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Human Immunodeficiency Virus-Specific CD8$^+$ T-Cell Activity Is Detectable from Birth in the Majority of In Utero-Infected Infants$^\nabla$

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Human immunodeficiency virus (HIV)-infected infants in sub-Saharan Africa typically progress to AIDS or death by 2 years of life in the absence of antiretroviral therapy. This rapid progression to HIV disease has been related to immaturity of the adaptive immune response in infants. We screened 740 infants born to HIV-infected mothers and tracked development and specificity of HIV-specific CD8$^+$ T-cell responses in 63 HIV-infected infants identified using gamma interferon enzyme-linked immunospot assays and intracellular cytokine staining. Forty-four in utero-infected and 19 intrapartum-infected infants were compared to 45 chronically infected children >2 years of age. Seventy percent (14 of 20) in utero-infected infants tested within the first week of life demonstrated HIV-specific CD8$^+$ T-cell responses. Gag, Pol, and Nef were the principally targeted regions in chronic pediatric infection. However, Env dominated the overall response in one-third (12/36) of the acutely infected infants, compared to only 2/45 (4%) of chronically infected children ($P = 0.00083$). Gag-specific CD4$^+$ T-cell responses were minimal to undetectable in the first 6 months of pediatric infection. These data indicate that failure to control HIV replication in in utero-infected infants is not due to an inability to induce responses but instead suggest secondary failure of adaptive immunity in containing this infection. Moreover, the detection of virus-specific CD8$^+$ T-cell responses in the first days of life in most in utero-infected infants is encouraging for HIV vaccine interventions in infants.

An association between human immunodeficiency virus (HIV)-specific CD8$^+$ and CD4$^+$ T-cell responses and control of viral replication has been well documented in acute adult infection (2, 4, 13–15), but it is less clearly established what role CD8$^+$ T cells play in control of HIV in acute pediatric infection, which occurs both in utero (IU) and intrapartum (IP). In adults, the appearance of HIV-specific CD8$^+$ T-cell immune responses in acute infection is temporally associated with a rapid decline in viremia from an average of 10 million copies/ml at peak to a median set point of 30,000 copies/ml (26, 33). In pediatric infection, there is typically no such rapid decline in viremia following acute infection, with viral loads remaining in an excess of 100,000 copies/ml over the first year of life, and decreasing only slowly over the next 2 to 3 years of life in survivors (31, 36).

A potential explanation for the absence of a dramatic decline in viremia in early pediatric infection is either low-frequency HIV-specific CD8$^+$ T-cell activity and/or ineffective CD8$^+$ T-cell activity in infancy. Previous studies of limited numbers of HIV-infected infants have demonstrated that HIV-specific CD8$^+$ T-cell responses can be detected at low frequency in some infants (5, 24, 35). Moreover, when detected in infancy, the CD8$^+$ T-cell responses generated had no immediate benefit in clinical outcome (5, 23, 32), and no studies have compared IU, IP, and chronic pediatric infection. Recent reports that CD8$^+$ T-cell responses in infants can exert selection pressure in vivo in known CD8$^+$ T-cell epitopes within the first few months of life (10, 20, 29) suggest that in some instances, at least, these responses may be functional. In addition, slow progression to disease has been well described in children who express HLA-B*27 or HLA-B*57 (10, 11), suggesting that CD8$^+$ T-cell responses can be important in pediatric infection as in adult infection.

The aim of these studies was to examine a large cohort of infants born to HIV-infected mothers and to determine both the age at which HIV-specific CD8$^+$ T-cell responses are in-

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duced in infected infants and the specificity of these responses using a panel of overlapping peptides spanning all the HIV proteins. These studies were undertaken in Durban, South Africa, a country in which its estimated that there are >100 newly infected infants born each day (http://www.avert.org/worldstats.htm).

