Deficiency of HIV-Gag-Specific T Cells in Early Childhood Correlates with Poor Viral Containment

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Deficiency of HIV-Gag-Specific T Cells in Early Childhood Correlates with Poor Viral Containment

SiHong Huang,†‡‡ Jacqueline Dunkley-Thompson,† YanHua Tang,* Eric A. Macklin,§ Julianne Steel-Duncan,† Indira Singh-Minott,† Elizabeth G. Ryland,* Monica Smikle, † Bruce D. Walker,*†‡ Celia D. C. Christie,† and Margaret E. Feeney2*‡§¶

Perinatal HIV infection is characterized by a sustained high-level viremia and a high risk of rapid progression to AIDS, indicating a failure of immunologic containment of the virus. We hypothesized that age-related differences in the specificity or function of HIV-specific T cells may influence HIV RNA levels and clinical outcome following perinatal infection. In this study, we defined the HIV epitopes targeted by 76 pediatric subjects (47 HIV-infected and 29 HIV exposed, but uninfected), and assessed the ability of HIV-uninfected infants, whereas responses among infected subjects increased in magnitude and breadth with age. Gag-specific immune response of infants. However, the exact nature of these likely attributable to developmental differences in the antiviral parities in HIV viral kinetics and clinical outcome are most progression to AIDS and death (3). These age-associated dis-

**Compared with HIV-infected adults, infants with perinatally acquired HIV infection maintain much higher levels of plasma viremia during the initial years of infection (1, 2) and are at substantially higher risk for rapid progression to AIDS and death (3). These age-associated disparities in HIV viral kinetics and clinical outcome are most likely attributable to developmental differences in the antiviral immune response of infants. However, the exact nature of these differences and their biological mechanisms remain poorly understood (4). Although T cell responses to HIV can be primed even during fetal life, as demonstrated by the presence of HIV-specific T cells in cord blood (5), early studies suggested that these responses are infrequent and remain narrowly directed against relatively few epitopes during infancy (5–8). However, recent studies using more sensitive assays have established that HIV-specific T cell responses are present in the majority of infants by 1 mo of age (9, 10), and that older children exhibit HIV-1-specific T cell frequencies comparable to those of chronically HIV-1-infected adults (11).

Yet major gaps in our knowledge of the infant T cell response to HIV remain. The majority of pediatric studies performed to date have assessed responses to a limited panel of optimal epitopes (9, 12–14) or to vaccinia constructs expressing whole HIV-1 structural genes (5, 6, 15), and were therefore unable to evaluate the full breadth and epitope specificity of the response. Moreover, assessment of CD8 T cell effector functions beyond IFN-γ production has been very limited, and even fewer data exist regarding the specificity and function of HIV-specific CD4 T cells in infants. The most comprehensive assessment of infant HIV-specific T cell responses performed to date suggested that the HIV proteins preferentially targeted by the T cell response may differ between early infancy and later childhood (10), but the impact of this difference upon containment of viral replication was not addressed. Such differences in T cell targeting during infancy could be of great consequence, because it has recently been demonstrated that CD8 T cells targeting different viral proteins have a divergent influence upon viral containment (16–18). Moreover, the quality of CD8 T cells, as assessed by their ability to exhibit multiple simultaneous effector functions, has been reported to correlate with control of viral replication (19).
We hypothesized that differences in the targets and/or functionality of HIV-specific T cells contribute to the efficacy of the antiviral immune response, and that age-related differences in these parameters may account for the inability of children to establish early containment of viremia. We examined CD8 and CD4 T cell responses to the full HIV-1 proteome in a cohort of infants and children born to HIV-positive mothers and assessed multiple effector functions by multiparameter flow cytometry. Our data indicate that HIV-specific T cell responses are present in the majority of HIV-infected infants, but that several qualitative features distinguish the responses observed in younger infants. Notably, Gag-specific T cell responses were less commonly detected in infants than in children older than 12 mo of age, and targeting of Gag was associated with significantly lower plasma HIV-1 RNA levels. CD8 T cells exhibiting multiple effector functions (IFN-γ, TNF-α, and degranulation) were also detected less frequently in younger infants, and HIV-specific CD4 T cell responses were of very low magnitude in nearly all pediatric subjects, and absent in the youngest infants. These qualitative differences in the CD8 response, coupled with a lack of CD4 T cell help, may be important contributors to the generally poor ability of young infants to restrict viral replication.

Materials and Methods

Study cohort

This study was performed in collaboration with the Kingston Perinatal AIDS Program, a large-scale initiative for the prevention and treatment of perinatal HIV-1 infection in Jamaica (20). The Kingston Perinatal AIDS Program provides antiretroviral therapy for the prevention of mother-to-child transmission of HIV-1, counsels HIV-positive mothers against breastfeeding, supplies free infant formula, and provides care and antiretroviral therapy for HIV-infected children in accordance with World Health Organization guidelines. The study was approved by the Institutional Review Board of the Massachusetts General Hospital and the Ethics Committee of the University Hospital of the West Indies in Kingston, Jamaica. A parent or legal guardian of each subject provided written informed consent before participation.

