Correlates of Delayed Disease Progression in HIV-1-Infected Kenyan Children

Rana Chakraborty, Anne-Sophie Morel, Julian K. Sutton, Victor Appay, Ruth M. Ripley, Tao Dong, Tim Rostron, Simon Ogola, Tresa Palakudy, Rachel Musoke, Angelo D'Agostino, Mary Ritter and Sarah L. Rowland-Jones


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Without treatment most HIV-1-infected children in Africa die before their third birthday (>89%) and long-term nonprogressors are rare. The mechanisms underlying nonprogression in HIV-1-infected children are not well understood. In the present study, we examined potential correlates of delayed HIV disease progression in 51 HIV-1-infected African children. Children were assigned to progression subgroups based on clinical characterization. HIV-1-specific immune responses were studied using a combination of ELISPOT assays, tetramer staining, and FACS analysis to characterize the magnitude, specificity, and functional phenotype of HIV-1-specific CD8+ and CD4+ T cells. Host genetic factors were examined by genotyping with sequence-specific primers. HIV-1 nef gene sequences from infecting isolates from the children were examined for potential attenuating deletions. Thymic output was measured by T cell rearrangement excision circle assays. HIV-1-specific CD8+ T cell responses were detected in all progression groups. The most striking attribute of long-term survivor nonprogressors was the detection of HIV-1-specific CD4+ Th responses in this group at a magnitude substantially greater than previously observed in adult long-term nonprogressors. Although long-term survivor nonprogressors had a significantly higher percentage of CD45RA+CD4+ T cells, nonprogression was not associated with higher thymic output. No protective genotypes for known coreceptor polymorphisms or large sequence deletions in the nef gene associated with delayed disease progression were identified. In the absence of host genotypes and attenuating mutations in HIV-1 nef, long-term surviving children generated strong CD4+ T cell responses to HIV-1. As HIV-1-specific helper cells support anti-HIV-1 effector responses in active disease, their presence may be important in delaying disease progression.


Aquired immunodeficiency syndrome is now the leading cause of death in sub-Saharan Africa and the fourth biggest killer worldwide (www.unaids.org/epidemic_update/report/index.html). More than 60% of the world’s HIV-infected subjects live in Africa, where transmission is commonly heterosexual and nearly half of all infected adults are women of childbearing age. As a result, the epidemic has particularly grave implications for African children. Child mortality has increased overall by 35–50% and by >100% in areas of high HIV-1 seroprevalence, thereby erasing the gains in child-survival rates made over the last 30 years (www.unaids.org/epidemic_update/report/index.html).

HIV-1 infection has a heterogeneous spectrum of clinical course following initial infection. Compared with HIV-1-infected children from Western cohorts, survival times are considerably shorter for African children who acquire the virus perinatally or during infancy (1). Early studies of perinatally infected children in developed countries before the era of highly active antiretroviral therapy (HAART) indicated that a subset of children (~25%) progressed very rapidly to AIDS (within 1 year). The median time to AIDS for the remaining 75% was ~7 years (2). However, a minority of HIV-1-infected infants remain clinically asymptomatic beyond childhood and into adolescence.

In adults, the mechanisms underlying delayed HIV-1 disease progression are heterogeneous and include viral attenuation, immunological factors, and host genetic determinants. More specifically, infection with nef/long terminal repeat-deleted HIV-1 resulted in slower disease progression among recipients from the Sydney Blood Bank Cohort (3). Differences in MHC class I-restricted HIV-1-specific CD8+ T cell responses variably impact on disease progression (2). These include maintenance of large expansions of oligoclonal or monoclonal memory CD8+ T cells targeted toward multiple conserved HIV-1 epitopes in a significant proportion of long-term survivors (LTSs). These responses may contribute to the asymptomatic state (4). Consistent HLA class I allele associations with delayed disease progression among Caucasians include HLA-A*0201 (5) and B27 (6). In adults, a cardinal feature of HIV-1 immunodeficiency is the early loss of HIV-1-specific CD4+ Th responses (7, 8), and preserved HIV-1-specific CD4+ Th responses are associated with delayed progression.

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2 Address correspondence and reprint requests to Dr. Rana Chakraborty, Pediatric Infectious Diseases Unit, 5th Floor Lanesborough Wing, St. George’s Hospital, Blackshaw Road, Tooting, London SW17 0QT, U.K. E-mail address: rchakrab@sghms.ac.uk

3 Abbreviations used in this paper: HAART, highly active antiretroviral therapy; LTS, long-term survivor; UTR, untranslated region; LTNP, long-term nonprogressor; LTSP, LTS progressor; EPI, early nonprogressor; TP, typical progressor; TRICE, T cell receptor rearrangement excision circle; SFU, spot-forming unit; PPD, purified protein derivative; ICS, intracellular cytokine staining.

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(9). Individuals heterozygous for the CCR5∆32 mutation have reduced cell surface expression of the CCR5 coreceptor for macrophage-tropic HIV-1 and exhibit delayed disease progression (10), whereas additional polymorphisms in CCR5 promoter positions and point mutations in the gene (3′-untranslated region (UTR)) for SDF 1α ligand may also modify disease progression (11). The relative contribution of individual host genetic factors to delayed disease progression in Africans has not been examined in detail, but a study of Kenyan sex workers showed that the protective polymorphisms in CCR5 and SDF-1α were rare (12) and that the CCR2-V64I mutation accounted for a significant proportion of long-term nonprogressors (LTNPs).

Biological determinants contributing to accelerated disease progression in infants and children may include relative immunological immaturity, thymic HIV-1-mediated destruction at a time of active thymopoiesis, and HLA class I sharing between mother and infant (2). There have been no previous studies investigating possible links between clinical outcome and immunological features in pediatric LTSs from Africa. Such studies may yield important information about HIV pathogenesis and protective immunity in HIV-1 infection. Therefore, we characterized HIV-1-specific T cell responses and determined T cell output and immunophenotypic among a cohort of HIV-1-infected children from Kenya in which a remarkable group of LTNPs have been identified.

