Pediatric HIV-1–Specific Cytotoxic T-Lymphocyte Responses Suggesting Ongoing Viral Replication Despite Combination Antiretroviral Therapy

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ABSTRACT: Human immunodeficiency virus-1 (HIV-1)–specific cytotoxic T-lymphocyte (CTL) responses are common in infected adults and usually exhibit rapid decay after combination antiretroviral therapy (ART). CTLs develop later in the first year of life, and the fate of HIV-1–specific responses in perinatally infected children after ART is less well described. HIV-1–specific CTL responses were measured in 17 perinatally infected children and adolescents (ages 3–20 y) receiving combination ART. Seven had prolonged viral suppression (<400 copies/mL) for 2.5–5.3 y and 10 had persistent viremia (median, 77,550 copies/mL). HIV-1–specific CTL responses were tested by interferon (IFN)-γ enzyme-linked immunospot (ELISpot) assays using 53 overlapping peptide pools spanning the entire HIV-1 proteome. HIV-1–specific CTL responses were detected in 14 of 17 individuals. Responses to one to four viral proteins were found in eight of 10 individuals with persistent viremia and six of seven with prolonged viral suppression. The magnitude and breadth of CTL responses were similar between groups. HIV-1–specific CTL responses were present in the majority of perinatally infected subjects, irrespective of viremia at evaluation. Because ART-treated infected adults usually have rapid decay of responses, these data suggest viral replication below the limits of detection is more persistent in combination ART-treated perinatally infected pediatric subjects. The long-term clinical implications of these findings remain to be determined. (*Pediatr Res* **61:** 692–697, 2007)

Previous studies of HIV-1–infected infants have shown either the presence or absence of HIV-1 RNA at birth, and the magnitude and duration of early viremia are predictive of the long-term clinical outcome (1,2). The long-term survivors of perinatal HIV-1 infection are a special group of chronically infected children and adolescents critical to enhanced understanding of perinatal HIV-1 infection. As in HIV-1–infected adults, combination ART can reduce morbidity and mortality through reduction of viral replication and preservation of CD4$^+$ T lymphocytes *in vivo*.

Virus-specific CD8$^+$ T lymphocytes or cytotoxic T lymphocytes (CTLs) represent a key immune response in HIV-1 infection. Strong HIV-1–specific CTL responses have been described to correlate with decreased viremia at the end of primary infection in adults (3–5); these responses persist throughout the chronic phase of infection and usually wane in late-stage disease, suggesting a role for controlling viremia. Experimental depletion of CTLs in the simian immunodeficiency virus (SIV) macaque model results in massive increases in viremia (6–8), further demonstrating their importance. As an arm of adaptive immunity, the expansion and maintenance of CTL are a response to antigen, which drives CTL proliferation. Intracellular antigens are processed via the proteasome pathway for presentation by cell surface human leukocyte antigen (HLA) class I for CTL T-cell receptor binding and consequent signaling for cytolysis, cytokine release, and proliferation. In the course of naturally cleared infections such as influenza and infectious mononucleosis, virus-specific CTL responses wane rapidly to leave low frequencies of resting memory cells. Although HIV-1 infection is generally persistent, this process of natural CTL decay is recapitulated in successful combination ART in HIV-1–infected adults. Pharmacologic reduction of viremia to undetectable levels is accompanied by loss of activated HIV-1–specific CTLs, which decay to low levels of resting memory cells (9,10).

In contrast to infected adults, CTL responses in perinatally HIV-1–infected individuals are less fully described. Infants have reduced immunocompetence to launch effective antiviral CTL responses and appear to have limited HIV-1–specific CTL responses in the first 3–6 mo of life (11–14). This lack of protective CTL due to immunologic immaturity may contribute to the higher levels of plasma viremia in contrast to

Abbreviations: ART, antiretroviral therapy; **CR,** complete responder; **CTL,** cytotoxic T lymphocyte; **ELISpot,** enzyme-linked immunospot; **Gag,** group-specific antigen gene; **ICR,** incomplete responder; **Pol,** polymerase; **Nef,** negative regulatory factor; **SFC,** spot-forming cell

adults. Consistent with this hypothesis is the finding that attenuated SIV yields protective immunity and no disease in adult macaques, but can cause acquired immunodeficiency syndrome (AIDS) in neonatal macaques. After this early period of unresponsiveness in HIV-1–infected humans, however, CTL responses develop and appear to have similar targeting as adults (15,16). However, the lack of correlation of currently available CTL measurements to *in vivo* antiviral efficacy makes it impossible to assess whether these responses are functionally equivalent to those in adults.

