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# Supranormal thymic output up to 2 decades after HIV-1 infection

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**Objectives:** AIDS is caused by CD4<sup>+</sup> T-cell depletion. Although combination anti-retroviral therapy can restore blood T-cell numbers, the clonal diversity of the reconstituting cells, critical for immunocompetence, is not well defined.

**Methods:** We performed an extensive analysis of parameters of thymic function in perinatally HIV-1-infected ( $n = 39$ ) and control ( $n = 28$ ) participants ranging from 13 to 23 years of age. CD4<sup>+</sup> T cells including naive (CD27<sup>+</sup> CD45RA<sup>+</sup>) and recent thymic emigrant (RTE) (CD31<sup>+</sup>/CD45RA<sup>+</sup>) cells, were quantified by flow cytometry. Deep sequencing was used to examine T-cell receptor (TCR) sequence diversity in sorted RTE CD4<sup>+</sup> T cells.

**Results:** Infected participants had reduced CD4<sup>+</sup> T-cell levels with predominant depletion of the memory subset and preservation of naive cells. RTE CD4<sup>+</sup> T-cell levels were normal in most infected individuals, and enhanced thymopoiesis was indicated by higher proportions of CD4<sup>+</sup> T cells containing TCR recombination excision circles. Memory CD4<sup>+</sup> T-cell depletion was highly associated with CD8<sup>+</sup> T-cell activation in HIV-1-infected persons and plasma interleukin-7 levels were correlated with naive CD4<sup>+</sup> T cells, suggesting activation-driven loss and compensatory enhancement of thymopoiesis. Deep sequencing of CD4<sup>+</sup> T-cell receptor sequences in well compensated infected persons demonstrated supranormal diversity, providing additional evidence of enhanced thymic output.

**Conclusion:** Despite up to two decades of infection, many individuals have remarkable thymic reserve to compensate for ongoing CD4<sup>+</sup> T-cell loss, although there is ongoing viral replication and immune activation despite combination antiretroviral therapy. The longer term sustainability of this physiology remains to be determined.

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**Keywords:** adolescents, CD4<sup>+</sup> T cells, immune reconstitution, perinatal HIV infection, recent thymic emigrant, T-cell receptor, thymopoiesis, T-cell receptor recombination excision circles

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## Introduction

The hallmark of HIV-1-induced immunosuppression leading to acquired immunodeficiency syndrome is CD4<sup>+</sup> T-cell depletion, which may be caused by direct cytopathic effects of infection, immune clearance of infected cells, persistent immune activation, and likely other factors [1]. In particular, immune activation is highly associated with the ongoing loss of CD4<sup>+</sup> T cells and believed to be the cause of increased T-cell turnover during chronic infection. The precise mechanisms for this inappropriate inflammatory state are unclear, but ongoing viral replication can be a major contributor even in persons with undetectable viremia [1–4].

Peripheral blood CD4<sup>+</sup> T-cell concentration is a widely used clinical predictor of the immunological status of an infected individual, with a level of less than 200/ $\mu$ l generally considered to reflect sharply increased risk for opportunistic infections that define AIDS [1]. However, this simple quantitative assessment does not precisely reflect immunocompetence. For example, recurrent bacterial pneumonias, malignancies, and AIDS-defining illnesses such as active cytomegalovirus infection and *Pneumocystis pneumonia* may occur at higher CD4<sup>+</sup> T-cell levels in children, adolescents, and adults [5–7]. It is very likely that the clonal diversity of the CD4<sup>+</sup> T-cell population and therefore breadth of pathogen recognition is also important [8].

Effective antiretroviral therapy (ART) suppresses HIV-1 replication, reduces immune activation, and increases peripheral blood CD4<sup>+</sup> T-cell concentrations [9,10]. However, the extent to which normalization of clonal T-cell diversity occurs is less well documented. In HIV-1-infected adults, the rise in CD4<sup>+</sup> T-cell levels seen after institution of ART is characterized by an initial rapid rise that is likely because of redistribution of total body memory CD4<sup>+</sup> T cells, followed by a slower and more prolonged increase in naive CD4<sup>+</sup> T cells [9,11]. By contrast, HIV-1-infected children demonstrate an early and sustained increase in naive CD4<sup>+</sup> T cells [12–16] that likely reflects greater baseline thymic function than adults, who tend to have age-related involution of thymic epithelial tissue and attrition of thymic function [17].

Supporting this concept, we previously demonstrated that adolescents and young adult survivors of perinatal HIV-1 infection on ART have markers of thymopoiesis that are comparable to uninfected age-matched controls, including concentrations of peripheral blood naive CD4<sup>+</sup> T cells and T-cell receptor recombination excision circles (TRECs) that reflect recent thymic emigrants [18]. Others have demonstrated that T-cell receptor CDR3 distribution perturbations are rapidly reduced in some children and adolescents during ART [19] suggesting that some degree of normalization of the TCR repertoire is possible. However, these measurements have not

excluded qualitative abnormalities in thymopoiesis that might result from the known impact of HIV-1 on the architecture of both the thymus and secondary lymphoid tissues [13–15,20–22].

Thus, it is unclear if CD4<sup>+</sup> T-cell clonal diversity is maintained in conjunction with recovered total CD4<sup>+</sup> T-cell numbers on ART, particularly in individuals who were infected before immunologic maturity. To address this uncertainty, we assess immune reactivity to HIV-1, thymopoiesis, and CD4<sup>+</sup> T-cell diversity in a cohort of long-term survivors of perinatal HIV-1 infection. These data address key questions as to whether infection early in life (during immunologic development), in conjunction with chronic infection (spanning more than 13 years), limit CD4<sup>+</sup> T-cell reconstitution.