Seventy-six infants and children (age 5 wk to 10 years) born to HIV-positive mothers were enrolled. Because molecular diagnostic HIV testing of infants had not been instituted in Jamaica at the time this collaboration was initiated, some infants <18 mo of age were of indeterminate HIV-1 infection status upon enrollment. Of these, 29 infants (age 5 wk to 17 mo) were later determined by PCR testing to be HIV negative, and therefore in screening assays on an automated peptide synthesizer by using Fmoc (9-fluorenylmethoxycarbonyl) chemistry. Previously described CD8-restricted HIV-1 optimal peptides (Los Alamos HIV Sequence Database www.hiv.lanl.gov) were synthesized by identical methods. Thirty-two overlapping peptides were added at a final concentration of 20 μg/ml per 106 cells were required to comprehensively screen all HIV-1 proteins using the overlapping peptide set described above. To minimize the cell number required for this comprehensive screening, we used a matrix approach that has been previously validated in both pediatric and adult cohorts studied in our laboratory (11, 22). The 411 peptides were pooled into five two-dimensional matrices such that each peptide was present in two different pools, and each pool contained between 7 and 11 peptides. The resulting 92 pools were plated at a final concentration of 20 μg/ml per well with 50,000–100,000 PBMC/well on a single 96-well plate; thus, only 5–10 × 104 cells were required to comprehensively screen all HIV-1 proteins. Each peptide within the positive pools was then tested individually in a separate ELISPOT assay the following day by using cells that had been incubated overnight at 10°C. A total of 411 overlapping peptides (18 mers with a 10-aa overlap) spanning the entire HIV-1 clade B consensus sequence was synthesized for use in screening assays on an automated peptide synthesizer by using Fmoc (9-fluorenylmethoxycarbonyl) chemistry. Previously described CD8-restricted HIV-1 optimal peptides (Los Alamos HIV Sequence Database www.hiv.lanl.gov) were synthesized by identical methods.

**Table I. Clinical and demographic characteristics of the study cohort**

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Sex (F)</th>
<th>Viral Load</th>
<th>CD4&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CD4%&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Treatment Duration (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected (n = 29)</td>
<td>7.6 (1.2–16.8)</td>
<td>41%</td>
<td>&lt;400 (all)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>HIV&lt;sup&gt;+&lt;/sup&gt; ARV&lt;sup&gt;+&lt;/sup&gt;-naive (n = 25)</td>
<td>13 (4–115)</td>
<td>60%</td>
<td>208,000 (2,670–893,000)</td>
<td>872 (42–2,240)</td>
<td>41 (7–52)</td>
</tr>
<tr>
<td>HIV&lt;sup&gt;+&lt;/sup&gt; on ARV (n = 22)</td>
<td>34.5 (13–96)</td>
<td>59%</td>
<td>29,050 (&lt;50–750,000)</td>
<td>1,255 (226–2,112)</td>
<td>41 (16–54)</td>
</tr>
</tbody>
</table>

<sup>a</sup> All data are presented as median and range values.

<sup>b</sup> CD4 data were available for 15 of the ARV-naive subjects and 14 of the subjects on ARV.

**IFN-γ ELISPOT assay**

Ninety-six-well polyvinylidene difluoride-backed plates (Millipore) were coated overnight with 2 μg/ml anti-IFN-γ mAb (Mabtech). Fresh PBMC were added into these precoated plates at 50,000–100,000 cells/well in 100 μl of R10 medium. Peptides were added at a final concentration of 20 μg/ml, and plates were incubated for 16 h at 37°C and 5% CO2, then processed by standard methods (21). Three negative control wells containing cells and medium alone and a positive control well containing PHA were included in each assay. Individual IFN-γ-secreting cells were counted using an AID ELISPOT reader system (Cell Technology). Results were calculated as the number of spot-forming cells (SFC) per million input cells (SFC/million) after subtraction of the background response (mean SFC of all no-Ag wells; in all cases, ≤30 SFC/million). A response was considered positive if it was both ≥50 SFC/million PBMC and ≥2 times the average of the negative control wells.

**Full genome ELISPOT screening for HIV-1-specific responses**

Subjects were screened by ELISPOT for responses to all translated HIV-1 proteins using the overlapping peptide set described above. To minimize the cell number required for this comprehensive screening, we used a matrix approach that has been previously validated in both pediatric and adult cohorts studied in our laboratory (11, 22). The 411 peptides were pooled into five two-dimensional matrices such that each peptide was present in two different pools, and each pool contained between 7 and 11 peptides. The resulting 92 pools were plated at a final concentration of 20 μg/ml per well with 50,000–100,000 PBMC/well on a single 96-well plate; thus, only 5–10 × 104 cells were required to comprehensively screen all HIV-1 proteins. Each peptide within the positive pools was then tested individually in a separate ELISPOT assay the following day by using cells that had been incubated overnight at 10°C.

