Human immunodeficiency virus type 1 (HIV-1) infection perturbs the T-cell receptor (TCR) Vβ repertoire. The TCR CDR3 length diversity of individual Vβ families was examined within CD45RA and CD45RO CD4 T cells to assess the impact of the virus on clonality throughout CD4 T-cell activation and differentiation. A cross-sectional and longitudinal cohort study of 13 HIV-infected and 8 age-matched healthy children and adolescents examined the Vβ CDR3 length profiles within CD4 T-cell subsets by the use of spectratyping. HIV-infected subjects demonstrated higher numbers of perturbations in CD4 CD45RA T cells (5.8 ± 4.9 Vβ families) than healthy individuals (1.6 ± 1.8 Vβ families) \((P = 0.04)\). Surprisingly, CD4 CD45RO central memory T cells from infected subjects showed no increased perturbations compared to the perturbations for the same cells from healthy subjects (2.9 ± 3.1 and 1.1 ± 1.8 Vβ families, respectively; \(P = 0.11\)). CD4 CD45RA TCR perturbations were higher among infected subjects with >25% CD4 cells than healthy subjects (mean number of perturbed Vβ families, 6.6 ± 5.4; \(P = 0.04\)). No correlations between perturbations in CD4 subsets and pretherapy age or viral load were evident. In contrast to CD8 T cells, HIV induces TCR disruptions within CD45RA but not CD45RO CD4 T cells. Therapy-induced viral suppression resulted in increases in thymic output and the normalization of the diversity of TCR within CD45RA CD4 T cells after 2 months of treatment. Perturbations occur prior to CD4 T-cell attrition and normalize with effective antiretroviral therapy. The impact of HIV on the diversity of TCR within naive, central memory, and effector memory CD4 T cells is distinctly different from that in CD8 T cells.

Human immunodeficiency virus type 1 (HIV-1) infection alters T-cell homeostasis by both impairing thymic output and inducing chronic T-cell activation. These disruptions are manifest by the increased level of expression of T-cell activation markers and decreased numbers of naïve T cells from the thymus (10, 12, 51). Oligoclonal T-cell expansion results in perturbations of the T-cell receptor (TCR) Vβ repertoire within both CD4 and CD8 T cells, with CD8 T cells being affected to a greater extent than CD4 T cells (7, 12, 16, 29, 50). Many of these abnormalities occur prior to CD4 T-cell attrition and are not fully reconstituted when viral replication is controlled by antiretroviral therapy (6, 17, 30). Multiple mechanisms have been postulated to contribute to this processes of aberrant T-cell activation and clonal expansion, including microbial translocation across the gastrointestinal tract as a result of virus-induced intestinal fibrosis (4, 5, 40) and the loss of immune regulation due to chronic HIV-induced antigenemia (8, 22).

CD4 and CD8 T cells are heterogeneous populations that differ functionally and in their expression of activation and differentiation markers, forming the basis of their classification as naïve, central memory (CM), or effector memory (EM) T cells (42). Isoforms of CD45 (CD45RA and CD45RO) are frequently used to subdivide CD4 and CD8 T cells into functional subsets (1, 13, 25, 44, 45). Oligoclonal expansions and deletions within T-cell subpopulations can be measured by analysis of the hypervariable CDR3 region of the TCR (37). CDR3 length variation reflects changes within the TCR Vβ repertoire during antigen-induced T-cell activation (24, 25, 34). Differences in CDR3 length diversity within the CD4 or the CD8 CD45RA or CD45RO subset enable assessments of disruptions of the TCR repertoire and the detection of oligoclonal expansion that would have been missed if the analyses were limited to unfractionated T cells (25, 26). While optimal control of viral replication by antiretroviral therapy (ART) corrects many T-cell abnormalities and slows the progression to AIDS, it is not clear if therapy completely restores the TCR repertoire or fully diminishes T-cell activation (7, 14, 27, 51). In the present study, we examined the relationship of TCR diversity, thymic output, and the expression of T-cell activation markers within the CD45RA and CD45RO subpopulations of CD4 and CD8 T cells before and after the initiation of ART to determine the extent to which the control of viral replication restores the TCR repertoire.