## Methods

### Study approval

Healthy control and HIV-1-infected study volunteers were enrolled under protocols approved by institutional review boards of the University of California Los Angeles and Children's Hospital Los Angeles. Written informed consent was received from all participants prior to inclusion in the study.

### Cohort and preparation of peripheral blood mononuclear cells

All study participants were enrolled from 2003 to 2006. Individuals with known hepatitis B or C infections were excluded. Twenty control participants and 20 HIV-1-infected participants were described in previous reports [18,23]. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density centrifugation gradient, washed twice with phosphate-buffered saline, and viably cryopreserved. Fresh umbilical cord blood was obtained from the UCLA CFAR Virology Core. For quantitative spectratyping and pyrosequencing studies, CD3<sup>+</sup>CD4<sup>+</sup>CD31<sup>+</sup>CD45RA<sup>+</sup> T-cells were purified from cryopreserved PBMC by fluorescence-activated cell sorting (FACSaria II using FACSDiva Version 6.1; Becton Dickinson, Franklin Lakes, New Jersey, USA). CD4<sup>+</sup> T cells from cord blood samples were isolated by negative selection (human CD4<sup>+</sup> T-cell enrichment mixture, RosetteSep, StemCell Technologies, Vancouver, Canada).

### Clinical laboratory tests

Complete blood counts and plasma HIV-1 RNA measurements were obtained through the Children's Hospital Los Angeles and UCLA clinical laboratories.

### Volumetric tomography of thymic tissue

Noncontrast helical computed tomography (CT) studies of the chest were performed with 3-mm collimation

extending from the thoracic inlet to the lung bases, using previously described methods [18]. All female participants had negative pregnancy tests confirmed prior to imaging. Volumetric CT scans were discontinued after 49 scans (29 HIV-1-infected and 20 uninfected controls) because an interim analysis indicated futility to detect statistically significant differences with the initially planned sample size.

### T-cell immunophenotyping by flow cytometry

Whole blood T-cell staining and flow cytometry was performed as described previously, with naive CD4<sup>+</sup> T cells defined as CD4<sup>+</sup>CD45RA<sup>+</sup>CD27<sup>+</sup> [18]. CD45RA<sup>-</sup>CD4<sup>+</sup> T cells were defined as memory cells (combined central and effector subsets). Staining was also performed to quantify the CD45RA<sup>+</sup>CD31<sup>+</sup> subset of CD4<sup>+</sup> T cells (recent thymic emigrants) [24] and the CD38<sup>+</sup>HLA-DR<sup>+</sup> subset of CD8<sup>+</sup> T cells (activated) [25]. Quantitation of naive, recent thymic emigrants, and memory CD4<sup>+</sup> T cells was not performed in one control participant and the percentage of CD38<sup>+</sup>HLA-DR<sup>+</sup> CD8<sup>+</sup> T cells was not determined for two other control participants. Because of the absence of a complete blood count, the concentrations (cells/ $\mu$ l) of T-cell subsets of one HIV-1-infected individual are absent from panels of Figs. 1 and 2.

### Detection of HIV-1-specific CD8<sup>+</sup> T-cell responses against HIV-1 by interferon- $\gamma$ ELISpot analysis

Peripheral blood HIV-1 specific CD8<sup>+</sup> T-cell responses in HIV-1-infected individuals with plasma HIV-1 levels of 400 RNA copies/ml or less at study entry were quantified by interferon (IFN)- $\gamma$  ELISpot analysis, as previously described [26]. In brief, purified CD8<sup>+</sup> T cells were screened against 53 pools of overlapping peptides spanning the total HIV-1 clade B consensus sequence proteome (NIH AIDS Reference and Reagent Repository) to determine the frequency of spot-forming cells (SFC) per added CD8<sup>+</sup> T cells. The frequency of HIV-1-specific SFC per volume of peripheral blood was calculated by multiplying the frequency of SFC in CD8<sup>+</sup> T cells and the number of CD8<sup>+</sup> T cells per volume of blood.

### Peripheral blood T-cell receptor recombination excision circles analyses

Cellular DNA was prepared from PBMCs and signal joint TRECs were quantified by real-time PCR as previously described [27–29], and reported as TREC/million cells. TREC were measured using isolated CD4<sup>+</sup> T cells (Rosette-Sep beads, StemCell Technologies) for most participants. The number of TREC<sup>+</sup> CD4<sup>+</sup> T cells per volume of peripheral blood was calculated by multiplying the frequency of TREC in isolated CD4<sup>+</sup> T cells and the concentration of CD4<sup>+</sup> T cells per volume of blood.