MATERIALS AND METHODS

Study subjects. Sixty-three HIV type 1 (HIV-1)-infected infants born to HIV-positive mothers were enrolled from St. Mary's and Prince Mshiyeni Hospitals in Durban, South Africa, from 2003 to 2005 (Table 1). HIV-1-seropositive mothers were recruited during the last trimester of pregnancy, and a single dose of nevirapine was given to the mothers during labor and to the infants within 48 h of birth, according to the HIVNET-012 protocol (16, 17). A total of 719 mothers were enrolled and screened, and 740 infants were born to the mothers; 623 infants were uninfected, 41 were untraceable, and only 75 were infected. Sixty-three of the 75 infants met the clinical criteria for enrollment into the study. Exclusion criteria comprised prematurity, intrauterine growth restriction, and congenital anomaly (28).

Forty-five HIV-1-positive, antiretroviral therapy-naïve children with chronic infection between ages of 2 and 12 years were recruited from the Pediatric HIV outpatient clinic at King Edward VIII Hospital and McCord Hospital, both in Durban, South Africa. The median absolute CD4 count in this cohort of children with chronic HIV infection was 559, with a median CD4 percentage of 17 and a median viral load of 110,000 copies/ml (Table 2). The mothers gave written informed consent for participation of their children in both studies. These studies were approved by all the participating Institutional Review Boards.

Diagnosis of HIV-1 infection in infants. Infants were diagnosed as HIV infected following detection of plasma HIV RNA by RNA PCR (Roche Amplicor assay). Blood was collected on day 1 and day 28 of life. A positive plasma viral load (>400 RNA copies/ml) on day 1 or day 28 was followed by a confirmation test before enrollment of the infant into the study. Infants with detectable virus on day 1 were defined as IU infected ($n = 44$), and infants with undetectable virus on day 1 but with detectable virus on day 28 were defined as IP infected ($n = 19$). Since the majority of infants were breast fed, it is possible that this "IP" group includes some early breast milk mother-to-child transmission.

Viral load and CD4 measurement. Plasma viral loads were measured using either the Roche Amplicor Monitor assay detection limit of 400 HIV-1 RNA copies/ml plasma) or the Roche Ultrasensitive assay detection limit of 50 RNA copies/ml plasma), according to the manufacturer's instructions. CD4 counts were determined from fresh whole blood using Tru-Count technology and analyzed on a four-color flow cytometer (Becton Dickinson) according to the manufacturer's instructions.

Isolation of PBMCs. Blood was collected in EDTA tubes and processed within 6 h of collection. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Histopaque (Sigma, St. Louis, MO) density gradient centrifugation and were used fresh in enzyme-linked immunospot (ELISPOT) assays.

Synthetic HIV-1 peptides. A panel of 410 overlapping peptides (18-mers with a 10-amino-acid overlap) spanning the entire HIV-1 clade C consensus sequence were synthesized on an automated peptide synthesizer (MBS 396; Advanced ChemTech) and used in a matrix system in screening assays.

ELISPOT assays. Screening for T-cell responses was done ex vivo using the gamma interferon (IFN-γ) ELISPOT assay as previously described (18). The individually recognized peptides within the pools were determined by the use of a second ELISPOT assay.

Freshly isolated PBMCs were plated in 96-well polyvinylidene difluoride-backed plates (MAIP S45; Millipore) that had been previously coated with 100 μl of anti-human IFN-γ monoclonal antibody 1-D1k (0.5 μg/ml; Mabtech) overnight at 4°C. Peptides were added at a final concentration of 2 μg/ml to a 96-well plate with 100 μl of R10 medium at 50,000 or 100,000 cells/well. Negative controls with cells and medium only were run in quadruplicate along with two positive controls containing phytohemagglutinin. The plate with contents was incubated overnight at 37°C with 5% CO_2 and then processed. Following overnight incubation, the plate was washed with cold phosphate-buffered saline (PBS), and 0.5 μg/ml of IFN-γ monoclonal antibody biotinylated secondary antibody (7-B6-1; Mabtech) was added and left for 90 min in the dark at room temperature. The plate was then washed with cold PBS, and 0.5 μg/ml of streptavidin-alkaline phosphatase conjugate antibody (Mabtech) was added and left for 45 min in the dark at room temperature. IFN-γ-producing cells were noted by direct visualization of the plate following development with alkaline phosphatase color reagents (Bio-Rad).