**Assessment of HIV-1-specific degranulation and cytokine production**

Whole blood (300 μl, obtained in sodium heparin tubes) was aliquoted with the costimulatory Abs anti-CD28 and CD49d (1 μg/ml each; BD Biosciences) and FITC-conjugated anti-CD107a Ab (BD Biosciences) into 15-ml polypropylene tubes. Overlapping HIV-1 peptides spanning the full viral genome were added at a final concentration of 2 μg/ml, in five separate pools: Gag (66 peptides); Nef (27 peptides); Pol (132 peptides); Env (113 peptides); and Rev, Tat, Vpr, Vif, and Vpu (72 peptides). Each assay included a positive control tube containing PHA (1 μg/ml) and a negative control tube with no peptide. Samples were incubated at a 5-degree slant at 37°C for a total of 6 h. The protein transport inhibitors brefaldin A (1 μg;
GolgiPlug; BD Biosciences) and monensin (0.6 µg; GolgiStop; BD Biosciences) were added after the first hour. Following this stimulation step, 40 µl of 50 mM EDTA solution was added to arrest cell activation and to remove adherent cells (15 min at room temperature). Simultaneous lysis of RBCs and fixation of nucleated cells were achieved with 3 ml of 3 × FACS lysing solution (BD Biosciences). Cells were washed in PBS containing 1% heat-inactivated FBS (Sigma-Aldrich), and then permeabilized using 500 µl of 1 × FACS Permeabilizing Solution 2 (BD Biosciences) for a 10-min incubation. Finally, cells were stained with a panel of fluorescently labeled Abs to CD3 (Pacific Blue), CD8 (allophycocyanin-Cy7), CD4 (allophtococyanin), IFN-γ (PE-Cy7), TNF-α (Alexa Fluor 700), and IL-2 PE (all from BD Biosciences) for 30 min at room temperature in the dark.

Flow cytometry acquisition and analysis

Samples were acquired within 24 h of staining on an LSR II flow cytometer using FACSData acquisition software (BD Immunocytometry Systems; v4.1.2), with bead compensation (CompBeads; BD Biosciences). Only samples from which at least 20,000 CD4⁺ and 20,000 CD8⁺ T lymphocytes were acquired were included in the analysis (median 50,000 CD4⁺ events and 54,345 CD8⁺ events). All data were analyzed using FlowJo software (Tree Star). A lymphocyte gate was set based on forward and side scatter, and CD3⁺ events within this gate were analyzed. CD8⁺ T cells were identified by first gating on CD3⁺CD4⁻ cells, and then gating on cells positive for CD8. Similarly, CD4⁺ T cells were identified by first gating on CD3⁺CD8⁻ cells, and then gating on cells positive for CD4. The frequency of CD4⁺ and CD8⁺ T cells producing each cytokine (or degranulating, in the case of CD107a) was determined by subtracting the percentage of cytokine-positive cells in the unstimulated CD28/49d control tube from that of each peptide-stimulated tube. For this single-parameter analysis, a response was considered positive if it was ≥3 times the negative control, and if it exceeded 0.01%. If there were at least 10 cytokine-positive events (corresponding to 0.02% if 50,000 CD4⁺ or CD8⁺ T cells were acquired). The highest background levels observed for any subject in the CD8⁺ subset were 0.03, 0.098, 0.028, and 0.18% for IFN-γ, TNF-α, IL-2, and CD107a, respectively. The maximum background levels observed in the CD4⁺ subset were 0.012, 0.067, and 0.041% for IFN-γ, TNF-α, and IL-2, respectively. Double-positive (IFN-γ⁻TNF-α⁻) and triple-positive (IFN-γ⁻TNF-α⁻CD107a⁻) cells were identified by sequential gating on these parameters; a response was considered positive if it exceeded 0.005% and if it met criteria for positivity for each of the individual parameters.

Statistical analysis

Data analysis was performed using SAS (version 9.1.3; SAS Institute). All statistical tests were two tailed, with p < 0.05 considered significant. Two-group comparisons of viral load and of HIV-specific T cell magnitude were performed with Wilcoxon rank sum test, with Bonferroni correction for multiple comparisons where indicated. Categorical variables were compared using Fisher’s exact test. The χ² test for trend was used to compare the frequency of responses to each viral protein across age strata. The correlation of age and viral load with immune response parameters (total magnitude of HIV-specific T cell response and the number of peptides targeted) was assessed using the Spearman rank correlation coefficient. Multiple regression controlling for log age was used to analyze the correlation between log-viral load and the breadth (number of peptides targeted) and magnitude (total SFC/million) of immune response and their interaction. The combined effects of immune response were evaluated as a 3 degree of freedom contrast. Models with rank and log transformation (specifically, log(no. peptides + 1), log(SFC/million + 1), and a separate indicator for SFC/million = 0 for all peptides) of immune response parameters were explored to reduce the influence of highly leveraged points, but our inference did not change. The influence of response to specific viral proteins was evaluated by including indicator variables for targeting of each viral protein in the model described above. A final model was selected by stepwise regression, retaining log age, and total immune response parameters at all steps. The relationship between viral load and magnitude of Gag vs non-Gag targeting T cells was tested in a multiple regression controlling for log age and total breadth of immune response. Pearson correlation coefficients are provided for all multiple regression analyses.