Materials and Methods

Study population and HIV-1 disease classification

The disease stage of 51 HIV-1-infected children residing in the Nyumbani orphanage for HIV-1-infected children in Nairobi, Kenya, was characterized. Although it was not possible to determine with certainty the timing and route of infection in this cohort, the children were all HIV-1 positive when adopted in infancy and are therefore assumed to have been infected in utero, during labor, or by breast-milk transmission of HIV-1 during the first year of life. By default, the home effectively selects for children with delayed disease progression because rapid progressors with early-onset Pneumocystis jirovecii pneumonia and other opportunistic infections most probably died in early infancy before adoption was possible.

In the absence of existing criteria for the definition of pediatric LTNPs, LTSs were defined as reaching the age of 8 years in the absence of HAART and were subclassified as either LTS nonprogressors (LTSNPs), CD4 T cell counts <500/mm³ (n = 9), or LTS progressors (LTPs), CD4 T cell counts < 500/mm³ (n = 9). Children under 8 years were subclassified according to age-specific CD4 T cell counts as either early nonprogressors (ENPs) (n = 6) in category 1 of the CDC classification of pediatric HIV-1 infection or typical progressors (TPs) (n = 22) in category 2 or 3.

In 2001, the home received a donation to provide HAART to the most immunocompromised children that included nonnucleoside and nucleoside reverse transcriptase inhibitors and protease inhibitors. Eight children from this study were selected to receive treatment. T cell receptor rearrangement excision circle (TREC) assays were performed on these children after HAART had been commenced (13).

Informed consent was obtained from the legal guardian of the children. Research ethics committees at the University of Oxford and the National Council of Science and Technology in Kenya approved all studies.

Cross-sectional immunologic and host genetic investigations

Initial investigations in Nairobi in 2000 included HLA typing and genotypic analysis of host coreceptor polymorphisms in all subjects (n = 51) and CD8+ T cell ELISPOT assays on fresh venous blood from 49 children. Peptide-HLA tetramer staining was performed to look for HIV-specific CD8+ T cell responses in six subjects with strongly positive HIV-specific CD8+ T cell responses by ELISPOT. In addition, HIV-specific CD4+ T cell responses were evaluated in 16 children by IFN-γ ELISPOT and further quantified by intracytoplasmic staining, as detailed below.

Frozen PBMCs from 2000 were thawed in London in 2001 from 39 children for additional staining by four-color FACS analysis for CD4, CD8, CD45RA, and CD45RO expression. The persistence of CD4+CD45RA+ T cells may reflect thymic activity. Therefore, we analyzed CD4+ T cell receptor rearrangement excision circle content and expression of CD103 and K67 on sorted PBMCs from 41 children.

The disparity in numbers of immunologic analyses performed on frozen PBMCs in 2001 is a reflection of limited sample availability and viability.

HIV-1 status and CD4+ T cell counts

A diagnosis of HIV-1 and infection was established and confirmed by repeated testing of sera for HIV-1 Abs by commercial ELISA. In children under 18 mo of age, plasma HIV-1 RNA copy number determined by RT-PCR (Roche) confirmed HIV-1 infection using assay kits with a high degree of sensitivity for nonclade B HIV-1. CD4+ T cell counts were determined by flow cytometry.

PBMCs were isolated by density gradient centrifugation and maintained through in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

HLA typing and coreceptor polymorphism

HLA typing was performed on genomic DNA extracted from PBMCs by amplification refractory mutation system-PCR (n = 51) using sequence-specific primers as described previously (14). Genotyping for previously described single nucleotide polymorphisms in the HIV coreceptors and the SDF-1α gene was performed using amplification refractory mutation system-PCR with sequence-specific primers (11).

ELISPOT assays

Forty-nine subjects were screened for virus-specific CD8+ T cell responses by ELISPOT assays (Mabtech) using a panel of predefined HLA class I-restricted HIV-1 epitopes optimized to represent clade A consensus sequences that had been shown previously to be immunogenic in HIV-1-infected adult cohorts in Kenya (15). HIV-1-specific IFN-γ release was reported as the number of spot-forming units (SFU)/10⁶ PBMCs, after subtracting background rates of spontaneous IFN-γ secretion. A positive HIV-1-specific ELISPOT result was defined arbitrarily as follows: a) IFN-γ release seen in response to 1:100 PHA μg/ml (positive control); b) ≥50 HIV-1-specific SFU/10⁶ mononuclear cells; and c) IFN-γ release in HIV-1 peptide wells exceeded background (spontaneous) rates of IFN-γ release by a factor of at least 2.

In 16 selected children (including 10 nonprogressors), CD4+ T cell responses to pooled HIV-1 gag peptides (p17 and p24), CMV lytase, and the recall Ag purified protein derivative (PPD) were screened by CD4+ ELISPOT on fresh venous blood, following magnetic bead depletion (Dynal Biotech) of NK and CD8+ T cells. The effectiveness of the CD8+ T cell depletion protocol as determined by flow cytometry was always >96%.

TRECs

CD8+ or CD4+ cells were sorted sequentially from 41 frozen PBMC samples using CD8+ and CD4-MACS microbeads (Miltenyi Biotech). The purity of each sorted sample was evaluated by FACS.

