 (SD, 2.0) × 10^{-3} versus mean, −0.6 (SD, 6.4) × 10^{-3}; $P = 0.06$), thus indicating that for a similar increase in TREC levels, the CD4 T-cell count increase was lower in the virological non-responders.

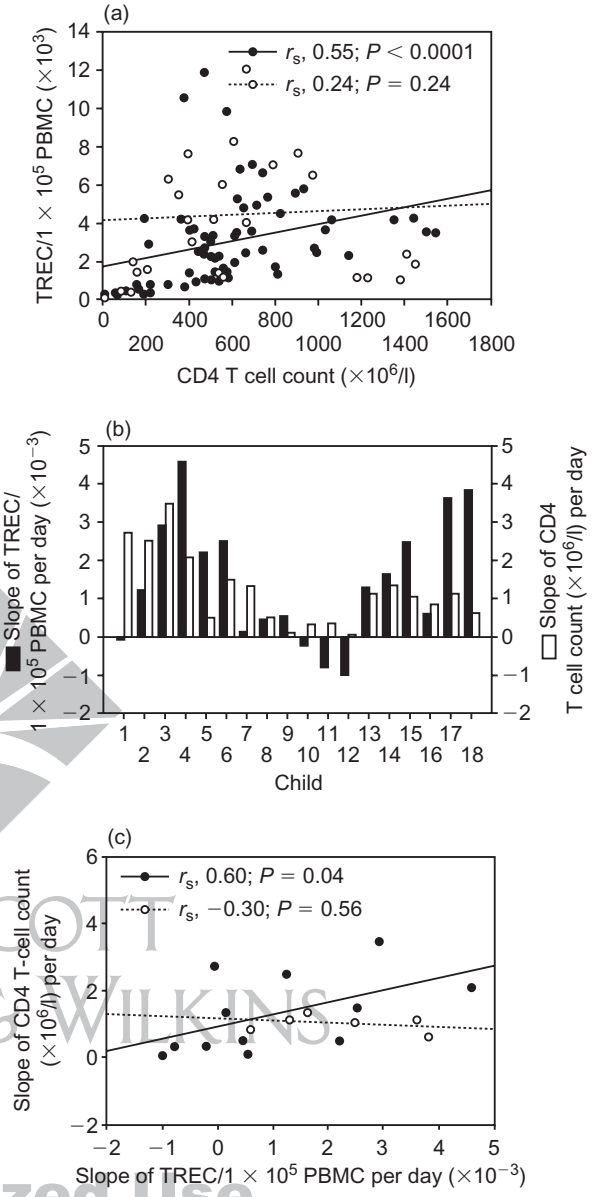

Fig. 4. Relationship between TREC and CD4 T-cell counts in virological responder and non-responder children during the first 12 months of therapy. (a) Relationship between TREC levels and CD4 T-cell counts in virological responder (filled circles) and non-responder (open circles) children. (b) Slopes of CD4 T-cell count (× 10^6/l) per day and TREC/1 × 10^5 PBMC per day for each virological responder (children 1–12) and virological non-responder (children 13–18). (c) Correlation between these two parameters in virological responders (filled circles) and non-responders (open circles). r_s, Spearman's correlation coefficient.

As the viruses that persisted in the non-virological responders are of R5 type (unpublished observations), and as memory CD45RO cells express higher levels of CCR5 molecules (the coreceptor of R5 isolates) [37] than naive CD45RA cells [38,39], a selective loss of these cells can be assumed. Immunophenotyping for CD4CD45RA and CD4CD45RO cells was available at baseline and at different time points during therapy in 10 children (six virological responders and four virological non-responders). CD4 T-cell counts positively correlated with both CD4 CD45RA and CD4 CD45RO cells in virological responders (r_s, 0.93; $P < 0.0001$ and r_s, 0.35; $P = 0.06$, respectively) and non-responders (r_s, 0.88; $P \leq 0.0001$ and r_s, 0.84; $P < 0.0001$, respectively). Furthermore, while TREC values did not correlate with CD4 CD45RO neither in virological responders nor in non-responders (r_s, -0.26; $P = 0.17$ and r_s, -0.04; $P = 0.85$, respectively), a correlation was found between TREC levels and CD4 CD45RA in virological responders, but not in non-responders (r_s, 0.4; $P = 0.03$, and r_s, -0.15; $P = 0.52$, respectively). These findings, albeit in a small number of cases, did not support a selective depletion of CD4 CD45RO cells, and suggested that a peripheral depletion of both CD4 CD45RA and CD45RO cells occurred. Taken together, these findings not only support that peripheral repopulation by recent emigrant thymic cells is a key event in the immune reconstitution after therapy, but also suggest that there is a persistent loss of peripheral CD4 T cells in virological non-responders. The observed increase in CD4 T cells, despite the lack of viral suppression may have been sustained by a high and persistent thymic output that overcame continuous CD4 T-cell depletion at the periphery.