Results

Absence of HIV-specific T cell responses among uninfected infants exposed to HIV in utero

Several studies have described HIV-1-specific CTL responses among highly exposed, but persistently seronegative adults (23–25) and among infants exposed to HIV in utero (26–29). However, other investigators have failed to detect such responses among uninfected infants (5, 8, 30, 31). We recruited 29 HIV-exposed, but uninfected infants and young children (age 5 wk to 17 mo) from a nonbreastfeeding population and comprehensively screened these subjects for T cell responses to HIV. All investigators were blinded to the infection status of these subjects at the time the assays were performed and analyzed, because no diagnostic testing results were available before enrollment and processing of samples. All subjects were subsequently documented to be HIV uninfected based on negative HIV DNA and RNA PCR assays. Comprehensive screening by ELISPOT for responses to a panel of overlapping peptides spanning the entire translated HIV proteome demonstrated that there was no detectable virus-specific IFN-γ response in any infant (Fig. 1). Six of these subjects were further screened for IFN-γ ELISPOT responses to optimal HIV-1 epitopes corresponding to their class I HLA alleles (7–46 peptides tested; median 24), which is the most sensitive method for detection of Ag-specific T cell responses. Again, no response to any optimal peptide was observed. To investigate the possibility that virus-specific T cells were present, but merely lacked the ability to produce IFN-γ, 17 of these HIV-negative subjects were assessed by multiparameter intracellular cytokine staining for IFN-γ, TNF-α, and IL-2 production and for degranulation (assessed by CD107a staining) following stimulation with overlapping peptide pools spanning all HIV proteins. Again, none of the subjects exhibited a response that significantly exceeded background. These data support multiple prior studies suggesting that HIV-negative infants exposed to HIV in utero do not mount durable T cell responses to the virus (5, 8, 30, 31).

The breadth and magnitude of the HIV-specific T cell response increase with age

To quantify the breadth and magnitude of the HIV-specific T cell response in young children and determine how these parameters change with age, we comprehensively screened 47 HIV-1-infected infants and children for responses to a set of overlapping peptides spanning all HIV proteins by IFN-γ ELISPOT. Thirty-six subjects (77%) exhibited an IFN-γ response to HIV, consistent with published pediatric studies (9, 10). Among those subjects in whom an HIV-specific response was detected, the total magnitude of response (determined by summation of all positive responses to individual screening peptides) varied widely from 50 to 15,540 SFC/million PBMC, with a median of 1,855 SFC/million (Fig. 1). The number of peptides targeted also varied widely, from 1 to 29 (median 8; data not shown). Infants who lacked any IFN-γ response to
HIV were generally <1 year of age (8 of 11 subjects) and exhibited a very high viral load (median 750,000 copies/ml). We analyzed the impact of age on the breadth and magnitude of the HIV-specific T cell response, limiting our analysis to subjects who were antiretroviral naive (n = 25) or who maintained a high level of HIV-1 viremia (≥50,000 RNA copies/ml) despite therapy (n = 9), because drug-mediated viral suppression has been shown to diminish HIV-specific CTL responses (11, 32, 33). Among this subset of subjects, there was a positive correlation between age and both the total magnitude of HIV-specific T cell response (Spearman’s r = 0.40, p = 0.02; Fig. 2A) and the number of peptides targeted (Spearman’s r = 0.52, p = 0.002; Fig. 2B). When the analysis was restricted to subjects who had never received antiretroviral therapy (n = 25), the correlation between age and both the magnitude of response (Spearman’s r = 0.40, p = 0.05) and the breadth of response (Spearman’s r = 0.53, p = 0.007) was essentially unchanged. Although these data were obtained cross-sectionally, they suggest that the antiviral T cell response increases throughout early childhood, recruiting more T cells and targeting more viral epitopes.