Purified cells were lysed in 100 μg/ml protease K (Boehringer Mannheim) for 1 h at 56°C and then 10 min at 95°C. Signal-joint TRECs and albumin were quantified in 5 μl of sorted CD8+ or CD4+ cell lysate by real-time PCR (16) using the 5′-nuclease (TaqMan). The following primers and probes were used: TREC forward primer, 5′-CACATCCCCTTCAAC CATGCT-3′, TREC reverse primer, 5′-GGCAAGCTCAGGTTTTAGG-3′, TREC probe, FAM-ACACCTCTGTTTTGTTAAGGTCGCACCT QSY7 (MegaBases), albumin forward primer, 5′-TGATGAGAAAA CGCCAGTAA-3′, albumin reverse primer, 5′-ATGGCTGCTTGC TTACCAA-3′, and albumin probe, FAM-TGACAGAAGTCACCAAATATGTCG ACAGAA-QSY7. PCR reactions contained 0.5 units of Platinum Taq polymerase (Invitrogen Life Technologies), 3.5 mM MgCl₂, 0.2 mM dNTPs, 500 nM of each primer, 150 μM probe, and Blue-636 reference (MegaBases). The reactions were run in an ABI7000 system (Applied Biosystems), and the conditions were 95°C for 5 min and then 95°C for 30 s and 60°C for 1 min for 40 cycles. Samples were analyzed twice in duplicates, which never varied by >10% from each other. TREC number per 5-μl sample was calculated according to a standard curve. As the cell number and purity of each sample varied, the TREC number was normalized on the albumin values and the respective percentages of CD4+ or CD8+ cells in the sample, yielding arbitrary TREC units (17).

Flow cytometric analysis

HLA class I PE-conjugated tetramers were used to stain PBMCs in six children with strongly positive HIV-specific CD8+ T cell responses by ELISPOT as described previously (18). The following tetramers were used: A2-ILKPVHGV, A2-SLYNTVATL, B53-QATQEVKNW, and B57-KAFSPEVIPMF. After incubation with whole blood and lysis of red cells,
lymphocytes were washed, fixed, permeabilized, and stained with the following Abs: FITC-conjugated anti-CD27 (BD Biosciences), anti-CD28 and anti-perforin and PerCP-conjugated anti-CD8 (BD Biosciences). CD8^{+} tetramer^{+} events were gated for analysis.

Using standard protocols, thawed PBMCs were stained for four-color FACS analysis in cold PBS containing 0.5% FCS and 0.05% sodium azide with the following mAbs: mouse anti-human CD4-PE, anti-CD8-PerCP, anti-CD45RO-FITC, anti-CD45RA-allophycocyanin, anti-CD95-FITC, and their corresponding isotype controls were obtained from BD Biosciences (n = 39). For analysis of putative recent thymic emigrants, sorted thawed CD8^{+} cells were stained for CD103 (BD; Beckman Coulter), CD8 (PE; BD Biosciences), and CD45RA (allophycocyanin; BD Biosciences). To estimate cell proliferation, sorted CD4^{+} or CD8^{+} thawed PBMCs were stained with anti-CD4 or anti-CD8-PE (BD Biosciences), fixed in ethanol, and stored at −20°C, according to the manufacturer’s protocol (BD Biosciences). They were then stained with unconjugated-Ki67 (BD Biosciences) or the isotype controlOX7 (mouse anti-rat/mouse CD90, IgG1; produced in-house), followed by rabbit anti-mouse FITC (DakoCytomation) (n = 41). Cells were analyzed on a FACSCalibur cytometer (Becton Dickinson) using the CellQuest software program.

Intracellular cytokine staining (ICS)

CMV- and HIV-1-specific CD4^{+} T cell responses were quantified by ICS, as described previously (19, 20). Briefly, 0.5 ml of whole blood aliquots was stimulated with either CMV whole viral lysate (Advanced Biotechnologies) (10 μg/ml), pooled HIV-1 gag p24 and p17 overlapping 20-mer peptides (each peptide at 5 μm) (National Institute for Biological Standards and Control), superantigen staphylococcal enterotoxin B (1 μg/ml) (Sigma-Aldrich), or saline for 6 h in the presence of costimulatory Abs anti-CD28 and anti-CD49d (BD Biosciences) at 1 μg/ml. Brefeldin A (Sigma-Aldrich) was added to prevent export of intracellular cytokines. Following red cell lysis, lymphocytes were fixed, permeabilized, and stained with FITC-conjugated anti-IFN-γ or anti-TNF-α, PE-conjugated anti-CD69, anti-CD38, or anti-IL-2 and PerCP-conjugated anti-CD4 (all from BD Biosciences). Cells were further washed and stored in 5% formaldehyde at 4°C. Samples were acquired using a FACSCalibur flow cytometer, and CD4^{+} events were gated for analysis using CellQuest software.

Statistical analysis

Differences in characteristics between children groups were analyzed using nonparametric tests. The Mann-Whitney U test was used to compare differences between individual groups studied, whereas one-way ANOVA was performed to detect significant variation across the groups. No linear relationship could be assumed between any of the variables studied; therefore, correlations between variables, i.e., regardless of the group, were calculated using Spearman’s r. Values of p are given for two-tailed analysis. All statistical analysis relating to TREC and FACS were performed using the SPSS for Windows software (SPSS).

The proportion of positive peptide-specific ELISPOT responses for each patient was compared between groups using a one-way ANOVA, with and without an empirical logit transform. Software functions used were aov, lm, and cor of S-PLUS (Insightful, 1988–2001).

Results

Cohort characteristics

Children were categorized into four groups according to age, clinical status, and CD4^{+} T cell count. The cohort demonstrated a characteristic East African distribution of HLA class I types (Table I), but because of small numbers, it was not possible to draw conclusions about the relationship between HLA type and disease state (14).