### Human leukocyte antigen and CCR5 genetic analyses

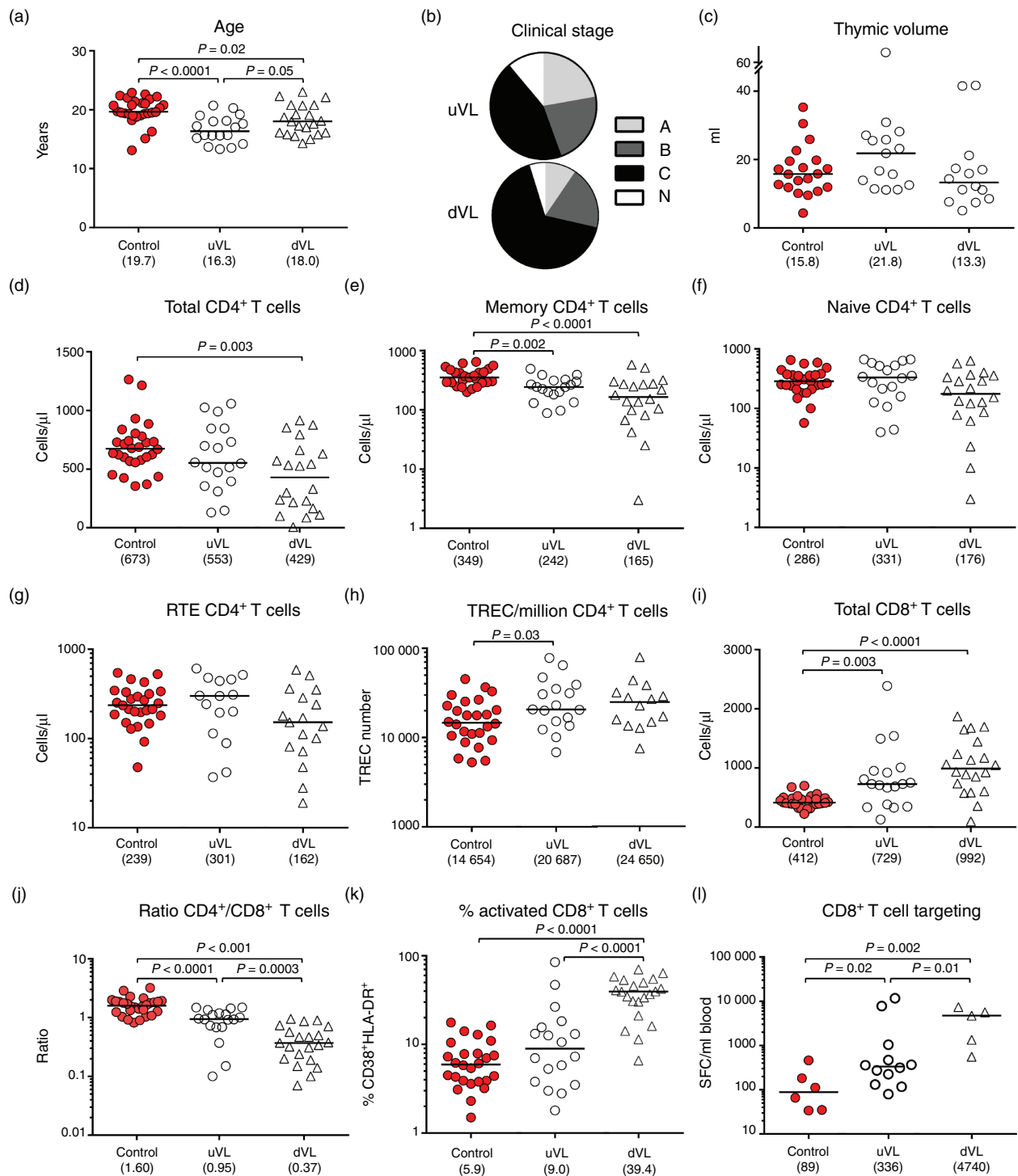
Using PBMC DNA, human leukocyte antigen (HLA) typing was performed by the clinical laboratory at the UCLA Immunogenetics Center, and PCR was used to determine if the  $\Delta$ 32 deletion was present at the CCR5 locus using oligonucleotide primers described by others [30].

### Quantitation of T-cell receptor beta variable family RNA transcripts

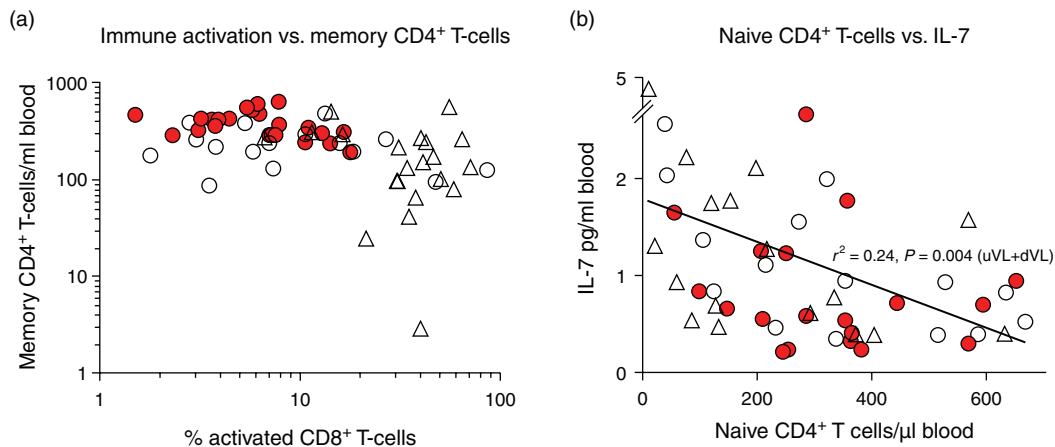
From 3 to 15 million cryopreserved PBMC from HIV-1-infected or control participants were stained and sorted to purify CD31<sup>+</sup>CD45RA<sup>+</sup>CD4<sup>+</sup> T cells, yielding 230 000–700 000 cells per individual. RNA was isolated from purified lymphocytes (RNeasy MiniKit; Qiagen, Valencia, California, USA), and reverse-transcribed to cDNA using random primers (High Capacity Reverse Transcription Kit; Applied Biosystems, Carlsbad, California, USA). Quantitative spectratyping was used to examine beta variable (BV) family usage as described previously [31]. In brief, RT-PCR was employed to determine the relative concentration of each BV gene family [IMGT nomenclature (<http://www.imgt.org>)], and capillary electrophoretic size resolution of each family yielded a profile of TCR sequence size distribution within each family.