The IFN-γ-secreting cells were quantified by counting the number of spots per well using an automated ELISPOT plate reader (AID ELISPOT reader system; Autoimmun Diagnostika GmbH, Strasburg, Germany). Results were expressed as number of spot-forming cells (SFC) per million PBMCs after subtractions of values for background wells. A response was defined as positive, using previously adopted criteria (1, 18), if it was ≥100 SFC/million PBMCs and ≥3 standard deviations above the mean for four background wells containing PBMCs but no peptide. The mean background levels in all assays were always less than 120 SFC/10^6 cells, with a range of 0 to 120 SFC/10^6 cells.

Quantitation of $CD8^+$ T-cell responses towards each HIV protein was undertaken from the ELISPOT assays as follows. Following subtraction of the background in each well, the numbers of SFC for each well containing peptides within a particular protein were summed; only positive wells with responses of ≥100 SFC were used to calculate responses to each protein. Wells with responses of <100 SFC were treated as negative and were assigned a value of 90 SFC for statistical analyses. To calculate the relative contribution of each protein to the total $CD8^+$ HIV-specific response for each study subject, the total response to all nine HIV proteins was summed, and the contribution of each individual protein was derived by dividing the protein-specific response by the total response. Any protein-specific response that was <100 SFC/million PBMCs represented 0% contribution to the total response.

Flow cytometric intracellular cytokine staining. Freshly isolated PBMCs (0.5×10^6) were incubated at 37°C with 5% CO_2 for 90 min with peptide pools at a final concentration of 2 μg/ml per peptide following stimulation with anti-CD28 and anti-CD49 antibodies (Becton Dickinson). Brefeldin (Sigma) was added, and cells were incubated for a further 4.5 h at 37°C with 5% CO_2. Cells were then stained with anti-human allophycocyanin-conjugated CD8 and anti-human phycoerythroerythrin-conjugated CD4 antibodies (Becton Dickinson), washed, fixed, and permeabilized as previously described (30) before addition of anti-IFN-γ–fluorescein isothiocyanate (FITC), anti-interleukin-2 (IL-2)–FITC, or anti-tumor necrosis factor alpha (TNF-α)–FITC. Following 20 min of incubation, the cells were washed, resuspended in 200 μl of PBS, and acquired on a FACSCalibur (Becton Dickinson). Duplicate negative controls with PBMCs alone together with a positive control containing PBMCs stimulated with phytohemagglutinin were included in the assays. For the infant cohort, a minimum of 150,000 events were collected per subject, and a minimum of 100,000 events were collected for the cohort of chronically infected children.

The total $CD4^+$ and $CD8^+$ T-cell responses were obtained after subtracting the mean of two negative controls. Reference ranges were obtained from intracellular cytokine staining for Gag IFN-γ in a total of 23 HIV-uninfected infants between the ages of 1 week and 17 months. A response was considered positive if it was above 0.07% for CD8 responses and above 0.02% for CD4 responses. Gag-specific $CD8^+$ responses ranged from 0.00 to 0.07% and $CD4^+$ responses from 0.00 to 0.02% in these 23 uninfected controls.

HLA typing. DNA for HLA typing was extracted using a Puregene DNA isolation kit for blood (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. HLA class I typing was done by DNA PCR using sequence-specific primers as previously described (18).

Statistical analysis. Fisher's exact test was used to compare proportions of IU- and IP-infected infants with early detectable responses and also to compare the numbers of responders among acutely and chronically infected children. The Mann-Whitney test was used to compare differences in the magnitude and contribution of each protein to the overall total response of the nine HIV proteins targeted by both acutely and chronically infected children. The Mann-Whitney test was also used to evaluate $CD4^+$ and $CD8^+$ T-cell responses measured by intracellular cytokine staining in acute and chronic children.