**MATERIALS AND METHODS**

**Subjects.** Thirteen HIV-infected children and adolescents, including four patients evaluated before and after the initiation of combination ART consisting of
two nucleoside reverse transcriptase inhibitors and a protease inhibitor or a nonnucleoside reverse transcriptase inhibitor, provided blood samples for analysis in this cross-sectional and longitudinal study. The control subjects included eight age-matched healthy subjects who had no underlying medical condition, recent illness, or immunizations. Informed consent for the collection and analysis of blood samples was obtained from the institutional review boards of the University of Florida and the University of South Florida/All Children’s Hospital.

The plasma HIV-1 RNA copy number was measured by an assay with the Amplicor (version 1.0) system (Roche Molecular Systems, Pleasanton, CA), which has a lower limit of detection of 400 copies/ml. T-cell subset analysis was performed by flow cytometry prior to and at 4-week intervals after the initiation of ART (46).

Isolation and purification of T-lymphocyte subsets. Peripheral blood mononuclear cells (PBMCs) were separated into T-cell subpopulations, as described previously (25, 44). Briefly, CD4 T lymphocytes were selected with magnetic multisort microbeads coated with anti-CD4 monoclonal antibodies (MABs) and a magnetic cell-sorting high-gradient magnetic separation column. After CD4 depletion, CD8 T cells were selected with microbeads coated with an anti-CD8 MAB (Miltenyi Biotech, Auburn, CA). Purified CD4 or CD8 cells were separated into the CD45RA and CD45RO subpopulations by using microbeads coated with the appropriate MAB (mouse immunoglobulin G1, clone L48, for CD45RA and mouse immunoglobulin G2a for CD45RO; Miltenyi Biotech). The purity of each T-cell subpopulation was >95%, as determined by flow cytometry and molecular analysis, with <1% contamination with the reciprocal subpopulation being detected (43, 44).

Assessment of TRECs in PBMCs. The frequency of signal-joining TCR receptor excision circles (TRECs) in PBMCs was quantified by real-time quantitative PCR of Vβ-specific TRECs, as described previously (51). Data were expressed as the log10 number of TREC copies per 10^6 PBMCs by using the mean values for triplicate TREC samples and duplicate ApoB TsqMan assays.

Flow cytometry analysis of CD4 T-cell subsets. PBMCs from the study subjects were incubated with the respective antibody combinations for 30 min in phosphate-buffered saline buffer containing 2% fetal calf serum and 0.1% sodium azide. The cells were then washed twice, fixed with 1% paraformaldehyde, and analyzed with a multiparametric LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ), as described previously (51). CD4+ T-cell subsets were defined as follows: naïve CD4 T cells, CD4+CD45RA+CD27−CD28+; EM CD4 T cells, CD4+CD45RA−CD27+CD28+; and CM CD4 T cells, CD4+CD45RO+CD27−CD28−; and CM CD4 T cells, CD4+CD45RO+CD27+CD28− (32, 39, 51).

Measurement of CD3R length variation. TCR CD3R for each Vβ family was amplified using 0.5 μl of 20 μl cDNA generated from mRNA extracted from the CD45RA or CD45RO subset of CD4 or CD8 T cells. Insufficient template mRNA resulted in an artificially distorted CD3R length distribution (Fig. 1A), characterized as CD3R lengths with a nontemplate sequence (GTTTCTT) and a blue fluorescent dye (6-carboxyfluorescein; Applied Biosystems, Foster City, CA) at a baseline and a missing length(s) (Fig. 1A, Vβ13) or the complete loss of a CDR3 length (Fig. 1A, Vβ18). A template with the complete loss of CDR3 peaks (Fig. 1A, Vβ13) or the complete loss of a CDR3 length (Fig. 1A, Vβ18) was translated into a probability distribution, Pj(i) − Aij(i)/Aij(j), where the letter j represents each sample, by using the fraction of the area (A) under the TREC family profile for each CD3R length from the minimal to the maximal length in steps of 3 nucleotides. Generally, there were 10 possible amino acid lengths (1 to 10) in each Vβ family. A control profile was established for each Vβ family by calculating the probability distribution, Pj(i) = (Σ Pj(i))/n, where n represents the number of healthy subjects, of the corresponding Vβ profiles. The resulting control profiles, Pj(i), conform to a Gaussian distribution.