Early initiation of combination ART in infants can suppress viremia, prevent or delay immunodeficiency, and generally dampen HIV-1–specific immune responses. Young infants may become HIV-1–seronegative and have decreased CTL responses if treated with combination ART before 3 mo of age (17). The data from adult-infected persons predict waning of HIV-1–specific CTL responses in the setting of combination ART suppression of viremia due to loss of the antigenic stimulation required for effector CTL expansion and persistence. In contrast to infants and adults, the fate of HIV-1–specific responses in perinatally infected children and adolescents after combination ART is less well described. Our hypothesis is that HIV-1 CTL responses vary with the degree of viral suppression in perinatally HIV-1–infected children and adolescents. Here we evaluate the HIV-1–specific CTL responses in a cross-sectional study of chronically infected children and adolescents perinatally infected with HIV-1.

MATERIALS AND METHODS

Perinatally HIV-1–infected subjects. Seventeen perinatally HIV-1–infected children and adolescents attending the Care 4 Families Clinic at Mattel Children's Hospital at the University of California, Los Angeles (UCLA) were evaluated after provision of informed consent under a UCLA Institutional Review Board–approved protocol. All subjects received antiretroviral drugs such as nucleoside reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors, or protease inhibitors (combination ART) as indicated in Table 1. In addition, all patients had monitoring of clinical parameters such as T-cell subsets and plasma viremia under the care of their physicians. Ten patients had at least two consecutive HIV-1 RNA values ≥400 copies HIV-1 RNA/mL within 2 y of evaluation, and will be referred to as incomplete responders (ICRs). The remainder ($n = 7$) will be referred to as complete responders (CRs).

Plasma HIV-1 RNA monitoring. The Roche Amplicor HIV-1 Monitor Test, v1.5 (Roche Molecular Diagnostics, Indianapolis, IN) was used to monitor quantitative plasma viremia. The lower limit of detection was 400 copies HIV-1 RNA/mL for the regular assay and 50 copies HIV-1 RNA/mL for the ultrasensitive assay. In earlier specimens, patients were evaluated with regular HIV-1 RNA monitoring assay, but when the ultrasensitive assay became available, the lower limit of plasma viremia was monitored at <50 copies HIV-1 RNA/mL. If results were below the level of detection, half of the assay cutoff was used for data calculation.

Measurement of HIV-1–specific CTL responses. HIV-1–specific CTL responses were assayed by IFN-γ ELISpot assays using a previously described protocol (18,19). In brief, polyclonally expanded CD8$^+$ T lymphocytes were derived from heparinized peripheral blood mononuclear cells (PBMCs) (fresh or cryopreserved). These were screened using standard ELISpot assays employing overlapping 15-mer peptides spanning the entire HIV-1 proteome (clade B consensus sequences) obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (20) in 53 pools of 12–16 peptides. Positive HIV-1–specific ELISpot responses were defined as those >100 spot-forming cells (SFCs)/10^6 CD8$^+$ T cells and