Table I. Detection of HIV-specific CD8^{+} T cell responses by ELISPOT from 16 subjects with previous virus-specific CD4^{+} T cell determinations (Fig. 4) and tetramer populations in children with strongly positive HIV-specific CD8^{+} T cell responses by ELISPOT

<table>
<thead>
<tr>
<th>Group</th>
<th>ID</th>
<th>Class I and II HLA Type</th>
<th>ELISPOT Response</th>
<th>HIV-1 Tetramer Peptide</th>
<th>SFU/10^6 PBMCs by ELISPOT for Tetramer Peptide</th>
<th>% Tetramer (+) Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTSNP NY2</td>
<td>A1,A6802,B4501,B53,Bw4,Bw6,Cw6 DRB1 07,DRB1 13,DRB3,DRB4,DRB14,DRB11,DRB15</td>
<td>Gag 2560, Pol 1000</td>
<td>B53-QATQEVKNW</td>
<td>4024</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>NY4 A26,A29,B44,B4501,Bw4,Bw6,Cw6,Cw14 DRB1 13,DRB3,DRB4,DRB11,DRB15</td>
<td>Gag 73, Pol 370, Env 85</td>
<td>680</td>
<td>0.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY8 A2,A31,B4501,B53,Bw4,Cw6 DRB1 1602 DRB1 1503</td>
<td>DRB4,DRB11,DRB15,DRB1503, DRB1503,DRB1 11,DRB3,DRB5,DQB1 02,DQB1 06</td>
<td>658</td>
<td>0.34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY18 A24,A74,B13,B53,Bw4,Bw6,Bw8 DRB1 03,DRB1 11,DRB3,DRB3,DRB4,DRB4,DRB11,DRB15,DRB1503</td>
<td>Gag 105, Pol 0, Env 15, Nef 0</td>
<td>209</td>
<td>0.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY27 A26,A29,B42,B4901,Bw4,Bw6,Cw7,Cw17 DRB1 17,DRB3 03,DRB3 01,DRB3 03, DRB3 04,DRB3 06</td>
<td>Gag 0, Env 500, Nef 0</td>
<td>840</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NY35 A24,A6601,B41,B301,Bw4,Bw6,Cw6,Cw17 DRB1 17,DRB3 03,DRB3 01,DRB3 03, DRB3 04,DRB3 06</td>
<td>Gag 335, Env 220</td>
<td>B53-QATQEVKNW</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY51 A29,A3601,B53,B58,Bw4,Bw6,Bw8 DRB1 13,DRB3,DRB3,DRB4,DRB4,DRB11,DRB15,DRB1503</td>
<td>Gag 62, Pol 50, Env 375</td>
<td>680</td>
<td>0.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY52 A23,A22,B301,Bw4,Bw6,Cw6,Cw16 DRB1 11,DRB3,DRB3,DRB4,DRB4,DRB11,DRB15,DRB1503</td>
<td>Gag 250, Pol 5, Env 105, Nef 10</td>
<td>209</td>
<td>0.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY6 A2,A6601,B18,B58,Bw4,Bw6,Cw6,Cw16 DRB1 03,DRB1 11,DRB3,DRB3,DRB4,DRB4,DRB11,DRB15,DRB1503</td>
<td>Gag 80, Pol 150, Nef 820</td>
<td>209</td>
<td>0.39</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NY6 A2,A30,B13,B58,Bw4,Bw6,Bw8 DRB1 05,DRB3,DRB3,DRB4,DRB4,DRB11,DRB15,DRB1503</td>
<td>Gag 413, Pol 87</td>
<td>209</td>
<td>0.39</td>
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<tr>
<td>NY8 A1,A6802,B4501,B53,Bw4,Bw6,Cw6 DRB1 11,DRB3,DRB3,DRB4,DRB4,DRB11,DRB15,DRB1503</td>
<td>Gag 15, Pol 76, Nef 0</td>
<td>209</td>
<td>0.39</td>
<td></td>
<td></td>
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<tr>
<td>TP NY20 A26,A6802,B7,B67,Bw6,Cw7,Cw12 DRB1 11,DRB1 13,DRB3,DRB3,DRB4,DRB4,DRB11,DRB15,DRB1503</td>
<td>Gag 1000, Pol 1463, Env 1253, Nef 1672</td>
<td>B57-KAFSEPVEIPMF</td>
<td>1075</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY58 A2,A31,B510,B47,Bw4,Bw6,Cw6,Cw16 DRB1 03,DRB1 04,DRB4,DRB4,DRB11,DRB15,DRB1503,DRB1 11,DRB3,DRB5,DQB1 02,DQB1 06</td>
<td>Gag 157, Pol 60</td>
<td>B57-KAFSEPVEIPMF</td>
<td>1075</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENP NY16 A2,A74,B301,B57,Bw4,Cw6,Cw7 DRB1 1 01 DRB3,DRB3,DRB4,DRB4,DRB11,DRB15,DRB1503</td>
<td>Gag 18, Pol 0, Env 0</td>
<td>1075</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY54 A30,A6602,B13,B4501,Bw4,Bw6,Cw6 DRB1 13,DRB3,DRB3,DRB4,DRB4,DRB11,DRB11,DRB15,DRB1503</td>
<td>Gag 767, Pol 76</td>
<td>1075</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY76 A2,B4501,B53,Bw4,Bw6,Cw6,Cw1601,Cw1602 DRB1 04,DRB3 12,DRB3,DRB4,DRB4,DRB11,DRB15,DRB1503</td>
<td>Gag 328, Pol 715, Env 5, Nef 20</td>
<td>1075</td>
<td>2.5</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* ELISPOT assays were carried out using optimized CTL epitopes according to donor HLA types. Responses to CTL epitopes are added together, grouped by gene products, and shown for each donor. The values are expressed as SFU/10^6 PBMCs. The background IFN-γ production (usually not higher than 20–30 SFU/10^6 PBMCs) has been subtracted.

* Responses >50 SFU/million PBMCs were considered significant.
HIV-1-specific CD8+ T cell responses are detected in all progression groups

CD8+ T cell responses were evaluated using a panel of optimized CTL epitopes in the IFN-γ ELISPOT assays and by FACS with selected peptide-HLA tetramers. ELISPOT assays identified large peptide-specific responses in children from all progression groups. Although the proportions of positive responses were higher in LTSNPs (55% compared with 38–40% in the other groups) (Fig. 1), this difference does not reach statistical significance (p = 0.24) and may reflect the small numbers of subjects in each group.