Viral fitness of primary isolates in virological non-responders

During therapy, the emergence of viral variants with mutations that confer resistance to drugs is a well-known phenomenon. It was observed that specific mutations associated with resistance to protease inhibitors can significantly reduce the catalytic activity of the viral protease, and consequently affect the replicative capacity of the virus [35,36,40]. A reduced replicative capacity and, in turn a reduced killing activity, of the drug-resistant viral variants was documented in association with the persistent improvement in CD4 T cells, despite the rebound of plasma HIV-1 RNA levels [30,41,42]. To explore the possibility that the emergence of viral variants with a reduced replicative capacity might have contributed to the CD4 T-cell count increase observed in virological non-responders, viral isolates obtained from plasma at baseline and at 12 months of therapy were sequenced in the protease region, and studied for their replicative capacity. As shown in Fig. 5, primary mutations in the protease region were identified in four cases: in three of them

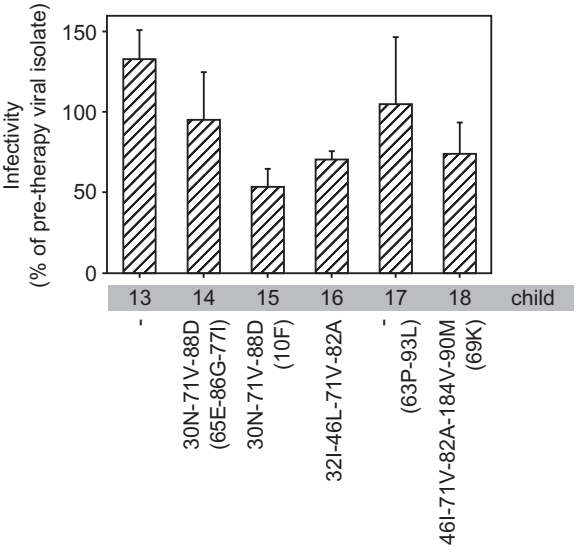

Fig. 5. Infectivity of viruses in virological non-responders at 12 months of therapy. Infectivity of recombinant viruses carrying pre-therapy and post-therapy HIV-1 protease sequences was tested on P4 cells, and the titre of post-therapy viruses was expressed as a percentage of the titre of pre-therapy viruses. The results presented are the mean (histograms), and the mean and SD (vertical bars) of three separate titration experiments. The primary and secondary (in parenthesis) mutations in the HIV-1 protease sequences are given below the patient identification number.

(15, 16 and 18, Fig. 5) the mutated viruses showed reduced infectivity compared with that of corresponding wild-type viruses at baseline. No correlation was found between rates of TREC increase, change in viral replicative capacity, and CD4 T-cell count increase, but it is noteworthy that child 16 had the lowest value of TREC increase (Fig. 4b), but an increase in CD4 T-cell count comparable to that of children with higher values of TREC increase (i.e., child 17, Fig. 4b); it is conceivable that reduced killing of CD4 T cells at the periphery might complement the thymic output in sustaining the CD4 T-cell count increase.

Discussion

Immune reconstitution in HIV-1-infected subjects following antiretroviral therapy may result from *de novo* generation of T cells from the thymus, peripheral T-cell redistribution and/or expansion, and decreased T-cell destruction. Thymus function is inversely correlated with age; so, while its impairment might lead to a more aggressive disease outcome in children than in adults, its restoration could influence the immune reconstitution process much more in children than in adults. In this study, we used TREC measurements as a

tool to investigate the role of thymic output in children showing an immunological response to therapy with and without a substantial suppression of HIV-1 (i.e., virological responders, and virological non-responders, respectively).