### Deep sequencing of T-cell receptor coding sequences

The cDNA (6.5–15  $\mu$ g) generated for spectratyping was PCR-amplified (Phusion High-Fidelity DNA Polymerase; New England BioLabs, Ipswich, Massachusetts, USA) for 35 cycles under the following conditions: initial denaturation 98°C (30 s), denaturation 98°C (10 s), annealing 62°C (30 s), extension 72°C (15 sec), and final extension (5 min). The PCR products were then purified (PureLink PCR Purification Kit; Invitrogen, Waltham, Massachusetts, USA) and further amplified using nested PCR (Phusion High-Fidelity DNA Polymerase) for 35 cycles under the following conditions: initial denaturation 98°C (30 s), denaturation 98°C (10 s), annealing 62°C (30 s), extension 72°C (15 s), and final extension (5 min). These PCR products were then separated in 2% agarose gels and cDNA from the appropriate bands was purified (QIA Gel Extraction Kit; Qiagen, Valencia, California, USA). Pyrosequencing of the nested PCR purified products using 454 FLX Titanium chemistry was performed according to the manufacturer's protocols (Roche Applied Science). The primers used for the three BV families were the same as those used in quantitative spectratyping analysis [31], but additionally tagged with multiplex identifier and primer key sequences (Supplemental Table S1, <http://links.lww.com/QAD/A863>). To check that diversity within the samples was retained during PCR amplification with the modified primers, the following control experiments were performed. First, three rounds of PCR



**Fig. 1. Clinical and immune parameters of study participants.** The HIV-1-infected participants included 39 persons, of whom 18 had plasma viremia less than 50 HIV-1 RNA copies/ml (uVL group, 11 male and 7 female) and 21 had plasma viremia 50 HIV-1 RNA copies/ml or more (dVL group, 11 male and 10 female), who were compared to a control group of healthy uninfected persons (10 male and 18 female) of similar ages (a). Most of the infected persons had had symptomatic disease in the past (b). Evaluated parameters included thymic volume (c), concentrations of blood CD4<sup>+</sup> T cells (d) and their characteristics (e–h), blood CD8<sup>+</sup> T-cell concentrations (i) and the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells (j), and CD8<sup>+</sup> T-cell activation (k) and HIV-1 targeting (l). Filled circles represent uninfected control participants; unfilled circles, represent undetectable viral load (uVL) participants with plasma HIV-1 RNA less than 50 copies/ml; open triangles represent detectable viral load (dVL) individuals with plasma HIV-1 RNA 50 to less than 400 copies/ml. Statistically significant results ( $P < 0.05$ ), as determined by Mann–Whitney U tests, are indicated. Bars indicate median values.



**Fig. 2. Relationship of immune activation to memory CD4<sup>+</sup> T-cell loss, and resulting homeostatic proliferation of CD4<sup>+</sup> T cells.** (a) Systemic immune activation, as reflected by CD8<sup>+</sup> T-cell coexpression of activation markers, is plotted against blood levels of memory CD4<sup>+</sup> T cells (CD45RA<sup>-</sup>, including central and effector memory subsets). (b) The relationship of blood levels of naive CD4<sup>+</sup> T cells to plasma levels of the homeostatic cytokine interleukin-7 is plotted. Linear regression line and its associated *P* value is indicated for relationship between naive T-cells and plasma interleukin-7 concentrations of HIV-infected participants. In both panels, filled circles represent uninfected control participants, unfilled circles represent uVL participants, and open triangles represent dVL individuals.

amplification were performed on an aliquot of an umbilical cord blood DNA sample, and spectratyping was performed after each round, showing that the TCR genes of the third round of amplification remained Gaussian in size distribution (Supplemental Fig. S1, <http://links.lww.com/QAD/A863>). Second, the PCR products from the third round of amplification were cloned and sequenced, showing polyclonality of TCRs in all cases (Supplemental Table S2, <http://links.lww.com/QAD/A863>), thus demonstrating no evidence of biased amplification.

### Statistical analyses

Clinical parameters analyzed as continuous variables were compared using two-tailed Mann–Whitney *U* test (except for the comparison of HIV ELISpot responses). Categorical variables were compared using Fisher's exact test. Pyrosequencing (454, Roche) of TCRs in nine samples of sorted CD31<sup>+</sup>CD45RA<sup>+</sup>CD4<sup>+</sup> T cells [from three HIV-1-infected participants receiving ART with suppressed viremia (uVL), three uninfected controls, and three umbilical cord blood (CB) Supplemental Table S3, <http://links.lww.com/QAD/A863>] yielded between 32 000 and 198 000 TCR sequences per sample. To compare the diversity of TCR sequences in these individuals, we examined TCR sequences in three specific BV families: BV03, BV19, and BV29 (IMGT nomenclature). These families were selected because they represented about 5% of total BV families in the CD31<sup>+</sup>CD4<sup>+</sup> T cells in HIV-infected individuals, control study participants, and cord blood specimens that were selected. Two samples with lower yields (control BN02 and infected participants CB13 with

33 000 and 32 000 sequences, respectively) were excluded from analyses that are especially sensitive to sample size.