Large populations of HIV-specific CD8+ T cells were identified ex vivo by tetramer staining in eight children with strongly positive HIV-specific CD8+ T cell responses by ELISPOT, regardless of progression group (Fig. 2 and Table I). These tetramer-staining populations exhibited the characteristic intermediate differentiation state (CD27high/CD28low/perforinlow) previously described in adults with chronic HIV-1 infection, irrespective of clinical status (21, 22). Although the level of perforin expression has been linked to delayed disease progression in adults (23), we found no significant differences in perforin expression between progressors and nonprogressors. However, given the small number of children studied (n = 8), these data may not be applicable for the whole cohort (Fig. 2 shows the largest populations specific for individual epitopes from two donors).

Nonprogressors are characterized by strong HIV-1-specific CD4+ T cell responses

IFN-γ release by HIV-1-specific CD4+ T cells was measured using ELISPOT assays and intracytoplasmic staining. Seven of seven LTSNPs and three of three ENPs exhibited strong HIV-1-specific CD4+ T cell responses to HIV-1 gag Ags in addition to maintaining responses to PPD, CMV, and other recall Ags. Populations detected by ELISPOT were confirmed by intracytoplasmic staining (Fig. 3). The largest response was observed in LTNP NY27 where 1.30% of CD4 T lymphocytes were gag-specific (Fig. 3) as determined by IFN-γ production (1.47% by TNF-α production). Overall, all seven LTSNPs had detectable IFN-γ or TNF-α anti-Gag cytokine responses. In contrast, HIV-1-specific CD4+ T cells could not be detected in three TPs and two of three LTSPs, although responses to recall Ags were preserved (Fig. 4). To the best of our knowledge, this is the first time such responses have been documented in HIV-1-infected children.

CD4+ T cells are predominantly of CD45RA+ phenotype in LTSNPs

Lymphocyte activation has been associated previously with an increased risk of disease progression and is predictive of the development of AIDS (24, 25). We have analyzed a variety of T cell surface markers in the cohort (Table II). In particular, CD4+CD45RA+RO− cell percentage was lower in TPs than in LTSNPs (p = 0.07), LTSPs (0.05), and ENPs (p = 0.062) (Fig. 5A). This skew was also observed for nonprogressors when analyzing CD4+CD45RA+ T cells per microliter of blood (p = 0.028 and 0.022 for LTSNPs and ENPs, respectively) (Fig. 5B). None of the other groups showed any statistically significant differences to LTSNPs. Therefore, as previously described, it appears that nonprogression is associated with a trend toward a higher percentage of CD4+CD45RA+RO− cells. There were no statistically significant differences in CD8+ populations between groups.

The detection of HIV-1-specific CD4+ T cells is not related to thymic output

The persistence of CD4+CD45RA+ T cells in LTSNPs may be indicative of a more active thymus. Therefore, we analyzed TREC...
Ki67 sorted T cell populations. The apparent higher mean and range of activation and proliferation, we measured Ki67 expression in the ANOVA.

Because TREC levels may be substantially affected by immune activation and proliferation, we measured Ki67 expression on CD4 cells in TPs reflects the paucity of CD4 cells among 

content in CD4\(^{-}\) and CD8\(^{-}\}-sorted PBMCs according to the protocol described. The same protocol was used successfully to determine thymic output from frozen human SCID patients PBMCs, with a lower detection limit of 7 TREC/10,000 PBMCs. Using this method, fresh and frozen PBMCs from normal control adults give comparable results.

CD4 TREC s were measured more readily than CD8 TREC s (Table II). In the 41 children tested, 19 had CD4 TREC s whereas 13 had CD8 TREC s, out of which only 4 did not show any CD4 TREC s simultaneously (Fig. 6). As expected, CD8 TREC s were associated with CD4 TREC s (\(p = 0.066\)). There were no significant differences in the number of children with measurable CD4 TREC s between the two groups or in the TREC levels themselves. Interestingly, not all LTSNPs had measurable TREC s, most notably none of the LTSNPs with preserved anti-CD4 responses in Fig. 4. We detected CD4 TREC s in five of the eight children who received a partial course of HAART (Fig. 6) (13).

Circulating CD4 or CD8 TREC levels did not correlate with viral load, as previously shown (26). However, CD4 TREC levels positively correlated with CD4\(^{+}\) T cell count (\(p = 0.5, p = 0.009\), CD4\(^{\%}\) (\(p = 0.375, p = 0.059\)), and CD4\(^{\%}\)CD45RA\(^{-}\) (\(p = 0.518, p = 0.007\)). CD8 TREC s also correlated with CD4\(^{\%}\)CD45RA\(^{-}\) (\(p = 0.45, p = 0.021\)) but not with any of the other CD8 surface markers. Given the variability in the quality of the samples, we were unable to calculate absolute TREC s per microliter of blood and could only perform comparison between groups. Despite the good correlation with CD4 count, there was no significant difference in TREC levels between groups as determined by one-way ANOVA.

Because TREC levels may be substantially affected by immune activation and proliferation, we measured Ki67 expression in the sorted T cell populations. The apparent higher mean and range of Ki67 CD4\(^{-}\) cells in TPs reflects the paucity of CD4\(^{+}\) T cells recovered from the frozen samples in such subjects (Table II), and there were no statistically significant differences between LTSNPs and TPs. There was no correlation either between CD4 TREC s and Ki67 expression on CD4\(^{+}\) T cells. In fact, all the children who had TREC s in the CD4\(^{+}\) T cells also expressed Ki67\(^{+}\).

We also analyzed the expression of CD103 on CD45RO\(^{-}\)CD8\(^{-}\) T cells because this population has been described previously as recent thymic emigrants (27). We could indeed measure such a population in all groups; however, there was again no measurable association with a particular group or variable (Table II).