To define the extent of perturbations in CD3R length, the distance (D) between the probability distributions of the samples and the average probability distribution for the healthy controls (μ) was calculated as Df(i) = Pj(i) − Pμ(i). The sum of the absolute difference, Df, was calculated for each Vβ family. Overall, the TREC length yields the perturbations of the TCR profile in percent. The average perturbation among all Vβ families studied for each individual j was calculated as an average distance (AD), ADj = (Σ Df(j))/m, where m is the number of all Vβ families examined. In this study, the average distance for a control sample is presented as ADμ = (Σ Dμ(j))/m. On the basis of the approximate Z test, a perturbation within each Vβ family was defined as ADj > (Σ ADμ(j))/n + 3 standard deviations (26).

Statistical analysis. Statistical analysis was performed with SAS (version 9.1) software (SAS Institute, Cary, NC). To accommodate the sample size and the nature of the pilot data, all variables were log10 transformed, and P values of <0.05 were considered significant. Comparison of the variables between the HIV-infected and the healthy individuals or within each group was performed by t test. The Pearson correlation coefficient was used to study the relationship of the number of perturbed Vβ families within the CD45RA and CD45RO CDR3 T-cell subsets with the clinical variables. The paired t test was used to compare the number of perturbed Vβ families within the CD4 CD45RA and CD45RO T-cell subsets in HIV-infected subjects before and after therapy.

RESULTS

TCR CD3R length profiles in CD4 T-cell subpopulations of healthy children. The TCR CD3R length profiles within CD4 CD45RA and CD45RO T cells from eight healthy children (median age, 6.5 ± 6.0 years) were evaluated to establish the normal CD3R diversity ranges. On the basis of the calculations for the healthy subjects, a perturbation in CD4 CD45RA T cells was defined as a Df value of >11.76% per Vβ family; that is, [Σ ADj(i) ]/n + 3 standard deviations = 7.74% (3 × 1.34%) = 11.76%, and similarly, a perturbation in CD4 CD45RO T cells was defined as a Df value of >19.87%.

Among the healthy children, the Vβ families within CD4 T cells displayed few perturbations (Fig. 2A). The mean number of perturbed Vβ families (NP) was 1.6 ± 1.8 in the CD45RA subset and 1.1 ± 1.8 in the CD45RO subset. No perturbations in either CD4 T-cell subset were detected in 11 of 21 (52%) Vβ
FIG. 1. Optimization of input template mRNA to avoid distortion of CDR3 length distribution resulting from template insufficiency. (A) CDR3 length distributions distorted by insufficient template were characterized as a warble baseline, low FI, and missing length peak of 201 bp in Vβ13 and a complete loss of length peaks in Vβ17. (B) Amplification of Vβ2 using serially diluted template mRNA at concentrations ranging from 10 ng (from 25.0 x 10⁴ purified T cells) to 0.6 ng (from 1.6 x 10⁴ purified T cells). The minimum amount of template mRNA able to generate a Gaussian distribution of CDR3 lengths was 1.3 ng. Two long CDR3 length peaks (**) diminished at the 0.6-ng level. (C) TCR CDR3 within CD45RA and CD45RO CD8 T cells from a healthy adult was amplified for 18 Vβ families by using 1.3 ng mRNA template. All Vβ families were amplified without a baseline oscillation and with the FI within the normal range, including Vβ families showing clonal expansion (Vβ15 within CD8 CD45RA T cells and Vβ11, Vβ14, Vβ15, and Vβ18 within CD8 CD45RO T cells). (D) Representative TCR CDR3 length distribution of all 21 Vβ families within CD45RA and CD45RO CD4 T cells from a 15-year-old healthy subject with the amount of template (4 to 20 ng mRNA) used for this study. The CDR3 lengths of all Vβ families showed Gaussian distributions.
CD4 T-cell subsets from 13 therapy-naive HIV-infected children and adolescents was examined prior to the initiation of ART (Table 1). The mean age of these children was 9.8 years, which was similar to the mean age of the corresponding healthy cohort (8.8 ± 2.5 years, which was similar to the mean age of the corresponding healthy cohort (P = 0.05, t test) (Fig. 2A). In contrast, there was no significant difference in NPs for CD45RO CD4 T cells between the HIV-infected cohort (mean NP, 2.9 ± 3.1) and the healthy cohort (P = 0.11, t test) (Fig. 2A). The CDR3 length perturbations differed within the CD4 CD45RA and CD45RO T-cell subsets. With TCR Vβ3 as an example (Fig. 2B), the Vβ perturbation fell into two types. One showed a single predominant length peak that represented more than 40% of the total area under the curve, as presented by patient P7, and the other showed multiple length peaks that differed statistically from a Gaussian distribution, as demonstrated by patient P12. Each formed a distinct contrast to the Gaussian distribution of TCR CDR3 lengths in the healthy controls.