RESULTS

HIV-specific $CD8^+$ T-cell responses are detectable from day 1 of life in infected infants. To determine whether IU HIV infection induces an adaptive immune response above the level of any response in exposed, uninfected infants, we enrolled 10 randomly selected HIV-infected mothers and tested their newborn infants' HIV-specific $CD8^+$ T-cell responses on day 1 of life, blind to diagnosis. Three of 10 had detectable $CD8^+$ T-cell responses on day 1 of life, and all subsequently proved to be

TABLE 1. Characteristics of the cohort of acutely infected infants at the time of first ELISPOT assay

Subject	Age (days)[a]	Transmission type[b]	HLA class I type	CD4 (%)	Viral load (RNA copies/ml)[c]
001-AC	4	IU	A0101/3001, B4201/8101, Cw1701/1801	75	<400
021-AC	1	IU	A0205/29, B4201/44, Cw0202/1701	18	23,900
046-AC	36	IP	A3002/6801, B5802/5802, Cw0606/0602	36	18,100,00
081-AC	92	IU	A29/68, B1516/44, Cw03/07	44	92
094-AC	34	IU	A2301/6801, B0702/5802, Cw0202/0602	41	>100,000
097-AC	44	IP	A0301/3601, B4501/5301, Cw04/1601	18	>750,000
102-AC	15	IU	A2301/6602, B07/4201, Cw07/1701	47	>647,000
114-AC	61	IP	A03/24, B07/08, Cw07/07	57	4,190
115-AC	42	IU	A29/6801, B1503/5802, Cw04/0602	44	1,540
127-AC	33	IU	A02/3402, B0801/1503, Cw02/04	46	436,000
133-AC	31	IU	A68/7408, B5802/8101, Cw04/0602	54	241,000
135-AC	69	IU	A01/3002, B08/8101, Cw07/18	13	>750,000
149-AC	21	IU	A3001/8001, B1503/18, Cw0202/0202	51	948
188-AC	32	IP	ND[d]	ND	2,330,000
197-AC	6	IU	A3001/3402, B44/5802, Cw04/0602	42	1,500
222-AC	26	IU	A29/6802, B0702/1302, Cw0602/07	48	<400
227-AC	5	IU	A0301/26, B1529/5201, Cw0202/07	ND	311
241-AC	56	IP	A23/23, B44/81, Cw0202/04	54	4,820
251-AC	13	IU	A29/6802, B1510/1516, Cw03/04	56	12,400
268-AC	27	IU	A3201/6802, B07/07, Cw0102/07	34	13,200
274-AC	4	IU	A6601/8001, B18/4201, Cw0202/1701	45	40,400
275-AC	55	IP	A23/6601, B1503/5802, Cw0202/0602	27	3,890
284-AC	36	IU	A3201/6801, B08/15, Cw04/07	38	3,030
298-AC	42	IU	A29/6802, B13/14, Cw06/08	35	339,000
304-AC	5	IU	A3402/6802, B1510/44, Cw03/07	44	14,200
312-AC	84	IP	A0301/6802, B1510/5802, Cw03/0602	27	3,690
