Antiretroviral Therapy Restores Diversity in the T-Cell Receptor Vβ Repertoire of CD4 T-Cell Subpopulations among Human Immunodeficiency Virus Type 1-Infected Children and Adolescents

Li Yin,1 Zhong Chen Kou,1 Carina Rodriguez,3 Wei Hou,2 Maureen M. Goodenow,1 and John W. Sleasman4*

Departments of Pathology, Immunology and Laboratory Medicine1 and Department of Epidemiology and Health Policy Research,2 Shands Cancer Center, University of Florida, College of Medicine, Gainesville, Florida, and Division of Infectious Diseases3 and Division of Allergy, Immunology, and Rheumatology,4 University of South Florida, College of Medicine, St. Petersburg, Florida

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Human immunodeficiency virus (HIV) type 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 occurred prior to CD4 T-cell attrition and normalized 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 subsets enable assessments of disruptions of the TCR repertoire and the detection of oligoclonal expansion that would have been missed if the analysis 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 microsorb beads 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 TREC levels in PBMCs. The frequency of signal-joining TCR receptor excision circles (TRECs) in PBMCs was quantified by real-time quantitative PCR with the primers and fluorogenic probe described previously (51). Data were expressed as the log_{10} number of TREC copies per 10^6 PBMCs by using the mean values for triplicate TRECs and duplicate ApoB TaqMan 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 2 flow cytometer (BD Biosciences, Franklin Lakes, NJ), as described previously (51). CD4+ T-cell subsets were defined as follows: naïve CD4 T cells, CD3+CD4+CD45RA+CD27+CD28-; EM CD4 T cells, CD3+CD4+CD45RA-CD27+CD28-; and CM CD4 T cells, CD3+CD4+CD45RO+CD27-CD28+. The CD8+ T cells were CD3+CD8+CD45RA-CD27+CD28-. The CD45RA and CD45RO subsets of CD4 or CD8 T cells were defined as a CD45RA or CD45RO subset of CD4 or CD8 T cells, CD3+CD4+ or CD3+CD8+. Insufficient template insufficiency because of a stable baseline and normal FIs. In this study, the TCR length yields the perturbations of the TCR profile in percent. The average perturbation among all Vf families studied for each individual j was calculated as an average distance (AD), AD = (Σ D_j)^n/m, where m is the number of all Vf families examined. In this study, the average distance of the control sample was calculated as 2D_j = D_j/n, where n represents the number of healthy subjects, of the corresponding Vf profiles. The resulting control profiles, P_j, conform to a Gaussian distribution.

To define the extent of perturbations in CDR3 length, the distance (Dj) between the probability distributions of the samples and the average probability distributions for the healthy controls (c) was calculated as D_j = P_j - P_c (25). The sum of the absolute distance, D = 100 Σ D_j, was calculated for each Vf family. Overall, the TCR length yields the perturbations of the TCR profile in percent. The average perturbation among all Vf families studied for each individual j was calculated as an average distance (AD), AD = (Σ D_j)^n/m, where m is the number of all Vf families examined. In this study, the average distance of the control sample is presented as AD_c = (Σ 2D_j)^n/m. On the basis of the approximate Z test, a perturbation within each Vf family was defined as AD_c > (Σ AD_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 log_{10} 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 Vf families within the CD45RA and CD45RO CD4 T-cell subsets with the clinical variables. The paired t test was used to compare the number of perturbed Vf families within the CD4 CD45RA and CD45RO CD4 T-cell subsets in HIV-infected subjects before and after therapy.

RESULTS

TCR CDR3 length profiles in CD4 T-cell subpopulations of healthy children. The TCR CDR3 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 CDR3 diversity ranges. On the basis of the calculations for the healthy subjects, a perturbation in CD4 CD45RA T cells was defined as a D^* value of ≥11.76% per Vf family, that is, [Σ AD_j(n)]/n + 3 standard deviations = 7.74% + (3 × 1.34%) = 11.76%, and similarly, a perturbation in CD4 CD45RO T cells was defined as a D^* value of ≥19.87%.

Among the healthy children, the Vf families within CD4 T cells displayed few perturbations (Fig. 2A). The mean number of perturbed Vf 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%) Vf

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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 2.5 × 10^5 purified T cells) to 0.6 ng (from 1.6 × 10^4 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.
families, including Vβ families 2, 3, 4, 6, 7, 8, 12, 13, 18, 20, and 22. The difference between the NPs in CD4 CD45RA and CD45RO T cells was not significant ($P = 0.17, t$ test).

TCR CDR3 length distributions in CD4 T-cell subpopulations from HIV-infected children before highly active ART. The TCR CDR3 length diversity in CD45RA and CD45RO 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 ± 7.9 years, which was similar to the mean age of the corresponding healthy cohort ($P = 0.25, t$ test). The mean CD4 T-cell counts pretherapy were in the normal range of 882 ± 833 cells/μl (31.5% ± 7.9%). Within the CD4 T cells, the absolute number and the percentage of the T-cell subsets were 666 ± 836 cells/μl and 66.8% ± 15.4%, respectively, for CD45RA T cells and 215 ± 73.4 cells/μl and 33% ± 15.3%, respectively, for CD45RO T cells. The absolute CD8 T-cell counts and percent-

ages were 906 ± 446 cells/μl and 37.5% ± 11.9%, respectively. The mean log10 HIV-1 RNA load was 4.2 ± 1.1 copies/ml.

Compared to the healthy individuals, the HIV-infected children and adolescents demonstrated a significantly higher number of perturbations within Vβ families of CD4 CD45RA T cells (mean NP, 5.8 ± 4.9; $P = 0.04, t$ 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).
(Fig. 4A, left panel). The mean pretherapy NP for CD4 CD45RO T cells was 3.3 ± 2.6, which decreased further to 0.3 ± 0.5 within 2 to 6 months of treatment (P = 0.06, paired t test) (Fig. 4A, right panel). The NP within either CD4 T-cell subset after 1 year of therapy was similar to the NP at 2 months posttreatment in patients P3 and P7. Figure 4B exemplifies the restoration of a Gaussian distribution of the CDR3 length repertoire after 2 months and 1 year of optimal viral suppression. In patient P7, there was a predominant CDR3 length peak in both Vβ3 and Vβ16 within the CD45RA and CD45RO CD4 T-cell subsets. These predominant peaks were identical in both subsets, on the basis of CDR3 lengths of 177 and 159 bp, respectively. By 2 months after the start of treatment, this oligoclonal expansion had resolved. The posttherapy NP within both subsets, on the basis of CDR3 lengths of 177 and 159 bp, respectively. 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One potential explanation is that viremia leads to a large proportion of activated HIV-specific cytotoxic T lymphocytes directed toward multiple antigenic epitopes (31, 35). The magnitude of HIV-specific cytotoxic T-cell responses does not predict the effectiveness of control of viral replication (10, 21). Multiple perturbations within the CD8 T-cell repertoire are associated with higher viral loads and lower CD4 T-cell counts (26) and could arise from viral immune escape as new antigenic epitopes emerge, resulting in less effective CD8 T-cell responses.

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 naïve T cells, as measured by the levels of TREC and expression naïve 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 naïve 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 naïve 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 naïve 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 log10 number of TREC copies/10⁶ 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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