Among the individuals in the infected population with relatively normal CD4 T-cell counts, the pretherapy NP within CD4 CD45RA or CD45RO T cells showed no correlation with age, the length of infection, the viral load, the percentage of CD4 or CD8 cells, or the proportion of CD45RA or CD45RO CD4 T cells. Perturbations within the CD4 CD45RA T-cell subsets in 10 HIV-infected subjects with CD4 T-cell counts of greater than 25% were compared to the perturbations within the CD4 CD45RA T-cell subsets in healthy subjects to determine if the TCR repertoire was disrupted prior to CD4 T-cell attrition. Among these subjects, CD4 CD45RA had a significantly higher NP in the infected group than in the healthy group (NPs, 6.6 ± 5.4 and 1.6 ± 1.8, respectively; P = 0.04, t test).

Comparison of TCR CDR3 length distribution in CD4 and CD8 T-cell naive and memory subpopulations in HIV-infected children before highly active ART. In six infected patients (patients P3, P4, P5, P6, P7, and P11), the CD4 and CD8 subpopulations were obtained at the same time point to examine the TCR CDR3 length diversity before the initiation of ART (Fig. 3). The mean NP within CD4 CD45RA T cells for these six patients was 7.5 ± 5.9, similar to the mean NP in CD8 CD45RA T cells (9.5 ± 4.8) (P = 0.41, t test) (Fig. 3A, upper panel). In contrast, the mean NP for CD4 CD45RO T cells was 2.5 ± 2.4, which was significantly lower than the NP for CD8 CD45RO of 14.0 ± 5.0 (P = 0.003, t test) (Fig. 3A, lower panel). Figure 3B shows the TCR CDR3 distributions within the CD4 and CD8 T-cell subpopulations in Vβ9, Vβ15, Vβ18, and Vβ22 in patient P3. All four Vβ families were perturbed in CD45RA CD4 and CD8 T cells and in CD45RO CD8 T cells but not in CD45RO CD4 T cells.