**CD95 expression is correlated to disease status and age**

Apoptosis, which plays a central role in depleting the CD4\(^{+}\) T cells from infected individuals, is finely regulated by the CD95 (APO-1/Fas) system (28). A robust increase in CD95 expression has been observed previously in T cells from HIV-1-positive children as compared with healthy controls, as well as increased sensitivity to CD95-mediated cell death (29). Therefore, we measured CD95 expression on CD4\(^{+}\) and CD8\(^{+}\) T cells by triple FACS staining. No clear correlations could be drawn from analysis of CD95 expression on CD8\(^{+}\) T cells. Although the number of LTSNPs and ENPs analyzed was small, CD95 expression on CD4\(^{+}\) T cells was clearly different between progression groups (Table II and Fig. 7). Previous studies have shown a strong correlation between loss of CD4\(^{+}\) lymphocytes and increased expression of CD95 (29); we could only find such a negative association among TPs (Spearman’s \(p = -0.627, p = 0.029\)) but not with LTSNPs. Because donor age was also shown to correlate with the levels of CD95\(^{+}\) cells (30), the increased expression in LTSNPs as compared with TPs could simply be attributed to the classification adopted for our study. However, within the older age group (LTSNPs and LTSPs), the percentage of CD95\(^{+}\) cells among CD4\(^{+}\) lymphocytes was significantly higher in progressors than

**FIGURE 3.** ICS. Fresh venous 0.5 ml of whole blood aliquots was stimulated with anti-CD28 and anti-CD49d and either no Ag, CMV, whole viral lysate (10 mg/ml), or combined HIV peptide pools (10 mg/ml final for each peptide). Samples were incubated for a total of 6 h with the Golgi apparatus inhibitor brefeldin A present for the final 5 h. Following lysis of erythrocytes, cells were fixed, permeabilized, and simultaneously stained for surface molecules and intracellular cytokine. Events shown are gated on CD\(^{+}\) lymphocytes in NY27 (the strongest responder) where 1.30\% of CD4 cells were Gag-specific as determined by IFN-\(\gamma\) production.

**FIGURE 4.** Responses Fifty SFU/million CD8\(^{+}\)-depleted PBMCs were significant. The stronger of the two responses to CMV and PPD is shown for each donor. Responses to the four pools of overlapping peptides representing clade A p24 and the two pools representing clade A p17 have been summed to produce the total Gag response. Seven LTSNPs (NY 2, 4, 18, 27, 35, 51, and 52), one LTSP (NY 1), and three ENPs (NY 16, 54, and 76) exhibited preserved HIV-1-specific CD4\(^{+}\) T cell responses to pooled HIV-1 Ags and to PPD and CMV. In contrast, HIV-1-specific CD4\(^{+}\) T cells were not detected among three TPs (NY 20, 58, and 74), although responses to the recall Ags remained intact.
nonprogressors \( (p = 0.041) \). This was not observed in children < 8 years old (TPs and ENPs).

**Coreceptor allele and genotype frequencies in the cohort**

Coreceptor genotyping revealed no children expressing either the CCR5 or the SDF-1 \(-\)UTR mutations associated with delayed disease progression in Caucasian cohorts.

**Defective nef genes are not identified in nonprogressors**

Phylogenetic analysis identified HIV-1 clades A, C, D, and a recombinant A/D subtype. All children harbored viruses with full-length nef sequences. Nucleotide sequences of the nef gene (>600 bp) derived from proviral DNA from 25 children were determined by direct sequencing. PCR amplification yielded products of the expected size for a full-length nef/LTR fragment corresponding to the wild-type strain by gel analysis (R. Chakraborty, M. Reinis, T. Rostron, S. Philpott, T. Dong, A. D’Agostino, R. Musoke, E. DeDeSilva, M. Stumpf, B. Weiser, H. Burger, and S. L. Rowland-Jones, manuscript in preparation) (deposited in GenBank under accession nos. AF538630 through AF538677).

**FIGURE 6.** TREC$ were detected equally in some but not all of the LTSNPs and TPs. In 2001, CD8 or CD4 cells were sorted sequentially from 41 PBMC samples frozen from 2000. TREC$ levels in the CD4-sorted and CD8-sorted PBMCs from treatment-naive children are indicated in open symbols and that of children who received a partial course of HAART in filled symbols. Key: diamonds, LTSNP; triangles, LTSP; squares, TP; and circles, ENP. CD4 TREC$ are positively correlated with CD4 count \( (p = 0.009) \). Correlation between CD8 TREC$ and CD4 count did not reach statistical significance.

**Table II.** Comparison of T cell phenotype, TREC$, and Ki67 in the 41 children studied according to the progression group$^a$