341-AC	64	IP	A3002/3201, B1510/5802, Cw03/0602	13	1,426,000
344-AC	52	IP	A29/6802, B44/5802, Cw0602/07	14	>750,000
349-AC	4	IU	A2301/4301, B4501/5802, Cw0602/0602	ND	33,950
355-AC	69	IP	A01/0301, B0801/5802, Cw0602/07	15	15,800
360-AC	47	IU	A02/3601, B3601/53, Cw0602/04	18	4,040,000
364-AC	23	IU	A29/3002, B4102/44, Cw07/1701	34	87,200
380-AC	9	IU	A2301/6801, B1510/1510, Cw03/08	56	16,100
385-AC	105	IP	A3001/3402, B1503/1503, Cw0202/0202	37	2,760
413-AC	4	IU	A01/3201, B07/8101, Cw07/1801	ND	5,650
423-AC	31	IP	A2301/2301, B18/41, Cw04/1701	38	14,040,000
433-AC	41	IU	A4301/6801, B1503/5801, Cw0602, 18	33	357,000
435-AC	2	IU	A02/3004, B4201/44, Cw0202/1701	45	91,400
446-AC	26	IU	A02/3001, B1503/4501, Cw0202/1601	20	3,730,000
447-AC	63	IP	A03/6802, B1401/4701, Cw0602/0802	34	8,860,000
458-AC	41	IP	A4301/6801, B1503/1510, Cw03/18	37	2,340,000
464-AC	44	IU	A29/6802, B15/18, Cw0202/03	28	5,720,000
468-AC	3	IU	A0202/6801, B5703/5801, Cw06/07	51	5,630
496-AC	13	IU	A0301/6801, B1503/5802, Cw0602/06	32	882,000
517-AC	31	IP	A3002/6802, B1510/4201, Cw03/1701	39	2,300,000
559-AC	4	IU	A2301/6802, B0801/5801, Cw07/07	61	731
562-AC	10	IU	A0205/3002, B1402/5801 Cw07/0802	36	2,580,000
568-AC	33	IP	A3001/6802, B07/18, Cw07/07	45	>750,000
576-AC	4	IU	A29/6802, B1401/44, Cw07/0802	48	94,500
579-AC	8	IU	A0202/3001, B4201/5703, Cw0701/1701	35	9,333
586-AC	10	IU	A0101/6802, B1510/8101, Cw08/1801	45	997
590-AC	8	IU	A02/68, B1503/5802, Cw0202/0602	33	>750,000
600-AC	7	IU	A26/3002, B0801/8101, Cw04/07	28	8,490
637-AC	14	IU	A29/6601, B1302/5802, Cw0602/0602	34	2,200
639-AC	7	IU	ND	31	31,600
641-AC	35	IP	A2301/3002, B0801/4501, Cw07/1601	43	>750,000
675-AC	5	IU	A30/6601, B3910/5802, Cw0602/12	49	2,040
698-AC	6	IU	A0205/24, B0702/1401, Cw07/08	52	1,150
720-AC	1	IU	A3001/30, B0702/18, Cw02/07	47	66,300
729-AC	103	IP	A29/6601, B1510/5802, Cw03/06	15	8,490
732-AC	6	IU	A3002/6602, B4201/45, Cw1601/1701	33	21,800
737-AC	69	IP	A0205/3001, B1510/4201, Cw08/1701	8	4,140,000
766-AC	1	IU	A03/3402, B5802/5802, Cw0602/0602	33	2,780,000