Impact of ART on T-cell activation markers and TCR CDR3 length diversity in CD4 CD45RA and CD45RO subpopulations. A longitudinal analysis of the changes in CDR3 length diversity within CD4 CD45RA and CD45RO T cells over the first 24 weeks of combination ART was performed for four children (patients P3, P4, P5, and P7) who fully suppressed viral replication by 2 months after the start of treatment (Fig. 4A). The mean NP for CD4 CD45RA T cells for the group prior to treatment was 10.5 ± 4.5, which declined to 3.5 ± 2.9 following 2 to 6 months of therapy (P = 0.10, paired t test)
To determine if changes in the TCR V\(\beta\) repertoire within CD4 CD45RO and CD45RO T cells correlated with changes in the TREC level and phenotypic shifts within CD4 T-cell subsets, the \(\log_{10}\) number of TREC copies/10\(^6\) PBMCs was evaluated by real-time quantitative PCR; and the absolute numbers and percentages of CD4 naïve, EM, and CM T cells in three of the suppressed subjects whose TCR V\(\beta\) repertoire was evaluated longitudinally were assessed by multiparameter flow cytometry analysis. The TREC levels increased in patients P3 and P7 and remained unchanged in patient P5, whose TREC level was normal for age before therapy (Fig. 4C, panel d), but the percentage remained similar and was unrelated to the changes in NP within total CD4 CD45RO T cells.

### DISCUSSION

There have been few assessments of the impact of HIV replication on the TCR repertoire within both CD4 and CD8 T subsets. The findings of this study, along with those of our previous studies, provide new insight into the extent that viral replication disrupts T-cell differentiation by measuring TCR diversity within the distinct naïve, CM, and EM T-cell subsets in HIV-infected individuals (25, 26, 51). Examination of the TCR repertoire within T cells at different stages of activation and differentiation enables sensitive assessment of how viral replication skews T-cell development by inducing oligoclonal T-cell expansion within EM and CM T cells. The study provides greater insight into perturbations within the TCR repertoire than analysis of unfraccionated CD4 or CD8 T cells (25).

Overall, the TCR V\(\beta\) repertoire is less perturbed in CD4 T cells than in CD8 T cells in healthy children and adolescents (16, 18, 33, 47). Our results show that in healthy children, CD8 T-cell perturbations are primarily the result of oligoclonal expansions within CD8 CD45RO T cells (26). In contrast, there are surprisingly few perturbations within circulating CD4 CD45RO T cells. The extent of perturbations in CD8 T-cell subsets is likely due to cytokine-driven memory CD8 T cells that are independent of continued antigen stimulation, which allows cells to persist in the blood over time (49). In contrast, preserved Gaussian distributions within CD4 CD45RO T cells could be the result of fewer clonally expanded HIV-specific CD4 T cells or antigen-induced homing of clonally expanded CD4 T cells to inflammatory sites or lymphoid tissues (6, 15, 23, 28).

HIV infection affects CD4 and CD8 T-cell subsets differently. In general, the degree of HIV-induced oligoclonal expansion in CD4 T cells is less than that in CD8 T cells (2, 16,
or CD4 T-cell attrition and TCR perturbations within CD4 T-cell subsets was found. However, the cohort selected for study had relatively normal total CD4 T-cell counts (range, 432 to 911 cells/µl), and in general, the individuals in that cohort were asymptomatic. We and others have shown that many HIV-infected children and adolescents have intact thymic output and normal proportions of naive T cells, as measured by the levels of TREC and expression naive T-cell surface markers (20, 33, 51). A novel finding in this study is the elevated level of perturbations within the CD4 and CD8 CD45RA subsets prior to the CD4 T-cell decline. The absence of perturbations within CD4 CD45RO T cells was particularly striking, particularly as this is the predominant subset that harbors HIV-1 in vivo (41, 43). Since HIV-specific memory CD4 T cells are preferentially infected, it would predict perturbations within this subset due to virus-induced expansion and apoptosis (11). It is likely that the small proportion of infected blood CD4 T cells results in a minimal impact on TCR diversity (2, 3, 6, 36).