**Age-related differences in the HIV proteins preferentially targeted by virus-specific T cells**

Recent population-based studies suggest that the efficacy of a CD8 T cell response may depend in part upon the HIV protein from which the targeted epitope is derived – in particular, CTL targets of Gag epitopes appear to be associated with superior viral containment (16–18). We therefore examined the distribution of T cell responses among all HIV proteins, and determined the relationship between age and the prevalence and magnitude of protein-specific responses. Thirty-four subjects who were antiretroviral naive or highly viremic despite therapy (≥50,000 HIV RNA copies/ml) were screened for responses to all HIV proteins by IFN-γ ELISPOT. Each HIV protein was targeted by at least one subject. Overall, the most frequently recognized protein was Nef (recognized by 74% of subjects), followed by Gag (56%) and Env (50%). To determine whether age influenced the distribution of virus-specific responses, subjects were stratified into three age groups, as follows: ≤12 mo (n = 12), 13–24 mo (n = 12), and ≥24 mo (n = 11), and the prevalence of a response to each HIV-1 protein was determined separately for each age stratum (Fig. 3A). Responses to the HIV-1-Gag protein were particularly infrequent during early infancy (present in only one of the six infants younger than 7 mo of age), but their frequency increased markedly with age from 33 to 58 to 82% across the three age strata (χ² test for trend, p = 0.011; Fig. 3A). No significant age-related increase in recognition was observed for the other viral proteins. When these data were analyzed as the relative proportion of the total HIV-specific response targeting each individual viral protein, an age-related increase in the contribution of Gag-specific responses was again noted (χ² test for trend, p < 0.001; Fig. 3B).

We next compared the total magnitude of the T cell response to Gag, Nef, and Env between older and younger children (Fig. 3C). Again, the total magnitude of Gag-specific responses among older children (>24 mo) significantly exceeded that observed among younger children (p = 0.014; Fig. 3C). The magnitude of Nef-specific and Env-specific T cell responses did not differ between...
the older and younger children. When analysis was restricted to younger children with a detectable response to each protein, there was trend toward higher frequencies of Gag-specific cells among the older age group (median 351 vs 1560 SFC/million), although this difference did not achieve statistical significance in the small sample of 19 responders ($p = 0.13$). Finally, subjects were categorized according to their dominant response, defined as the HIV protein eliciting the highest frequency of responding T cells. A dominant response to Nef or Env was observed more frequently among children <24 mo of age than among the older children (83 vs 40%; $p = 0.07$), 60% of whom mounted a dominant response to Gag. Together, these cross-sectional data suggest that the ability to mount responses to various viral proteins may differ by age, with preferential targeting of Nef and Env during infancy and a shift to targeting of Gag only during later childhood. Alternatively, there may be a survival bias favoring infants with Gag-specific T cell responses.

**Total HIV- and Gag-specific T cell frequencies correlate inversely with viral load**

The relationship between plasma viral load and the frequency of virus-specific T cells in HIV-infected individuals is complex. Although several studies have failed to detect a correlation between viral load and the total HIV-specific CD8 T cell response (11, 22, 34), some investigators have reported a negative correlation of viral load with responses to particular viral epitopes or proteins (16, 18, 35, 36), whereas others have reported a positive correlation (16, 34, 37). These conflicting findings have led to speculation that HIV-specific T cells of differing protein specificity may vary in their anti-viral efficacy. We performed a cross-sectional analysis of the relationship between viral load and virus-specific immune responses in antiretroviral-naive pediatric subjects ($n = 25$), looking first at the total HIV-1-specific T cell response and then at responses to individual viral proteins.

We found a highly significant inverse correlation between HIV viral load and the total breadth of the HIV-specific T cell response (Spearman’s $r = -0.71$, $p < 0.001$; Fig. 4A), as well as the total magnitude of this response (Spearman’s $r = -0.63$, $p < 0.001$; Fig. 4B). Because HIV RNA levels are known to decline over time in young children, we repeated this analysis, controlling for age using multiple regression. The breadth and magnitude of the CD8+ T cell immune response remained inversely correlated with viral load after adjustment for age ($p = 0.027$).

We next assessed whether targeting of particular HIV-1 proteins was associated with control of viremia by comparing plasma HIV-1 RNA levels among responders and nonresponders to each viral protein. Children who mounted a T cell response to Gag had significantly lower viral loads than to those who did not ($p = 0.005$; Fig. 5). No significant difference in viral load was observed between responders and nonresponders to the other HIV-1 proteins. The association of viral load with a T cell response to Gag remained significant after Bonferroni correction for multiple comparisons ($p = 0.04$). In light of this finding, we extended our multiple regression model to include indicator variables signifying the presence or absence of a response to each individual HIV-1 protein, to assess whether targeting of particular proteins was associated with differences in HIV-1 viral load. Stepwise regression yielded a model that indicates that targeting of Gag was associated with lower HIV-1 viral load ($p = 0.009$), whereas targeting of Nef was associated with higher viral load ($p = 0.02$). Finally, we stratified all T cell responses into Gag and non-Gag responses and analyzed the relationship of the summed magnitude of these responses to viral load after adjustment for log age and total breadth. This analysis revealed that the total magnitude of Gag-specific responses correlated inversely with viral load ($p = 0.037$; Fig. 4C), whereas no significant correlation was observed between viral load and the total magnitude of non-Gag responses (data not shown). Together, these findings provide additional support for an important role for Gag-specific T cell responses in the control of HIV-1 viremia. Alternatively, the ability to mount Gag-specific T cell responses may be impaired in the setting of high-level HIV-1 viremia.