[a] Time point of the initial ELISPOT assay in the acutely infected infant cohort.

[b] IU, the viral load was detectable at >400 RNA copies/ml on the first day of life; IP, the viral load was undetectable (<400) on day 1 of life but was detectable on day 28 of life.

[c] Viral load measurement at the time point of the initial ELISPOT assay, several days following intake of single-dose nevirapine (viral loads at birth for 001-AC, 081-AC, 222-AC, and 227-AC were 1,370, 22,400, 142,000, and 7,940 RNA copies/ml, respectively). The initial viral load test for IU-infected infants was done at a median of 1 day of life (range, day 0 to 7), and IP-infected infants were tested at a median of day 28 of life (range, day 28 to 36). None of the infants were on treatment at the time of the first ELISPOT assay.

[d] ND, not determined.

TABLE 2. Characteristics of the cohort of chronically infected children at the time of the first ELISPOT assay at baseline visit

Subject	Age (yr)	HLA class I type	CD4 (%)	Viral load (RNA copies/ml)
001-CC	5	A02/74, B1510/44, Cw0202/03	26	92,000
002-CC	3	A29/74, B15/35, Cw04/04	23	14,000
003-CC	2	A0301/2301, B1503/5802,Cw0202/0602	27	670,000
004-CC	5	A24/68, B0702/1510, 0304/08	24	58,000
005-CC	2	A23/29, B08/44, Cw03/1403	23	35,000
006-CC	4	A02/29, B44/44, Cw07/07	8	320,000
007-CC	6	A23/29, B14/4201, Cw0802/1701	15	39,000
009-CC	2	A4301/6802, B15/15, Cw03/1801	20	210,000
010-CC	7	A29/3001, B4201/57, Cw07/1701	18	14,000
011-CC	6	A0301/3001, B1503/5802, Cw0202/0602	22	4,200
012-CC	3	A3001/6601, B4202/5802, Cw0602/1701	18	1,000,000
013-CC	11	A02/29, B1302/1401, Cw0602/08	10	83,000
014-CC	6	A03/03, B4501/5802, Cw0602/06	12	12,000
015-CC	8	A2301/4301, B1503/5802, Cw0202/0602	16	84,000
016-CC	6	A01/3402, B1503/8101, Cw0202/1801	18	49,000
017-CC	2	A23/3203, B1503/1503, Cw0202/0202	17	70,000
018-CC	2	A02/2301, B1510/5801, Cw07/1601	24	170,000
019-CC	4	A23/66, B1510/5802, Cw0602/1601	29	220,000
020-CC	5	A0202/4301, B1503/5703, Cw07/18	13	120,000
021-CC	3	A2301/6802, B1510/44, Cw03/03	12	150,000
022-CC	7	A6802/7408, B14/41, Cw0802/1701	20	50,000
023-CC	7	A3001/3201, B5703/5802, Cw1801/0602	17	270,000
024-CC	5	A02/2301, B0801/1503, Cw02/04	26	230,000
025-CC	5	A3402/6802, B1503/44, Cw04/04	20	6,600
026-CC	5	A02/33, B1516/4201, Cw1601/1701	13	21,000
027-CC	11	A2301/3201, B1503/1503, Cw0202/03	22	71,900
028-CC	12	A23/6802, B0801/4201, Cw03/1701	17	4,300
029-CC	8	A23/23, B0801/1503, Cw0202/03	12	24,000
030-CC	5	A03/23, B0801/41, Cw07/1701	18	110,000
064-CC	8	A29/6802, B1510/4403, Cw0701/0304	4	1,670,000
065-CC	8	A2301/74, B1503/5301, Cw0202/0401	8	1,300,000
082-CC	10	A29/66, B58/5802, Cw0602/06	5	454,000
091-CC	4	A2301/4301, B1510/1503, Cw16/18	16	4,060,000
094-CC	9	A2301/24, B07/07, Cw07/07	17	100,000
098-CC	8	A2301/3402, B0801/44, Cw0202/0701	23	123,000
110-CC	4	A29/6801, B0702/5802, Cw0602/07	14	123,000
118-CC	5	A02/33, B4501/53, Cw04/1601	15	647,000
167-CC	7	A2301/3402, B4201/44, Cw04/1701	25	34,400
184-CC	12	A0205/74, B5801/35, Cw04/07	6	233,000
185-CC	9	A29/74, B1503/1510, Cw0202/16	15	100,000
205-CC	6	A0301/29, B0801/44, Cw07/07	28	355,000
261-CC	7	A24/3402, B0801/44, Cw04/07	25	4,820
299-CC	10	A3002/4301, B08/1503, Cw0202/07	10	141,000
314-CC	7	A0205/3001, B1510/4201, Cw08/1701	12	280,000
419-CC	11	A2301/4301, B14/5802, Cw0602/0802	23	133,000

HIV infected by plasma HIV RNA determination (Fig. 1A). The remaining seven subjects who all had undetectable $CD8^+$ T-cell responses (<100 SFC/million PBMCs) proved to be HIV uninfected.