Table 1. *Patient characteristics*

Subject	Age, y	Sex	HIV RNA (copies/mL)	CD4, %	CD4 Abs (cells/mm^3)	CD8, %	CD8 Abs (cells/mm^3)	Combination ART
CR ($n = 7$)								
A	6.8	M	Undetectable	35	955	29	780	NFV, D4T, DDI
B	14.4	M	Undetectable	37	979	39	1019	EFV, D4T, 3TC
C	20.3	M	Undetectable	21	502	25	572	NFV, D4T, 3TC
D	13.4	F	Undetectable	34	1352	27	1073	D4T, EFV, NFV
E	12.1	M	Undetectable	35	781	40	893	AZT/3TC, NFV
F	18.9	M	Undetectable	34	742	33	720	D4T, 3TC, NFV
G	16.1	F	Undetectable	34	314	32	296	NFV, D4T, EFV, 3TC
Median	14.4			34	781	32	780	
Range	6.8–20.3			21–37	314–1352	25–40	296–1073	
ICR ($n = 10$)								
H	6.6	M	70,232	15	545	59	2232	AZT, 3TC, AMP, RTV
I	7.7	F	1,261	33	1309	39	1551	D4T, 3TC, NVP
J	14.5	M	426	20	269	49	670	ABC, 3TC, LPV/r
K	6.1	M	297,559	35	1659	51	2417	AZT, DDI, RTV
L	16.5	F	246,343	1	3	20	58	T-20, TNF, SQV, RTV
M	14.7	F	1,392	33	406	44	597	AZT/3TC, RTV
N	9.3	F	1,770	45	1754	24	834	D4T, 3TC, NFV
O	3.1	M	750,000	26	811	30	1052	AZT, 3TC, LPV/r
P	12.1	M	Undetectable	37	859	39	909	EFV, FTC, TNF
Q	9.7	M	84,867	20	475	57	1354	AMP, RTV, EFV, D4T
Median	9.5		36,001	30	678	42	981	
Range	3.1–16.5		Undetectable to 750,000	1–45	3–1754	20–59	58–2417	
All ($n = 10$)								
Median	12.1		200	34	781	39	893	
Range	3.1–20.3		Undetectable to 750,000	1–45	3–1754	20–59	58–2417	

F, female; M, male; abs, absolute count; ZDV or AZT, zidovudine; 3TC, lamivudine; D4T, stavudine; DDI, didanosine; NVP, nevirapine; NFV, nelfinavir; EFV, efavirenz; ABC, abacavir; RTV, ritonavir; LPV/r, lopinavir/ritonavir; AMP, amprenavir; T-20, enfuvirtide; TNF, tenofovir; SQV; saquinavir; FTC, emtricitabine. HIV RNA (copies HIV-1 RNA/mL), CD4$^+$ and CD8$^+$ absolute count. For the lower limit of detection, half of the assay cutoff was used for calculation. Subject J had viral suppression for 6 y, followed by 3 mo of viremia due to nonadherence. After a switch of combination ART, he had 1 y of undetectable viremia before evaluation. Subject P started a new regimen 3 mo before evaluation.

at least 3 SDs over background determined using triplicate no peptide controls. For those responses that were below the level of detection, half of the limit of detection was used for data calculation. The HIV-1 viral genes evaluated were group-specific antigen gene (Gag), polymerase (Pol), envelope (Env), negative regulatory factor (Nef), trans-activator of transcription (Tat), regulator of virion (Rev), viral protein R (Vpr), viral protein U (Vpu), and viral infectivity factor (Vif).

Statistical analysis. Continuous variables were analyzed with means, SDs, 95% confidence intervals (CIs), medians, and ranges. Means were compared using *t* tests. Data with non-normal distributions was assessed using Wilcoxon rank sum tests. HIV-1–specific ELISpot responses were log transformed. Analysis was performed with JMP version 5.1 and SAS software release 8.02 (SAS Inc., Cary, NC).

RESULTS

Study participants. We studied 17 perinatally HIV-1–infected children and adolescents on combination ART. The median age of all participants at HIV-1–specific CTL evaluation was 12.1 y of age (range, 3.1–20.3 y). Overall, subjects had received combination ART for a median duration of 5.3 y (range, 1.5–8 y). CRs received combination ART for a median of 5.3 y (range, 2.9–6.6 y) and had undetectable viremia for a median of 4.3 y (range, 2.5–5.3 y), whereas ICRs were treated for a median of 5.3 y (range, 1.5–8.0 y). Our patient population had severe immunosuppression and clinical disease as defined by Centers for Disease Control and Prevention (CDC) Classification (21). At nadir, 53% of patients had symptomatic infection (CDC Class C), 41% had CDC Class B symptoms and one patient had CDC Class A symptoms (6%). Comparing the groups, 43% of CR had CDC Class C clinical disease compared with 60% of ICRs; 43% of CRs and 40% of ICRs had CDC Class B symptoms, but only 14% of CRs were in Class A. No ICR had CDC Class A symptoms. The lowest level of immunosuppression ever achieved by subjects was CDC immune category 3 in 70%, CDC immune category 2 in 12%, and CDC immune category 1 in 18% of patients. At the time of evaluation, clinical parameters remained comparable in the CR and ICR groups, except for the presence or absence of recent viremia (Table 1).