**FIGURE 4.** Total and Gag-specific T cell responses correlate inversely with viral load. Cross-sectional analysis of HIV-1-specific T cell responses among 25 antiretroviral-naive subjects demonstrated a highly significant inverse correlation between HIV viral load and the total breadth (A) of the HIV-specific T cell response (Spearman’s $r = -0.71$, $p < 0.001$), as well as the total magnitude (B) of this response (Spearman’s $r = -0.63$, $p < 0.001$). The breadth and magnitude of the HIV-specific T cell immune response remained inversely correlated with viral load after controlling for age in a multivariate regression model ($p = 0.027$ for the aggregate effect of breadth and magnitude). C. The total magnitude of the Gag-specific T cell response correlated inversely with viral load after adjusting for known covariates ($p = 0.037$).

**FIGURE 5.** Responders to HIV-1 Gag exhibit lower viral loads. Subjects with Gag-specific T cell responses ($n = 14$, median VL = 703,000 copies/ml) had significantly lower viral loads than Gag nonresponders ($n = 11$, median VL = 102,090 copies/ml; $p = 0.005$). Bars indicate median values.
We examined production of IFN-γ by virus-specific CD8 T cells in the pediatric population, and whereas infants have been shown to exhibit HIV-1-specific T cell responses even in cord blood (5, 8–10), it is not known whether such responses are functionally mature. Polyfunctional CD8 T cells were present in the majority of pediatric subjects. HIV-specific CD8 T cells producing both IFN-γ and TNF-α were observed in 64% of subjects overall. Triple-positive (IFN-γ+TNF-α+CD107a+) cells were present in 50% of subjects, and were observed in response to all five peptide pools. Quadruple-positive cells (IFN-γ+TNF-α+CD107a+IL-2+) were not detected in any subject, given the rarity of IL-2-producing CD8 T cells. Age appeared to influence the ability to mount a polyfunctional response, as CD8 T cells exhibiting three effector functions (IFN-γ+TNF-α+CD107a+) were detected less frequently in children <24 mo of age (n = 8) compared with older children, although this difference did not achieve statistical significance (p = 0.11, χ² test). Of note, such responses were not detected in any subjects who were <12 mo of age (Spearman’s r = 0.586, p = 0.014; dotted line indicates 95% confidence band).

Functionality of CD8+ T cells in perinatally HIV-infected subjects

It has been reported previously that control of HIV-1 viremia in adults is associated with the presence of polyfunctional HIV-1-specific CD8+ T cells – that is, cells that exhibit the capacity to degranulate and to produce multiple cytokines simultaneously (19). However, no prior studies have assessed simultaneous production of multiple cytokines by virus-specific CD8 T cells in the pediatric population, and whereas infants have been shown to exhibit HIV-1-specific T cell responses even in cord blood (5, 8–10), it is not known whether such responses are functionally mature. We examined production of IFN-γ, TNF-α, and IL-2, and degranulation (assessed by staining for CD107a) following stimulation with five pools of overlapping HIV peptides using 9-parameter flow cytometry (Fig. 6A). All 15 subjects were antiretroviral therapy naïve, and they ranged in age from 4 to 120 mo (median 23 mo). HIV-specific CD8 T cell responses were detected in 93% of the subjects assessed (14 of 15), with individual subjects targeting a median of three of the five peptide pools. Production of IFN-γ was the most commonly observed effector function, and it was very rare for a subject to exhibit other functions in the absence of IFN-γ production. The highest frequencies of IFN-γ-producing CD8 T cells were elicited by the Gag pool (Fig. 6B; 2.756%, median 0.403%, n = 9), Env (0.073–1.215%, median 0.388%, n = 9), Pol (0.012–1.206%, median 0.214%, n = 11), and Rev/Tat/Vif/Vpr/Vpu (0.014–0.791%, median 0.122%, n = 8). Similar results were obtained for CD107a staining, as there was a very high degree of correlation between IFN-γ production and degranulation (Fig. 6C; Spearman’s r = 0.87, p < 0.0001). HIV-specific production of TNF-α was observed in a subset of patients, generally at lower frequencies than IFN-γ (Fig. 6B; median response ranging from 0.047% for Nef to 0.084% for Rev/Tat/Vif/Vpr/Vpu). IL-2 production was quite rare, observed in only three subjects and at low frequencies (0.0102–0.0612%).

Polyfunctional CD8 T cells were present in the majority of pediatric subjects. HIV-specific CD8+ T cells producing both IFN-γ and TNF-α were observed in 64% of subjects overall. Triple-positive (IFN-γ+TNF-α+CD107a+) cells were present in 50% of subjects, and were observed in response to all five peptide pools. Quadruple-positive cells (IFN-γ+TNF-α+CD107a+IL-2+) were not detected in any subject, given the rarity of IL-2-producing CD8 T cells. Age appeared to influence the ability to mount a polyfunctional response, as CD8+ T cells exhibiting three effector functions (IFN-γ+TNF-α+CD107a+) were detected less frequently in children <24 mo of age (n = 8) compared with older children, although this difference did not achieve statistical significance (p = 0.11, χ² test). Of note, such responses were not detected in any subjects who were <12 mo of age (Spearman’s r = 0.586, p = 0.014; dotted line indicates 95% confidence band).
infant before 12 mo of age, although degranulation and production of TNF-α and IFN-γ were observed as individual functions in much younger infants. These cross-sectional data suggest that, whereas young infants are capable of multiple CD8 effector functions, their capacity to mount a polyfunctional CD8 T cell response may be impaired. Alternatively, our findings are consistent with a survival bias favoring infants with polyfunctional CD8 cells.