In the current study, no correlation between the viral burden or CD4 T-cell attrition and TCR perturbations within CD4 T-cell subsets was found. However, the cohort selected for study had relatively normal total CD4 T-cell counts (range, 432 to 911 cells/µl), and in general, the individuals in that cohort were asymptomatic. We and others have shown that many HIV-infected children and adolescents have intact thymic output and normal proportions of naive T cells, as measured by the levels of TREC and expression naive T-cell surface markers (20, 33, 51). A novel finding in this study is the elevated level of perturbations within the CD4 and CD8 CD45RA subsets prior to the CD4 T-cell decline. The absence of perturbations within CD4 CD45RO T cells was particularly striking, particularly as this is the predominant subset that harbors HIV-1 in vivo (41, 43). Since HIV-specific memory CD4 T cells are preferentially infected, it would predict perturbations within this subset due to virus-induced expansion and apoptosis (11). It is likely that the small proportion of infected blood CD4 T cells results in a minimal impact on TCR diversity (2, 3, 6, 36).

Overall, comparison of the perturbations within the CD45RA and CD45RO subpopulations emphasizes the differences in cellular dynamics between CD4 and CD8 T cells. CD8 CD45RA phenotypes within HIV-infected individuals display downregulated CCR7, CD27, and CD107 and increased levels of expression of activation markers, such as CD38, HLA-DR, and CD11a (1, 7, 19, 48, 51). Increased perturbations within CD8 CD45RA T cells correlate with CD4 T-cell suppression and a higher viral burden. This correlation is not evident when the TCR Vβ repertoire in CD4 CD45RA or CD45RO T cells is examined.

The viral suppression caused by ART results in the rapid reestablishment of a Gaussian distribution of CDR3 lengths in both the CD45RA and CD45RO CD4 T-cell subsets. The normalization is likely due to increases in thymic output, as demonstrated by increases in the levels of TREC and CD4 T cells bearing a naive phenotype (Fig. 4C, panels a and b), and represents a fundamental difference in T-cell immune reconstitution in infected young individuals (9, 16, 26, 51). The rapid reestablishment of CDR3 length diversity within CD4 CD45RA T cells in children and adolescents treated with combination ART is distinctly different from the kinetics of change in TCR diversity in treated adults (9, 16). The therapy-induced normalization of TCR diversity in both T-cell populations contributes to improved T-cell function (9, 16, 26, 38), reflecting the increased capacity of thymic output in children and adolescents. However, in the limited number of children and adolescents evaluated before and after ART, the absolute numbers of naive and central memory T cells increased, yet the numbers of effector memory CD4 T cells were unchanged. While these results are consistent with those of previous studies of the cellular dynamics of the T-cell immune reconstitution in children, further studies are needed to examine the relationship of viral replication and virus-induced T-cell activation on EM CD4 T cells (46). In particular, the therapy-induced changes in TCR diversity in EM T cells following therapy need to be examined. We speculate that the therapy-induced normalization of TCR diversity in CD45RA T cells is most likely related to increases in naive T cells from the thymus. In conclusion, this study is one of the first comprehensive evaluations of TCR diversity within CD4 and CD8 T cells along their differentiation pathways. It included children infected perina-
tally as well as adolescents infected through sexual transmission. The results reveal the precise points in T-cell development that HIV disrupts the TCR repertoire and shows how quickly the control of viral replication results in its normalization.

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FIG. 4. Changes in TCR CDR3 Vβ profiles within CD45RA and CD45RO CD4 T cells and changes in TREC levels and the numbers of CD4 T-cell subpopulations after combination antiretroviral therapy. (A) Decrease in NPs within both CD45RA and CD45RO CD4 T cells from the baseline (BL), 2 to 6 months (M), and over 1 year (>1Y) posttherapy in patients P3, P4, P5, and P7. (B) Restoration of a Gaussian distribution of the TCR CDR3 length repertoire in Vβ3 and Vβ16 within CD45RA and CD45RO CD4 T-cell subsets from patients P7 at 2 months (2M) and 1 year (>1Y) after therapy. *, baseline oligoclonal expansions of 177- and 159-bp lengths within CD45RA and CD45RO T cells. (C) Change in log_{10} number of TREC copies/10^6 PBMCs (a) and changes in the number of naïve (b), EM (c), and CM (d) CD4 T cells (number of cells/μl) in three HIV-infected individuals (▲, patient P3; ●, patient P5; ■, patient P7) 48 weeks after therapy.
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