HIV-1–specific CTL responses are present in both CR and ICR groups. To determine whether suppression of detectable viremia by combination ART had an impact on the frequency of HIV-1–specific CTL responses in perinatally infected subjects, these participants were screened using IFN-γ ELISpot assays for responding $CD8^+$ T lymphocytes (Fig. 1). As might be expected due to the persistence of antigen (as reflected by ongoing viremia), the ICR group had detectable CTL responses in eight of 10 (80%) persons, recognizing a mean of 2.0 viral proteins. Screening of the CRs, however, also demonstrated that the majority had detectable CTL responses against HIV-1, six of seven (86%) recognizing a mean of 1.57 proteins. These data suggest that loss of detectable viremia after combination ART does not result in complete decay of CTL responses as in infected adults.

The HIV-1–specific CTL responses in ICR and CR groups are similar in magnitude and breadth. To assess whether the degree of treatment response had an impact on the quantity of HIV-1–specific CTLs, the responses between groups were compared for their magnitude and breadth of HIV-1 targeting (Fig. 2 *A* and *B*). The total magnitude of HIV-1–specific CTLs
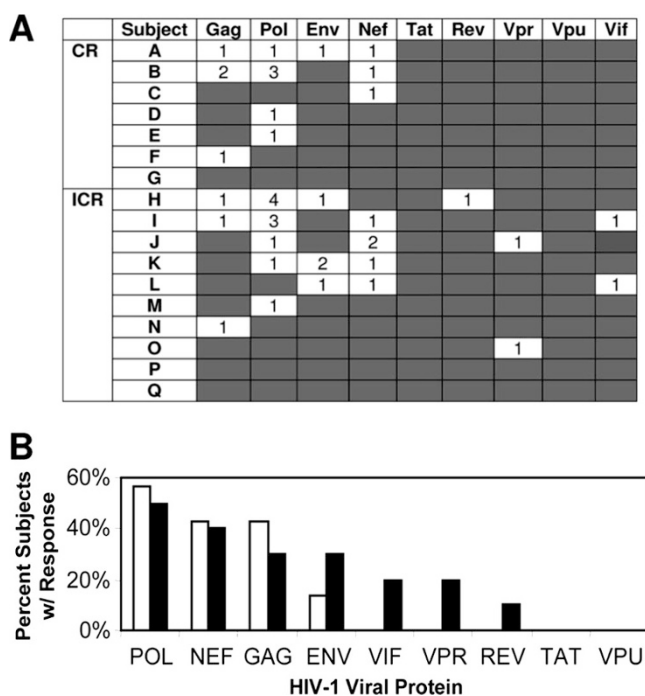

Figure 1. (*A*) HIV-1–specific CTL responses. Subjects are categorized according to virological response to combination ART. HIV-1–specific CTL responses to viral proteins are listed under each column; numbers indicate the number of viral peptide pools detected. (*B*) HIV-1–specific CTL responses according to virological response to combination ART. Pol, Nef, and Gag were the most frequent responses detected among all subjects. HIV-1 viral proteins are presented on the *x* axis from the most frequently recognized to least recognized. CRs (□), ICRs (■). There was no significant difference between the CR and ICR responses to Pol, Nef, Gag, Env, Vif, Vpr, or Rev by Fisher's exact test.