HIV-1-specific CD4 T cell responses are of low frequency during infancy

Functionally preserved CD4+ T cells are important for the maintenance of effective virus-specific CD8+ T cells (38–40). However, prior studies suggest that infants and young children may have an age-related impairment in their ability to mount CD4+ T cell responses to HIV and other viruses such as CMV (10, 41–43). We assessed the frequency, functionality, and protein specificity of HIV-specific CD4 T cells in 15 antiretroviral naive children, all of whom had detectable HIV viremia, by using multicolor flow cytometry to detect production of IFN-γ, TNF-α, and IL-2 following stimulation with five overlapping peptide pools spanning the entire HIV genome. Consistent with prior studies of HIV-infected adults, our analysis revealed that the majority of CD4 T cell responses targeted the HIV-Gag protein, with 8 of 15 subjects demonstrating an IFN-γ response. However, most of these responses were of very low magnitude (0.0141–0.145% of CD4 T cells, median 0.033%) compared with Gag-specific CD4 T cell frequencies previously reported in untreated HIV+ adult cohorts (44, 45). Overall, there was a statistically significant increase in the frequency of Gag-specific IFN-γ-producing CD4 T cells with age (Fig. 6D; Spearman’s r = 0.586, p = 0.014), with the four youngest infants failing to mount any significant IFN-γ response to Gag or any other viral protein. IFN-γ responses were also observed to the Env (3 subjects), Pol (2), Nef (2), and Rev/Tat/Vif/Vpr/Vpu pools (1), but these were of similarly low magnitude. Among the eight children with Gag-specific IFN-γ responses, simultaneous production of TNF-α was detected in five (62.5%), and in each case double-positive IFN-γ+TNF-α+ cells accounted for the majority of IFN-γ-producing cells. No subject responded to Gag by producing TNF-α alone. Production of TNF-α was also observed in response to each of the other viral protein pools, at frequencies similar to that observed for IFN-γ (0.0198–0.152%). Significant levels of IL-2 production by CD4 T cells were observed in only two subjects. Interestingly, both of these antiretroviral-naive subjects demonstrated exceptionally good control of HIV-1 viremia compared with age-based norms; subject 00244C1, whose viral load was 2670 copies/ml at 16 mo of age, demonstrated an IL-2 response to Pol, whereas subject 04035C1, whose viral load was 7930 copies/ml at 10 years of age, demonstrated IL-2 responses to both the Gag and Rev/Tat/Vif/Vpr/Vpu pools. Each of these IL-2-producing CD4 T cell populations was triple-positive (IFN-γ+TNF-α+IL-2+). Together, these data, which provide the first comprehensive assessment of the CD4 response to all HIV proteins performed in children, indicate that virus-specific CD4 T cells are of strikingly low magnitude during early infancy.

Discussion

Studies performed early in the HIV/AIDS epidemic indicated that HIV-specific T cells were infrequent and narrowly targeted in infants (46, 47), but subsequent data suggest that these quantitative deficiencies may be largely attributable to the early stage of infection, because acute HIV infection is now known to induce a relatively narrow T cell response in adults as well as children (48). Indeed, our data support other recent pediatric studies demonstrating that HIV-specific CD8 T cells can be detected in most infants during the first months of life (10, 49), and further indicate that this response progressively broadens throughout childhood, with HIV-specific T cell frequencies among older children approximating those of adults (11). Thus, there do not appear to be substantial quantitative differences in the CD8 response to HIV during infancy. However, in the present study, we describe several age-related qualitative differences in the HIV-specific T cell response that may contribute to the inability of infants to restrict viral replication. These differences include a propensity of infant CD8 T cells to target variable proteins such as Nef or Env rather than Gag, a lower degree of functionality among infant CD8 T cells, and a paucity of virus-specific CD4 T cell help during early infancy.