Pyrosequencing is typically associated with significant sequencing errors [32,33], but this will alter comparisons of diversity estimates if the error statistics do not differ across samples. Differences in the diversity of TCR coding sequences were evaluated by methods commonly employed in ecologic studies including Shannon index of diversity, sample size-corrected Shannon index, rarefaction curves, and analysis of the fraction of singleton species (that occur only once in the sample). We also used a histogram shape estimation technique using an 'unseen estimator,' which uses the observed distribution of species in a sample to estimate the total number of unique species missed in sampling, as well as the full species distribution [34].

As an additional control, sequences were also clustered with two different algorithms, as described by others [35]. Finally, the clusters were translated into stop codon-free amino acid sequences with verified BV and J $\beta$  flanking regions.

## Results

### Cohort characteristics

The study participants included 39 persons who were infected with HIV-1 as infants (22 male and 17 female) and an uninfected control group of 28 individuals (10 male and 18 female), ranging from 13.3 to 23.0 and 13.1 to 22.9 years of age respectively at the time of study (Fig. 1a). Most

infections (85%) were from mother to child transmission (including one by breastfeeding from a mother who acquired infection postpartum by blood transfusion), and the remainder (15%) were from blood transfusions in 1982 and 1983, including twin brothers who were described extensively in an earlier report [23]. Among the infected individuals, none had the CCR5  $\Delta$ 32 mutation; one and three respectively had HLA-B\*27 and HLA-B\*57 genotypes associated with slower disease progression [36], and one and none respectively had HLA-B\*3502 and HLA-B\*3503 associated with accelerated disease progression. All infected participants were receiving combination ART at the time of study; 18 had plasma viremia less than 50 HIV-1 RNA copies/ml (uVL group, 11 male and 7 female) and 21 had plasma viremia 50 HIV-1 RNA copies/ml or more (dVL group, 11 male and 10 female). Most (77%) of the HIV-1-infected individuals had clinical or laboratory evidence of immunodeficiency (CDC class B or C) in the past (Fig. 1b), although imaging revealed relatively normal thymic size overall (Fig. 1c). Thus, the infected individuals represented a group of long-term survivors of whom most had sustained clinically significant immunodeficiency because of HIV-1 infection at some point.

### Many long-term survivors of perinatal HIV-1 infection have relatively normal total and naive CD4<sup>+</sup> T-cell concentrations on ART, despite generally depressed levels of memory CD4<sup>+</sup> T cells

At enrolment, peripheral blood CD4<sup>+</sup> T-cell levels were lower overall in the HIV-1-infected persons versus uninfected controls (mean 514 versus 686 cells/ $\mu$ l blood, respectively), although the uVL group had levels similar to the controls (mean 601 versus 686 CD4<sup>+</sup> T cells/ $\mu$ l, respectively, Fig. 1d). Examining the CD4<sup>+</sup> T-cell population phenotypically, both HIV-1-infected groups exhibited significant depletion of the memory (CD45RA<sup>-</sup>) subset (Fig. 1e). By contrast, the naive (CD45RA<sup>+</sup>/CD27<sup>+</sup>) subset was relatively normal to elevated in the uVL group and slightly reduced in the dVL group (neither statistically significantly different; Fig. 1f). More detailed analysis of the naive CD4<sup>+</sup> T-cell population suggested overall normal levels of recent thymic emigrants (CD45RA<sup>+</sup>/CD31<sup>+</sup>) [24] in the uVL group and normal to reduced levels in the dVL group compared to controls (no statistically significant differences, Fig. 1g). Furthermore, the frequency of the total CD4<sup>+</sup> T-cell population with TRECs was elevated in both uVL and dVL groups compared to controls (statistically significantly only for the uVL group; Fig. 1h), suggesting higher percentages of cells produced in the thymus (versus peripheral homeostatic proliferation). As a whole, these data demonstrate that these long-term survivors of perinatal HIV-1 infection had depleted levels of memory CD4<sup>+</sup> T cells, but generally exhibited quantitative restoration of naive T-cell populations via increased thymic output on ART.

### Despite suppression of viremia by treatment, perinatally infected individuals have evidence of ongoing HIV-1-driven immune activation

Compared to controls, both groups of HIV-1-infected participants had significantly elevated blood CD8<sup>+</sup> T-cell levels (Fig. 1i). Examining the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells, it was apparent that the relative increase of CD8<sup>+</sup> T cells and decrease of CD4<sup>+</sup> T cells was especially marked in the dVL group (Fig. 1j), suggesting an association between abnormality in the CD8<sup>+</sup> and CD4<sup>+</sup> T-cell compartments. Additionally, CD8<sup>+</sup> T-cell activation (CD38<sup>+</sup>/HLA-DR<sup>+</sup>) was increased in both infected groups versus the control group, significantly greater in the dVL versus uVL group (Fig. 1k). Finally, screening of participants with <400 HIV-1 RNA copies/ml plasma (12 from the uVL group and five from the dVL group) for CD8<sup>+</sup> T-cell responses against the whole HIV-1 proteome [26] (Fig. 1l) revealed persisting responses (predominately targeting Gag and Nef proteins similarly to infected older adults [37]) (not shown) in most persons despite undetectable or low viremia (between 50 and 400 copies/ml). Lower blood levels of memory CD4<sup>+</sup> T cells were seen in infected individuals with higher levels of CD8<sup>+</sup> T-cell activation (Fig. 2a), and there was a significant inverse correlation between the number of naive CD4<sup>+</sup> T cells and the plasma concentration of interleukin-7 in the HIV-1-infected group (Fig. 2b); no such correlation was seen in the uninfected control group. These results suggest persistent generalized immune activation was present and was associated with ongoing loss of memory CD4<sup>+</sup> T cells and secondary enhanced homeostatic proliferation of naive CD4<sup>+</sup> T cells in addition to the enhanced thymic output suggested by the aforementioned data.