<table>
<thead>
<tr>
<th>No of patients</th>
<th>LTSNP</th>
<th>TP</th>
<th>LTSP</th>
<th>ENP</th>
<th>HAART</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral load ( (\text{copies/ml}) )</td>
<td>402,302 ((1,080–496,575))</td>
<td>582,149 ((12,502–1,810,786))</td>
<td>667,683 ((53,824–1,999,385))</td>
<td>279,440 ((8,682–442,694))</td>
<td>474,411 ((371–100,994))</td>
</tr>
<tr>
<td>CD4 (^{+}) (% of PBMC)</td>
<td>624 ((428–1,872))</td>
<td>393 ((12–781))</td>
<td>299 ((17–516))</td>
<td>69 ((18–120))</td>
<td>100 ((66–1,235))</td>
</tr>
<tr>
<td>CD4 (^{+}) CD45RA (^{+}) (% of PBMC)</td>
<td>72 ((2–381))</td>
<td>22 ((0–106))</td>
<td>90 ((63–117))</td>
<td>69 ((18–120))</td>
<td>33 ((25–49))</td>
</tr>
<tr>
<td>CD4 (^{+}) CD45RO (^{+}) (% of PBMC)</td>
<td>41 ((7–114))</td>
<td>14 ((0–55))</td>
<td>69 ((18–120))</td>
<td>33 ((25–49))</td>
<td>15 ((2–32))</td>
</tr>
<tr>
<td>TRECs in CD4 (^{-}) (% of PBMC)</td>
<td>5 positives ((0–154))</td>
<td>6 positives ((0–422))</td>
<td>2 positives ((0–1320))</td>
<td>3 positives ((0–1280))</td>
<td>5 positives ((0–92))</td>
</tr>
<tr>
<td>Ki67 (^{+}) CD4 (^{+}) (% of CD4 (^{+})) ( (p = 0.009) )</td>
<td>2 ((0–6.2))</td>
<td>7.3 ((0.7–20))</td>
<td>3.3 ((0.9–3.7))</td>
<td>3.6 ((0–6.1))</td>
<td>12.9 ((0.8–55.6))</td>
</tr>
<tr>
<td>TRECs in CD8 (^{-}) (% of PBMC)</td>
<td>16 ((0–73))</td>
<td>275 ((0–3,438))</td>
<td>0 positives</td>
<td>4 positives ((0–62))</td>
<td></td>
</tr>
<tr>
<td>Ki67 (^{+}) CD8 (^{+}) (% of CD8 (^{+})) ( (p = 0.009) )</td>
<td>2.4 ((0.8–1.1))</td>
<td>2.5 ((0.9–5.5))</td>
<td>2.5 ((1.6–4.5))</td>
<td>3.1 ((2.6–4.3))</td>
<td>3.6 ((0.5–10.7))</td>
</tr>
<tr>
<td>CD8 (^{+}) CD103 (^{+}) (% of PBMC)</td>
<td>2.7 ((0.2–8.2))</td>
<td>1.08 ((0.4–2.1))</td>
<td>1.2 ((0.5–2.1))</td>
<td>2 ((0.1–4.8))</td>
<td>1.4 ((0.2–2.1))</td>
</tr>
<tr>
<td>CD95 (^{+}) CD4 (^{+}) (% of CD4 (^{+})) ( (p = 0.009) )</td>
<td>8.3 ((0.2–58.6))</td>
<td>2.89 ((0.3–17.2))</td>
<td>13.1 ((8.5–15.8))</td>
<td>1.55 ((0.9–0.22))</td>
<td>9.25 ((3.9–16.3))</td>
</tr>
<tr>
<td>CD95 (^{+}) CD8 (^{+}) (% of CD8 (^{+})) ( (p = 0.009) )</td>
<td>4.03 ((0.3–35.2))</td>
<td>0.22 ((0–0.07))</td>
<td>5.9 ((3.2–13.6))</td>
<td>0.15 ((0.1–0.2))</td>
<td>2.48 ((0.2–8.7))</td>
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</tr>
</tbody>
</table>

$^a$ The means are indicated, and the range is shown in brackets.

**FIGURE 5.** Reduced expression of CD4 \(^{+}\) CD45RA \(^{+}\) CD45RO \(^{-}\) T cells from TPs. In 2001, CD4, CD8, CD45RA, and CD45RO expressions were studied by four-color FACS in PBMC samples frozen from 2000 from 39 children. CD4 \(^{+}\) CD45RA \(^{+}\) CD45RO \(^{-}\) T cells were gated within the PBMCs and are expressed as a percentage of total cells \( (A) \) or related to the CD4 T cell count \( (B) \). The individual dots represent the outliers. The \( p \) value for the Mann-Whitney \( U \) test is indicated.
Discussion

Pediatric LTNP are rare in Caucasian cohorts, and none have so far been described in Africa where the course of HIV-1 infection in children appears to be particularly rapid (1). Accelerated HIV disease progression in African children may be a consequence of many factors, including limited access to proper nutrition, clean water, and health care, as well as exposure to a high prevalence of respiratory and gastrointestinal pathogens from HIV-infected parents and in the general community. The high quality of care in Nyumbani orphanage minimizes these cofactors in our study (31), allowing us to identify correlates of delayed HIV disease progression in children raised in the same environment.

In contrast to the majority of adults, CD4+ T cell depletion develops early in most untreated vertically infected children (32). Plasma HIV-1 RNA levels are uncontrolled in infants and do not decrease <10^5 copies/ml until at least the third year of life, with a continued reduction in plasma HIV-1 RNA levels observed in some vertically infected children until the age of 5–6 years (33). This prolonged elevation of plasma HIV-1 RNA levels may reflect the large pool of cells that are permissive to viral replication in the child during a time of high thymopoiesis. Other investigators have postulated that virus-specific immune responses are deficient in young children (34), although recent data in congenital CMV infection have shown that infants are able to mount a CD8+ T cell response with the same functional phenotype as adults (35). The factors that determine the eventual viral set point in HIV-1-infected children during clinical latency are unknown, but it may be reduced by efficient HIV-specific CD8+ lymphocyte and Th cell responses (36). It is unclear whether differences in viral set points correlate with disease progression in children (33, 37, 38).

Factors associated with delayed disease progression in HIV-1-infected adults include strong cellular immune responses with preserved HIV-1-specific CD4+ T cell responses (9) and host gene polymorphisms, particularly in genes encoding the chemokine receptors used by HIV-1 for cell entry and their ligands. However, the protective CCR5Delta32 mutation and the 3'-UTR polymorphism in the gene for SDF-1 are uncommon in Africa (12) and were not identified in any children in this study.

The nef gene encodes an accessory protein expressed early in the life cycle of HIV/SIV, which supports viral infectivity and replication by a number of mechanisms. These include interference with signal transduction pathways and down-regulation of the cell surface receptors CD4 and HLA class I (A and B not C) (39). Nef interacts with proteins in the TCR-signaling pathway (40). Its association with the TCR-ζ chain, for example, results in the up-regulation of Fas ligand and the induction of apoptosis in HIV-1-infected T-lymphocytes (40). In clinical practice, the biological effects of nef have been observed most strikingly among primate and human non- or slow progressors infected with an attenuated viral phenotype with in-frame stop codon mutations, deletions, or frameshift mutations at conserved positions (3, 41). No large sequence deletions were detected in nef gene sequences from any of the 25 children, including the LTNSPs in this study, and the sequences of functionally active regions showed no significant changes from relevant clade reference sequences. In the absence of host genetic and virologic factors, strong cellular immune responses may have played a major part in delayed disease progression in our nonprogressor cohort.