in the ICR group ranged from 50 to 4825 SFC/10^6 $CD8^+$ T lymphocytes [mean, 1120.5 ± 1493.0 (95% CI: 52.7–2188.3)], and the breadth (as reflected by the number of recognized peptide pools of the 53 screened) ranged from 0 to 7 (mean, 2.7 ± 2.5). In comparison, the magnitude and breadth of HIV-1–specific CTLs in the CR group were similar. The magnitude ranged from 50 to 3220 SFC/10^6 $CD8^+$ T lymphocytes [mean 786.4 ± 1145.6 (95% CI: 0–1845.0)], and the breadth ranged from 0 to 6 recognized peptide pools [mean CRs, 2 ± 2.16 (95% CI: 0.002–4.0)]. Thus, the CTL responses in these perinatally infected subjects were quantitatively similar regardless of viremia suppression above or below the limits of detection by combination ART.
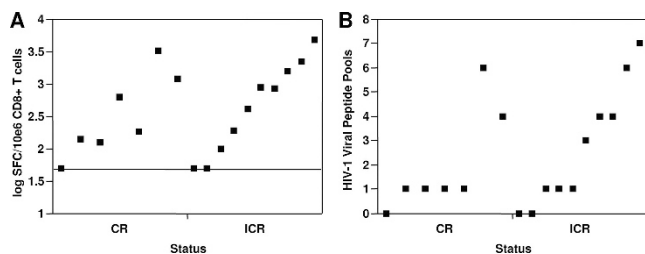

Figure 2. HIV-1–specific CTL targeting according to virological response. Magnitude and breadth of HIV-1–specific CTL targeting according to log SFC/10^6 CD8+ T cells (*A*) and HIV-1 viral peptides (*B*). The *solid line* (log 1.7) represents the lower limit of detection for log SFC/10^6 $CD8^+$ T cells (midpoint used for calculations).
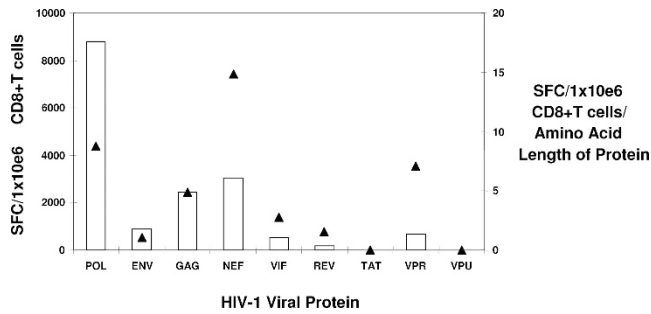
Figure 3. HIV-1–specific CTL targeting adjusted according to amino acid length of viral protein. *Columns* represent the total magnitude of HIV-1–specific CTL responses SFC/10^6 CD8$^+$ T cells of each viral protein for all patients; *triangles* represent the ratio of SFC/10^6 CD8$^+$ T cells/amino acid length of protein. HIV-1 viral proteins are represented on the *x* axis, from largest protein with the greatest number of amino acids to least (left to right).

HIV-1–specific CTL responses in ICR and CR groups have similar viral targeting. HIV-1–specific CTL responses to individual viral proteins were detected in the majority of patients, 14 of 17 (82%), as displayed in Figure 1. The most commonly recognized HIV-1 viral protein in all subjects was to Pol in nine of 17 (53%), followed by Nef in seven (41%) and Gag in six (35%). There were no detectable responses to Tat or Vpu in any subjects. Considering the magnitude of HIV–1-specific CTL responses *versus* the size of the targeted viral proteins, the density of targeting varied, as seen in Figure 3. The magnitude of responses was normalized by calculating the density of target proteins based on the amino acid length for each HIV-1 viral protein. Overall, CR and ICR had similar targeting, with Nef and Pol being the most densely targeted viral proteins.

DISCUSSION

Studies in adults have demonstrated vigorous HIV-1–specific CTL responses that develop at the latter stage of primary infection and persist throughout chronic infection. These responses are driven by the persistence of viral antigen. Clearance of antigen after suppressive combination ART has been shown to interrupt this process, with decay of CTL responses to low memory levels in adults (9,10,22). Broad HIV-1–specific CTL responses have been detected in acutely and chronically infected adults, but no correlation was determined between CD8$^+$ T cell responses and viremia (23). Fewer data about treated pediatric subjects are available. Perinatally infected children appear to have limited CTL responses in the first 3–6 mo of life (11,13,14,24), but then develop responses similar to those seen in adults (16,25–29).

In contrast to treated adults, our data demonstrate persisting HIV-1–specific CTL responses in the majority of perinatally infected subjects on combination ART, with similar magnitude and breadth regardless of viremic suppression on treatment. These findings are different from our original hypothesis. Interestingly, we found that even among patients with highly suppressed viremia, HIV-1–specific CTL responses were still present in this group of long-term survivors. These results agree with previous reports of CTL responses detected in older pediatric subjects on combination ART (16,26–31).