### The long-term survivors of perinatally HIV-1 infection exhibit increased CD4<sup>+</sup> T-cell receptor diversity and breadth

To evaluate thymopoiesis more qualitatively, we examined the TCR repertoire of the CD31<sup>+</sup> subset of CD4<sup>+</sup> T cells, which represent thymic emigrants and their early progeny because CD31 is lost after a few cycles of homeostatic proliferation. Quantitative spectratyping analysis [23,38] revealed Gaussian distributions of TCR size populations for three control umbilical cord blood samples, as expected for unperturbed native populations [31] (Supplemental Figure S2, Panel A, <http://links.lww.com/QAD/A863>). TCR repertoires of control participants and uVL individuals also showed generally Gaussian distributions (data not shown), suggesting grossly diverse TCR production.

To better define the diversity of TCR production, we performed deep sequencing analysis of TCR families BV03, BV19 and BV29 (IMGT nomenclature), selected for having relatively consistent representation of ~5% of total BV families in CD31<sup>+</sup>CD4<sup>+</sup> T cells from representative uVL and control study participants and

three CB specimens (Supplemental Table 3, Supplemental Table 4 and Supplemental Figure S2, Panel B, <http://links.lww.com/QAD/A863>). The Shannon entropy index (S) for raw sequences (initially excluding BN02 (an uninfected control)) and cord blood CB13, which had insufficient sampling), ranged from 14.3 to 16.3 for CB, 11.3 to 14.1 for controls, and 13.6 to 14.1 uVL. The Shannon index of the estimated histogram from filtered sequences ranged from 16.8 to 17.2 for CB, 13.2 to 13.5 for uninfected controls, and 13.8 to 14.1 for uVL (Table 1), indicating that TCR diversity was greatest in CB and least in controls ( $P < 0.01$ ). The two samples initially excluded (control BN02 and cord blood CB13) were also consistent with this pattern (Table 1).

As sample sizes could have biased estimates of Shannon index even after simple corrections, we also analyzed the TCR repertoire using rarefaction curves plotting the number of unique species found in random subsamples of the total sequence population of all three BV families (Fig. 3), which revealed the same relative pattern of TCR diversity being highest in CB, intermediate in uVL participants, and least in control participants ( $P = 0.018$ ). These differences also held true for BV families considered individually ( $P = 0.041$ ; Supplemental Figure S3, <http://links.lww.com/QAD/A863>).

As a third approach to confirming the diversity comparisons between groups, we also performed analyses using random subsamples 24 000 sequences (corresponding to the smallest sampling size, obtained for CB13) from each individual sequence set. The number of discrete sequences, Shannon index, and fraction of singletons (number of sequences observed only once divided by the total number of observed sequences) were assessed (Supplemental Table 5, <http://links.lww.com/QAD/A863>). Again, all parameters revealed the pattern of highest TCR diversity in CB followed by uVL, both greater than uninfected control persons ( $P < 0.01$ ). The HIV-1-infected individuals had overall more species and a higher fraction of singleton sequences. Additional analyses examining the abundance of rare and common sequences (Supplemental Figures S4 and S5, <http://links.lww.com/QAD/A863>) corroborated these results, supporting the overall conclusion that the recent thymic emigrant CD4<sup>+</sup> T cells of uVL participants had a broader TCR repertoire than uninfected participants.

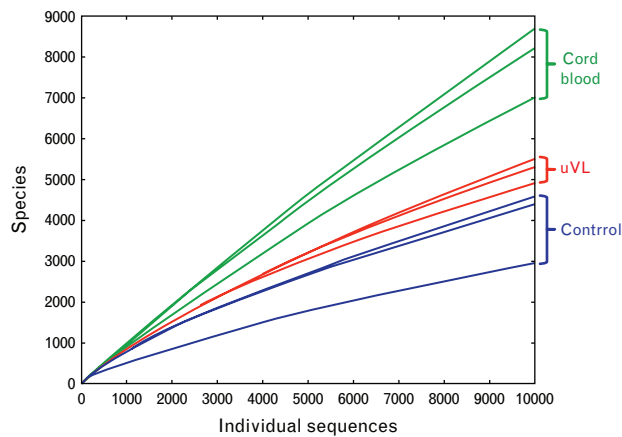
## Discussion

Immune reconstitution following initiation of ART of HIV-1 infection clearly differs between children and adults. In children, expansion of naive T-cell populations begins soon after initiation of ART, whereas redistribution and expansion of memory T-cell populations initially predominate in adults, in whom increases in naive CD4<sup>+</sup>

Table 1. Numbers of TCR sequences obtained by deep sequencing and Shannon Diversity Indexes (parentheses).