In order to further define adaptive immune responses in acute infant infection, we studied an expanded cohort of infants who were HIV infected on day 1 of life (defined as IU infected) and other infants who were HIV negative on day 1 but became HIV positive when retested on day 28 (defined as IP-infected infants). A total of 63 infected infants were identified from 719 mothers enrolled in the study; 70% of these infants (44/63) were IU infected, and 30% (19/63) were IP infected (Table 1). The initial IFN-γ ELISPOT assays were performed as soon after diagnosis as feasible, before antiretroviral therapy was initiated, at a median of 9 days of age (range, 1 to 92 days) for IU-infected infants and at a median of 55 days of age (range, 31 to 105 days) for IP-infected infants. $CD8^+$ T-cell responses were detectable in 39/63 infants (29 of 44 IU infected and 10 of 19 IP infected). In three of these infants, all of whom had detectable responses, peptides within Env and Vif were not included in the assay because of a paucity of PBMCs.

HIV-specific $CD8^+$ T-cell responses were detected in 14 of 20 IU-infected infants tested within 1 week of birth, and the majority of IU-infected infants tested after 4 weeks of life had detectable responses (Fig. 1B and data not shown). Overall, a greater proportion (29/44; 65%) of IU-infected infants had detectable cellular immune responses compared to IP-infected infants (10/19; 52%) (not significant), with a median follow-up of the IP-infected infants of 52 days (range, 31 to 105 days). By comparison, all of 45 chronically infected children had detectable $CD8^+$ T-cell responses when initially tested at year 2 of life or greater (median, 580 SFC/million PBMCs).

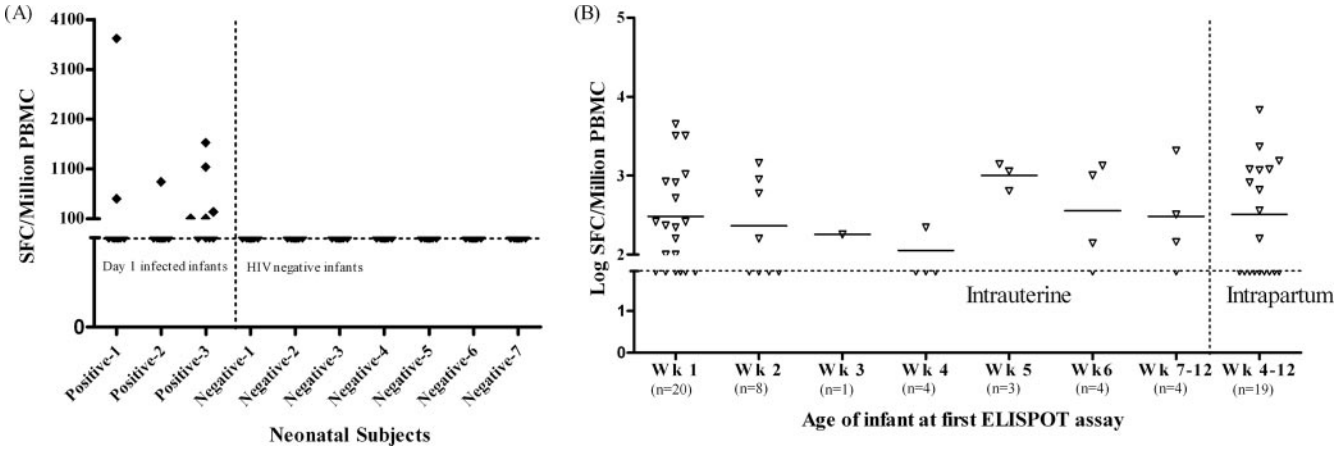

FIG. 1. Early detection of CD8+ T-cell responses in HIV-infected infants. (A) CD8+ T-cell responses on day 1 of life in 10 infants born to HIV-positive mothers. (B) Detection of HIV-specific CTL responses in acutely infected infants. Infants were grouped by weeks depending on the earliest time that the first assay could be done. For both plots, infants with CD8+ T-cell responses above the horizontal dotted line (≥100 SFC/million PBMCs) were defined as having positive responses and those below as having a negative response. The vertical dotted line divides IU- and IP-infected infants. Responses of <100 SFC were assigned 90 SFC and treated as negative.