However, some investigators have found a correlation between CD8$^+$ T-cell responses and viremia in those on combination ART (25,26,29) and greater CTL responses were in children with persistent viremia after treatment (16,27,30). Our findings are similar to the CTL responses found in subjects with both detectable and undetectable viremia except for their finding of the significantly lower response noted in subjects with viral suppression and greater overall magnitude in CTL responses in both groups (16). Because CTL proliferation and persistence are driven by antigen, these findings suggest that persistence of viral replication is more common in these perinatally infected subjects who started combination ART later in life compared with adults with primary infection. The reason for this discrepancy compared with treated adults is unclear. However, given the nature of this cross-sectional study, these CTL responses are a measurement of one point in time for a specific group of pediatric patients.

Our current study differs from previous studies in the methodology to assess HIV-1–specific CTL responses. We evaluated the entire HIV-1 proteome using 53 overlapping peptide pools with clade B consensus sequences from National Institutes of Health (20) as did Feeney *et al.* (16). The findings in that study were different from ours as previously mentioned. Previous investigators have used recombinant vaccinia vectors expressing single specific viral proteins but not all HIV-1 proteins at once (15,17,25–28,30,31) or tetrameric complexes (17,29,30). Most authors have measured IFN-γ by ELISpot assays, as we did, with the exception of those who used chromium release assays to measure CTL responses (15,17,31).

A major difference between adults and perinatally infected children is that adults are infected after immunologic maturity, whereas children face infection during immunologic maturation. This raises the possibility that HIV-1 infection fundamentally alters some developmental property of the immune system, such as immune regulation (*e.g.* dysregulation causing inappropriate immune activation that favors ongoing viral replication) or effector competence to contribute to containment of viral replication during drug treatment. Interestingly, very early treatment of infected infants can yield highly effective suppression of viremia that can prevent antibody responses (17), making the latter possibility less likely. Additional evidence that very early treatment is more effective than later treatment for suppressing viremia in these patients comes from the observation that infants treated early (<3 mo) with combination ART have a higher rate of sustained viral suppression after 4 y (32). Moreover, another cohort of perinatally infected subjects who started combination ART later in life, as in our study, also demonstrated persisting HIV-1–specific CTL responses (28).

In addition, it is well established that viremia set points in children are higher than adults, and achieving undetectable viremia in perinatally infected subjects is only 56% after 12 mo (33) and 52% and 18% successful after 12 and 24 mo of treatment, respectively (34), which is much lower than adults. Pediatric subjects on combination ART have been reported to reach undetectable levels of viremia <400 and <50 copies HIV-1 RNA/mL by median of 4 and 20 wk, respectively (35).

Of those patients who reach undetectable (<400 copies HIV-1 RNA/mL), 83% remained suppressed for 6 mo.

It is known that a "latent reservoir" of replication competent virus allows HIV-1 persistence even in the setting of suppressive combination ART (36,37). This reservoir serves as a source of continued low-grade viral replication even in adults with treatment and undetectable viremia. Supersensitive methods reveal persisting viral transcription in these persons (38). The existence of the latent reservoir in perinatally infected subjects from our group (39) and other cohorts (40) also has been demonstrated. Thus, the continuing presence of latent viral reservoirs even among children with highly suppressed viremia may potentially be the reason why CTL responses are preserved.

In summary, our data indicate that HIV-1–specific CTL responses persisted in our group of perinatally infected children and adolescents, indicating persistence of viral replication. This suggests that alteration of immunity due to infection before immunologic maturity renders a change that increases basal HIV-1 replication and/or reduces clearance of HIV-1 during combination ART. Further delineation of the HIV-1–specific CTL responses in relationship to disease progression and control of viremia should be investigated in HIV-1–infected pediatric subjects on combination ART. Our studies are limited in a cross-sectional study, but further prospective evaluation is warranted to investigate HIV-1–specific CTL responses influenced by timing of ART, CD4 recovery and function, latent reservoirs, and virological suppression.

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