At baseline, TREC levels correlated with the number of CD4 T cells; this finding, in agreement with a recent study performed in children [22], but not with previous observations in adults [21], might support the greater contribution of the thymus to the peripheral T-cell population in children than in adults. The increases in TREC levels during the first 12 months of therapy were inversely correlated with both baseline TREC and CD4 T-cell counts, and no increase in TREC was observed in children whose baseline TREC values were comparable to those of normal age-matched children; thus, rather than an overall increase in thymic output, a restoration of thymic function seems to occur during therapy. As the thymus is known to express high levels of CXCR4 [43,44], the major entry coreceptor for syncytium-inducing X4 HIV-1 strains [37], and given that X4 viruses have a higher cytopathic effect than R5 isolates [45,46], it has been argued that individuals with lower TREC levels might harbour X4 isolates; viral suppression and a restriction of coreceptor usage after therapy [47] might greatly reduce thymic cell destruction, thus leading to a more pronounced recovery of thymic output. However, this hypothesis was not confirmed by our finding of an R5 isolate in most of the children studied (unpublished observation), nor by the results of Zhang *et al.* [21] showing that many individuals with low TREC levels had no detectable X4 viruses in their blood. In addition, this hypothesis was refuted by the evidence that an increase in thymic output occurred in patients who showed an increase in CD4 T-cell counts despite the persistence of high levels of HIV-1 viraemia.

Such a dissociated response to therapy is observed in a significant proportion of adults and children treated with HAART [24–28]. Our finding of a significant increase in TREC levels in children who showed a CD4 T-cell count increase without a substantial decline in HIV-1 plasma viraemia (i.e., virological non-responders) is in agreement with recent data. A gain in TREC was reported by Chavan *et al.* [22] in children with a similar dissociated response to therapy. Furthermore, Lecossier *et al.* [29] found an increase in thymic output, assessed by measurement of TREC, in HAART-treated adults who showed an improvement in CD4 T-cell counts despite virological failure. It has been advanced that the rebound in TREC levels after antiretroviral therapy, rather than a recovery of thymic function, might reflect a decrease in the cell division index due to HIV-1 suppression [48]. This suggestion does not explain the changes in TREC that we and others observed in patients who showed an increased CD4 T-cell count without virological suppression; therefore, while the increase in TREC levels in virological responders might reflect both an increase in thymic output and a decrease in T-cell activation, in virological non-responders it could be due mainly, if not only, to the increase in thymic output, thus supporting a role for the thymus in immune reconstitution.

The comparison between children with concordant (both immunological and virological) and dissociated (only immunological) responses to therapy provided some new and interesting insights. It emerged that the TREC increase correlated with the CD4 T-cell count increase in virological responders, but not in non-responders. Furthermore, the ratios between TREC and CD4 T-cell counts tended to be higher in virological non-responders than in responders, thus indicating that for a similar increase in CD4 T-cell count a higher thymic output was required in the former. This might imply that a greater peripheral cell loss persists in children with a dissociated response to therapy, compared with virological responder children. The notion of a selective loss of CD4 T cells with a memory phenotype is conceivable in view of the persistence of R5 type viruses in virological non-responders, and the high levels of CCR5 molecules on CD4 CD45RO cells [37], but was not supported by our findings; instead, the lack of correlation between TREC levels and CD4 CD45RA cells observed in this group of children suggested that a depletion of these cells also occurred at the periphery.

It was demonstrated that viruses carrying specific mutations that confer resistance to protease inhibitors have an impaired replicative capacity [35,36,40], and a reduced viral fitness has been reported in association with a gain in CD4 T cells [41,42]. We found protease-resistant isolates with a reduced viral infectivity in three out of six virological non-responders. It is conceivable that a reduced killing of peripheral CD4 T cells might complement the thymic output in sustaining the CD4 T-cell count increase. Furthermore, it was recently reported that protease-resistant viruses isolated from the peripheral blood compartment may have a greater impairment in their replicative capacity in human thymocytes than in PBMC [30]; this may explain the recovery of thymic function that took place in these patients despite the persistent, albeit reduced, HIV-1 replication and CD4 T-cell depletion at the periphery. Nonetheless, we found an increase in thymic output even in the absence of detectable drug-resistant viruses in the peripheral blood compartment. The thymus may harbour HIV-1 variants different from those present in the peripheral blood compartment [49]; that antiretroviral therapy might have a different impact on thymic and peripheral blood quasispecies viral variants is an attractive hypothesis.

In conclusion, our study indicates that thymic output is a key event in the immune reconstitution process occurring in children on antiretroviral therapy, and suggests that an increase in CD4 T-cell count in spite of the lack of HIV-1 suppression is sustained by a continuous thymic output which helps to overcome the persistent peripheral CD4 T-cell loss that, in turn, might be slowed down by the emergence of viruses with reduced replicative capacity. Further studies are needed to investigate the mechanisms that underlie the dichotomy between the restoration of thymic function, and the persistence of deleterious HIV-1 effects at the periphery.

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