Virus-specific CD4+ T cell responses are pivotal in maintaining effective immunity to a number of chronic viral infections (9, 42) but are usually undetectable during chronic HIV-1 infection, probably because these cells are preferentially targeted by HIV-1 (43). Preserved HIV-1 gag-specific CD4+ Th responses have been described in adult nonprogressors (9), but this is the first such description in pediatric slow/nonprogressors.

We speculated that the persistence of HIV-specific CD4+ T cell responses in children with delayed disease progression might be due to lack of thymic infection in some children together with preserved thymic output. However assays of thymopoietic activity showed no correlation with preserved HIV-1 CD4+ T cell responses in LTNSPs. Furthermore CMV-specific CD4+ T cells were detected consistently among most children from the cohort irrespective of disease progression and measured thymic activity (J. Sutton, V. Appay, S. Seneviraine, T. Dong, C. Liesnard, A. Marchant, A. Cross, R. Musoke, A. D’Agostino, S. Rowland-Jones, and R. Chakraborty, manuscript in preparation). These data suggest that the mechanisms underlying long-term maintenance of HIV-1-specific CD4+ T cells in children have yet to be defined.

HIV infection has been associated with accelerated involution of the thymus. A number of investigators have demonstrated both in adults and children that peripheral TREC are decreased during HIV-1 infection and increase after HAART, suggesting recovery of the thymic function upon viral suppression (44, 45). Thymic volume itself has also been shown to increase after HAART in adults with depleted baseline TREC levels and in children and has a strong predictive value for the immunological effect of HAART (46). In children, repopulation of the Th lymphocytes after HAART involves mainly naive CD4+ T cells, with only a small increase in memory CD4+ T cells (47) reflecting the greater regenerative capacity of the thymus in children. Peripheral CD4 TREC were also detected in most of our children after commencement of HAART, even with structured interruption and very advanced immunosuppression (13). CD4+ naive T cell populations were similarly detected in five of the treated children.

In HIV-infected treatment-naive children from a Western cohort, long-term nonprogression was linked to preservation of thymic function. These included almost normal TREC and IL-7 values among long-term asymptomatic children, compared with lower levels among rapid progressors (48). In the present study, LTNSPs had a higher number of CD45RA+CD4+ cells. However, in contrast with previous studies and despite a strong correlation between levels of CD4+ TREC and number of CD45RA+CD4+ cells, TREC levels did not directly correlate with long-term survival at the time of analysis. We could not identify differences in TREC between progression groups, and some of the children maintained...
thymopoiesis despite active viral replication. Because no longitudinal data are available at present for this cohort, we cannot study TREC levels in parallel with medical history and offer a reasonable explanation for the lack of detectable TRECs in some of the children. Although thymic output is affected during HIV infection (44) and might still play a role in fighting the virus, our results suggest that other factors control disease progression, such as the persistence of HIV-1-specific CD4+ T cell responses.

Apoptosis may be pivotal in depleting CD4+ T lymphocytes; however, only one study has previously documented the increased expression of CD95 on CD4+ T cells and increased sensitivity of PBMCs to CD95-induced apoptosis during pediatric HIV infection (30). We found no clear correlation with CD45RA or TRECs but observed a significant correlation with age. Although we could not analyze apoptosis per se, older children (≥8 years, LTSPs) that were progressing had a higher proportion of CD95+ CD4+ T cells than the nonprogressors from the same age group (LTSPNs). These data suggest precocious aging of lymphocytes within the progressor group compared with children with slow and nonprogressive infection and that CD95 overexpression might parallel the immunologic progression of the disease. Whether CD4+ T cells from progressors are more sensitive to apoptosis as a consequence of CD95 overexpression remains unclear but could explain the presence of preserved HIV-1-specific CD4+ Gag responses in LTSPNs.

The reasons for long-term survival and maintenance of HIV-1-specific CD4 T cell responses in the cohort are unclear. HIV-infected treatment-naive adolescents have been shown to harbor greater perturbations in their TCR Vβ repertoire in comparison with HIV-negative subjects, and these were negatively associated with CD4 TREC levels (49). Different abilities in maintaining a diverse T cell repertoire between LTSPNs and TPs might explain the persistence of good HIV-1-specific CD4+ T cell responses.

The mechanisms whereby HIV-1-specific CD4+ T cells contribute to effective antiviral immunity may relate to enhanced effector CD8+ T cell activity by priming CD8+ T cell responses (50), maintaining CD8+ T cell memory (51), and maturing CD8+ T cell function (52). HIV-1-specific CD8+ T cell responses are thought to be important in suppression of viral replication during acute and chronic HIV-1 infection by direct cytosis of infected cells and by secreted antiviral cytokines (22, 23). Strong HIV-1-specific CD8+ T cell responses have been detected in LTSPNs (16, 53), but in adult cohorts, the magnitude of responses did not differ between LTSPNs and progressors (54). In our study, strong HIV-1-specific CD8+ T cell responses and low levels of perforin expression in tetramer-stained HIV-specific CD8+ T cells may have been common to all progression groups.

In summary, strong HIV-1-specific Th cell responses were detected in a selected group of pediatric LTSS infected with nonclade B virus from East Africa. In the absence of protective coreceptor polymorphisms and viral attenuation in the nef gene, these responses may have contributed to delayed disease progression. Preserved gag-specific CD4+ Th responses were not related to measured thymic output. Identifying factors that contribute to the maintenance of protective immune responses will be important for therapeutic and prophylactic vaccine development.

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Disclosures
The authors have no financial conflict of interest.

References