	uVL			Uninfected			Cord blood		
	AP04	AP22	CP04	CN13	CN02	BN02	CB12	CB11	CB13
BV03	27 537 (10.79)	32 125 (12.62)	37 942 (11.84)	112 979 (11.71)	54 912 (11.23)	25 396 (9.63)	47 801 (15.06)	82 367 (15.95)	10 987 (14.23)
BV19	25 337 (12.27)	13 015 (12.81)	23 497 (11.69)	72 688 (12.99)	30 868 (11.02)	4 734 (10.46)	10 868 (14.01)	73 392 (15.00)	11 325 (16.86)
BV29	38 031 (14.07)	3 377 (14.23)	24 832 (14.45)	12 789 (14.08)	33 727 (12.92)	2 883 (11.77)	29 607 (16.81)	19 827 (14.66)	9 627 (15.59)
Overall	90 905 (14.11)	48 517 (13.78)	86 271 (14.14)	198 456 (13.50)	119 507 (13.23)	33 013 (10.79)	88 276 (17.15)	175 586 (16.82)	31 939 (16.86)

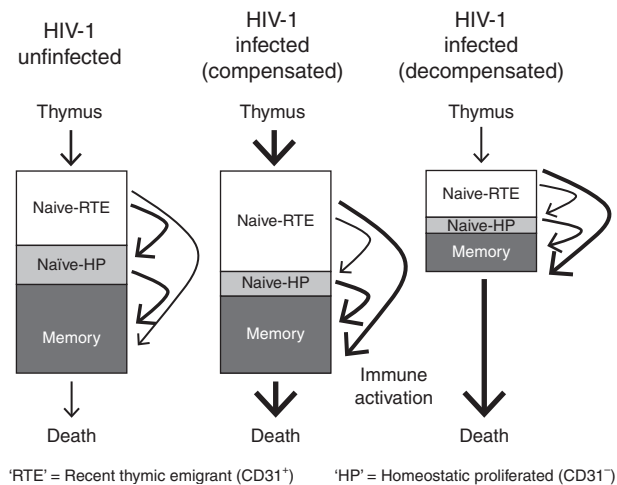




**Fig. 3. Rarefaction analysis of TCR species in CD31<sup>+</sup> naive CD4<sup>+</sup> T cells.** Rarefaction curves are plotted for TCR sequences isolated from each sample, indicating that diversity is greater in the uVL participants compared to uninfected control participants (considering the number of species at  $x = 9809$ ,  $P = 0.02$ ).

T cells are typically seen only months after therapy [9,11–15,39]. To investigate the nature of these differences, we performed an extensive survey of thymic function markers and found evidence of robust thymopoiesis in long-term survivors of perinatal infection (>13 years) receiving ART, compared to healthy controls of similar age. The majority in the HIV-1-infected group had evidence of abundant thymic tissue and active thymopoiesis, with naive CD4<sup>+</sup> T-cell levels and markers (TRECs) suggesting elevated production compared to uninfected persons. This was further supported by deep sequencing of TCRs in the naive CD4<sup>+</sup> T-cell population, which demonstrated not only preserved but also enhanced diversity in these long-term survivors of perinatal infection versus uninfected persons. These findings are consistent with previous studies of immune reconstitution during ART [12–16], and observations that ‘thymic rebound’ (expansion of histologically normal thymic tissue occurring after illness, stress, and cancer chemotherapy) is more common in children than adults, likely underlying age-dependent recovery of lymphocyte populations after cancer chemotherapy [40]. Of note, a recent report demonstrated that restoration of naive T-cell populations may be impaired in adult individuals with advanced HIV-1 infection, possibly because of loss of the normal stromal fibroblastic reticular cell network in lymphoid tissue [41]. Given the long average duration of infection (~17 years) and histories of AIDS-defining illness in more than half of our study participants, this underscores the likely importance of age in HIV-1-induced damage to secondary lymphoid tissues and/or its reversal during ART.

As a whole, the data presented earlier suggest a model in which HIV-1 replication (and/or secondary immune dysregulation) drives loss of memory CD4<sup>+</sup> T cells,



**Fig. 4. Schematic model of CD4<sup>+</sup> T-cell homeostasis in long-term survivors of perinatal HIV-1-infection.** Box depicts the partitioning of CD4<sup>+</sup> T cells into three discrete populations following emigration from the thymus: recent thymic emigrant CD31<sup>+</sup> T cells (Naive-RTE) that have not undergone peripheral expansion, naive T cells that have undergone homeostatic proliferation (CD27<sup>+</sup> CD31<sup>-</sup>), and memory (CD45RA<sup>-</sup>) cells. In the infected persons receiving ART, loss of memory (and possibly naive) CD4<sup>+</sup> T cells is associated with enhanced thymopoiesis (thicker bold lines) and possibly less homeostatic proliferation in the naive subset. The naive T-cell population is retained. In some individuals, this compensatory results in resulting in an increased fraction of Naive RTE cells with relatively increased TREC content, and enrichment of TCR diversity.

leading to compensatory supranormal thymic output of naive CD4<sup>+</sup> T cells (Fig. 4) in these youths. Evidence of ongoing immune activation and replication is provided by the high fraction of CD38<sup>+</sup> HLA-DR<sup>+</sup> CD8<sup>+</sup> T cells (Fig. 1k) [25] and the persistence of CD8<sup>+</sup> T-cell responses to HIV (Fig. 1l) which indicate ongoing HIV antigen production. We note that the persistence of broad antiviral responses to HIV during cART has previously been observed in other perinatally infected persons and contrasts with the situation in older adults, in whom complete decay of these responses is common [37,42–45].