Our results in clade B-infected infants and young children support and extend those of another recent pediatric study that described age-related differences in the distribution of responses at the viral protein level among clade C-infected African infants (10). This prior study found that Env-specific responses predominated during the first 6 mo of life, whereas responses to Gag were very uncommon. We observed a similar paucity of Gag-specific T cell responses, with only 17% of infants in this age group exhibiting a Gag-specific response. However in our cohort, responses to Nef were slightly more common than to Env during early infancy, perhaps reflecting differences in class I HLA allele frequencies or HIV-1 subtypes of the populations studied. Interestingly, both Nef- and Env-specific responses have previously been associated with higher levels of HIV viremia in clade C-infected Africans (16), and a similar association of Nef responses and poor viral control was observed in our multivariate model. Both Nef and Env are characterized by a relatively high degree of sequence variability, making them potentially elusive targets in a rapidly evolving virus, in contrast to Gag, which is relatively conserved. Despite this sequence heterogeneity, preferential targeting of Nef was previously observed in a cohort of acutely infected adults, whose dominant responses shifted after prolonged Ag exposure to include Gag, Pol, and Env (50). The cross-sectional nature of our study did not permit us to differentiate whether the observed age-related differences reflect a temporal shift, in which acute-phase responses to Nef or Env are lost or eclipsed by late-emerging Gag-specific responses, or instead reflect a survival bias favoring infants who mount T cell response to Gag. The prospective longitudinal studies that would be required to distinguish these two possibilities may not be ethically feasible in the antiretroviral therapy era, because infants are at high risk for rapid progression to AIDS without early initiation of highly active antiretroviral therapy (51).

The infrequent recognition of Gag by infant T cells may have important implications for viral control. In the present study, subjects who recognized at least one Gag epitope exhibited significantly lower HIV RNA levels than those who did not, and the frequency of Gag-specific T cells correlated inversely with viral load after adjustment for age. These findings are in agreement with other recent studies indicating that Gag-specific T cell responses are associated with better control of HIV viremia in adults (16–18). The potential mechanism underlying this association remains uncertain. The gag sequence is highly conserved relative to other HIV proteins, and the virus may be less able to escape from Gag-specific T cells due to functional constraints on the Gag protein, resulting in more durable antiviral T cell responses. Indeed, the inability of HIV to tolerate sequence changes within highly conserved Gag epitopes without great cost to viral replicative fitness has been postulated as an explanation for the association of HLA-B*27 and B*57 with spontaneous viral containment and long-term nonprogressive HIV disease (52–55). An alternative explanation for the superior efficacy of Gag-specific CTL is that HIV-infected
cells have been demonstrated to present Gag-derived CD8 T cell epitopes within 2 h of infection, before viral integration, transcription, and Nef-mediated down-regulation of HLA (56). This phenomenon appears to be unique to Gag epitopes, owing to cross-presentation of the abundant Gag protein particles present in internalized virions, and could confer an ability to lyse infected target cells before the release of progeny virions, which would be highly advantageous for viral containment.

Our data add to mounting evidence that there is a generalized impairment of CD4 responses to HIV and other viruses such as CMV during early childhood (10, 41–43, 57). Previous studies have shown that virus-specific proliferative responses to both CMV (43) and HIV (42) are largely absent during the first 5 years of life. Perinatally CMV-infected infants exhibit a deficiency of virus-specific IFN-γ production by CD4, but not CD8, T cells when compared with adults with acute CMV infection (41, 57). The results of the present study indicate that there is a similar temporal pattern in the emergence of CD4-mediated IFN-γ responses to HIV among infants. We observed a virtual absence of HIV-specific CD4+ T cells during the first year of life, despite high-frequency CD8+ responses in many of these infants, followed by a linear increase in the frequency of Gag-specific CD4+ T cells during early childhood. These results are in agreement with prior studies, mostly limited to measurement of Gag-specific IFN-γ production, which found HIV-specific CD4+ T cells to be absent during the first year of life (10), and detectable in older children, but generally at lower frequencies than those reported in adults (44, 45, 58). Although our study is more comprehensive in that it assessed the frequency of multiple cytokines in response to all HIV proteins, responses were nonetheless absent or of extremely low magnitude in the majority of infants. A generalized deficit in T cell help during infancy could have important consequences for the ability of infants to control viral infections, because it might be expected to compromise CD8 effector function and/or memory potential, similar to the “unhelped CD8 T cell” phenotype described following vaccination of CD4-depleted mice (39, 40, 59, 60). This impairment of T cell help during early infancy may help to explain the lower frequency of polyfunctional CD8 T cells observed among younger infants in the present study. It remains to be determined whether this age-related deficiency in virus-specific CD4 responses is due to developmental differences in APCs, costimulatory pathways, or CD4 T cells themselves, or is instead due to active suppression by CD4 T regulatory cells (61), which are particularly abundant during infancy.

Mounting empiric evidence for the superior antiviral efficacy of Gag-specific T cells, combined with theoretical considerations, such as the high degree of Gag sequence conservation and the kinetic advantage of early Gag epitope presentation, make HIV-Gag a particularly attractive vaccine target. The mechanism underlying the inefficient targeting of Gag by infants is unclear, and solving this puzzle will most likely require a better understanding of the general determinants of immunodominance in the setting of viral infections, which may in turn lead to approaches to manipulate the hierarchy of epitopes targeted by vaccine-induced immune responses. Our data indicate that optimal immunization of neonates may require strategies to skew the immunodominance hierarchy away from that observed in natural pediatric infection, and toward induction of Gag-specific responses.

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Disclosures
The authors have no financial conflict of interest.

References