The Naive CD4<sup>+</sup> T-cell population is composed of both CD31<sup>+</sup> and CD31<sup>-</sup> CD45RA<sup>+</sup> cells and homeostatic proliferation is thought to transform the former into the latter, resulting in a decrease in the concentration of TREC in CD4<sup>+</sup> T cells [24,46]. Within the naive CD4<sup>+</sup> T-cell population, the population size of the recent thymic emigrant (CD31<sup>+</sup> CD45RA<sup>+</sup>) CD4<sup>+</sup> T cells (Naive RTE) is maintained in most individuals studied (Fig. 1g), suggesting increased thymic output occurs to replace loss of naive (CD27<sup>+</sup>CD45RA<sup>+</sup>) cells that differentiate into memory CD4<sup>+</sup> T cells. The hypothesis that supranormal thymopoiesis occurs is also supported by

our observation of enrichment of TREC in CD4<sup>+</sup> T-cells of uVL participants (Fig. 1h) and the enhanced diversity of TCRs in the CD31<sup>+</sup> CD4<sup>+</sup> T-cell population observed by pyrosequencing.

TCR breadth is likely an important clinical factor, as severe AIDS-defining illnesses may occur years after blood CD4<sup>+</sup> T-cell concentrations have 'reconstituted' to seemingly safe levels in both children and adults receiving ART [5–7]. Our detailed analysis of TCR repertoire substantiates and extends prior studies of HIV-1-infected adults in whom ART does not generally restore CD4<sup>+</sup> T-cell numbers to normal or fully normalize skewing of the TCR repertoire, as assessed by various tools ranging from relatively indirect to more precise measures of diversity such as CDR3 size distributions ('spectratyping' or 'immunoscoping'), DNA hybridization kinetics ('Amplicot'), multiplex amplification of V-J segments, and CDR3 sequencing [8,23,47–50], and one study in children/adolescents indicating that perturbations in TCR diversity of naive cells begin to resolve within several months of therapy [19]. Because HIV-1 infection is typically associated with disrupted thymic architecture, involution of the thymic cortical epithelial space, and fibrosis of the peripheral lymph nodes that are required for expansion of thymic emigrants [41,51–53], the novel finding of substantially increased TCR breadth in the recent thymic emigrant CD4<sup>+</sup> T-cell compartment of our HIV-1-infected participants was surprising. Supporting this observation, recent trials of administering recombinant human interleukin-7 to infected persons on ART have demonstrated enhanced naive CD4<sup>+</sup> T-cell production accompanied by indirect measures of increased TCR diversity [54].

Our study has several limitations, including its cross-sectional nature and the ~2.3 year average age difference between the groups of HIV-1-infected and control individuals. These concerns are mitigated by evidence that thymic architecture and function change little over this short age span [39,51,55,56]. Moreover, we found no evidence of a correlation between age and the number of CD31<sup>+</sup> T cells in any of the three groups studied (Controls, uVL or dVL individuals; data not shown), consistent with earlier reports indicating that CD31<sup>+</sup> T cells decrease less than 50% between 20 and 60 years of age [24,46]. We also observed stability of thymopoiesis parameters over 1–3 years in our cohort (TREC, number and fraction of blood CD31<sup>+</sup>CD4<sup>+</sup> T cells; manuscript in preparation). Our assessment of TCR repertoire was limited to three BV families representing about 5% of naive CD4<sup>+</sup> T cells, and may not reflect the total functional repertoire, although there is no reason to suspect BV family-specific differences. Despite these limitations, the composite data support the sanguine view that thymic function and naive T-cell homeostasis may be restored by prolonged ART in adolescent and young adult survivors of perinatal infection.

Overall, our study suggests that thymic function is resilient in most persons, even ~17 years after HIV-1 infection that occurred when immunologically immature. Despite prior clinically significant immunosuppression (including AIDS defining illness and conditions indicative of moderate immune deficiency), ART appears to allow recovery of an apparently adequate TCR repertoire in many survivors of perinatal infection who have reached young adulthood, which is encouraging in light of numerous studies showing damaging effects of HIV-1 on the thymus. This appears to differ from persons infected as adults, and it is unclear whether the difference is simply because of better age-related regenerative potential and immunologic reserve, or perhaps a difference in viral persistence or reservoirs specific to infection when immunologically immature. While the data are hopeful that long-term survivors of perinatal infection are well compensated immunologically, there remain questions about whether the supraphysiologic TCR repertoire could in fact reflect an abnormality such as reduced stringency in thymic T-cell negative selection, and whether heightened thymic output will remain sustainable over longer periods of time if persistent HIV-1-driven memory CD4<sup>+</sup> T-cell loss continues. Indeed, there appeared to be some persons in our cohort with low memory and naive CD4<sup>+</sup> T-cell levels. Additional studies will be needed to determine the extent to which normalization of TCR diversity in and other T-cell parameters is indicative of true restoration of normal immune function in the setting of prolonged HIV-1 infection, and to examine the impact of detectable HIV replication and residual HIV-specific immune responses on these processes.

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Authorship Contributions: CRA-S helped design and carried out pyrosequencing studies and manuscript preparation. P.K., M.B., and J.C. were responsible for the organization of the cohorts, participant recruitment

and specimen collection, interactions with institutional review boards, and manuscript preparation. P.K. also supervised all technical aspects of the study, apart from ELISpot and spectratyping. O.Y. collaborated in design and implementation of the pyrosequencing studies and manuscript preparation, supervised spectratyping and ELISpot analysis. P.C. and D.C. performed flow cytometry and spectratyping studies. M.I.B. supervised and interpreted CT scan analysis, blinded to participant status. N.J. and P.L. helped analyze the pyrosequencing data.

### Conflicts of interest

CRA-S owns Roche stock. The other authors have no conflicts of interest.

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