Frequent targeting of Env in early pediatric infection and of Nef in chronic infection. To identify the proteins principally targeted in early and chronic pediatric infection, the magnitudes of the responses to each of the nine HIV proteins were documented from the first time point at which assays were performed for the 36 acutely infected infants (Fig. 2A) and 45 chronically infected children (median age of 6 years) (Table 2; Fig. 2B) who had detectable responses upon comprehensive screening in ELISPOT assays. There was a greater number of responders to Gag, Pol, Vif, Nef, and Vpr ($P = 0.0011$, 0.0001, 0.0051, 0.0008, and 0.0051, respectively, by Fisher's exact test) among chronically infected children than among acutely infected infants. Further analysis confirmed a larger magnitude of responses to Gag, Pol, and Nef ($P = 0.0012$, 0.0002, and 0.0410, respectively, by Mann-Whitney test) in chronically infected than in acutely infected children. There were no significant differences between acute and chronically infected children in responses to Vpu, Tat, Env, and Rev.

We next compared the proportional contribution of each protein to the overall total response per child in both acute and chronic pediatric infection (Fig. 3A). The overall contribution of the Env and Rev responses was greater in acute infection than in chronically infected children ($P < 0.0001$ and $P = 0.0074$), and in chronically infected children the contribution of the Nef-specific response to the total response was greater than in acute infection ($P = 0.0027$ by Mann-Whitney test) (Fig. 3B). Of particular significance is the predominant targeting of Env in acute infection: in one-third (12/36) of infants Env was the dominant target, compared to 4% (2/45) of the chronically infected children ($P = 0.00083$ by Fisher's exact test) (Fig. 3A).

To determine whether the initial responses observed in children are maintained over time we tracked the responses longitudinally in four infected infants (Fig. 4). These were the only 4 of 20 followed longitudinally from birth who did not meet the clinical and immunological WHO criteria to receive highly active antiretroviral therapy (HAART) within the first 12 months of age. Two of the infants, 349-AC and 447-AC, illus-

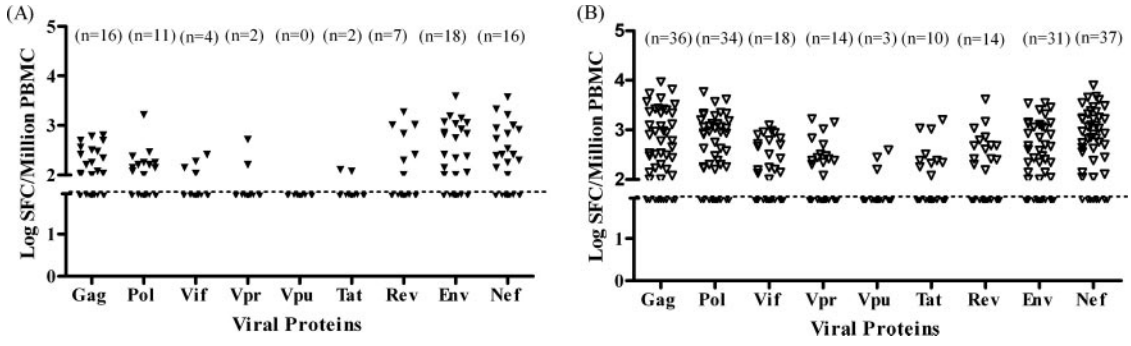

FIG. 2. Hierarchy of responses to HIV proteins targeted by acutely (A) and chronically (B) infected children. Analysis was made from the 36 acutely infected infants ($n = 39$; 3 were excluded due to insufficient cells to carry out full matrix screen) and 45 chronically infected children, all of whom made responses to at least one of the nine HIV proteins on the first ELISPOT assay. The first ELISPOT assay was undertaken in infants at a mean of 31 days of age (range, 1 day to 105 days; interquartile range, 7 to 46 days). The number of infants showing positive responses to each protein is shown in parentheses. Negative responses were assigned 90 SFC/million PBMCs (dotted line); values of ≥100 SFC/million PBMCs were defined as positive responses and those of <100 SFC/million PBMCs negative (see Materials and Methods).

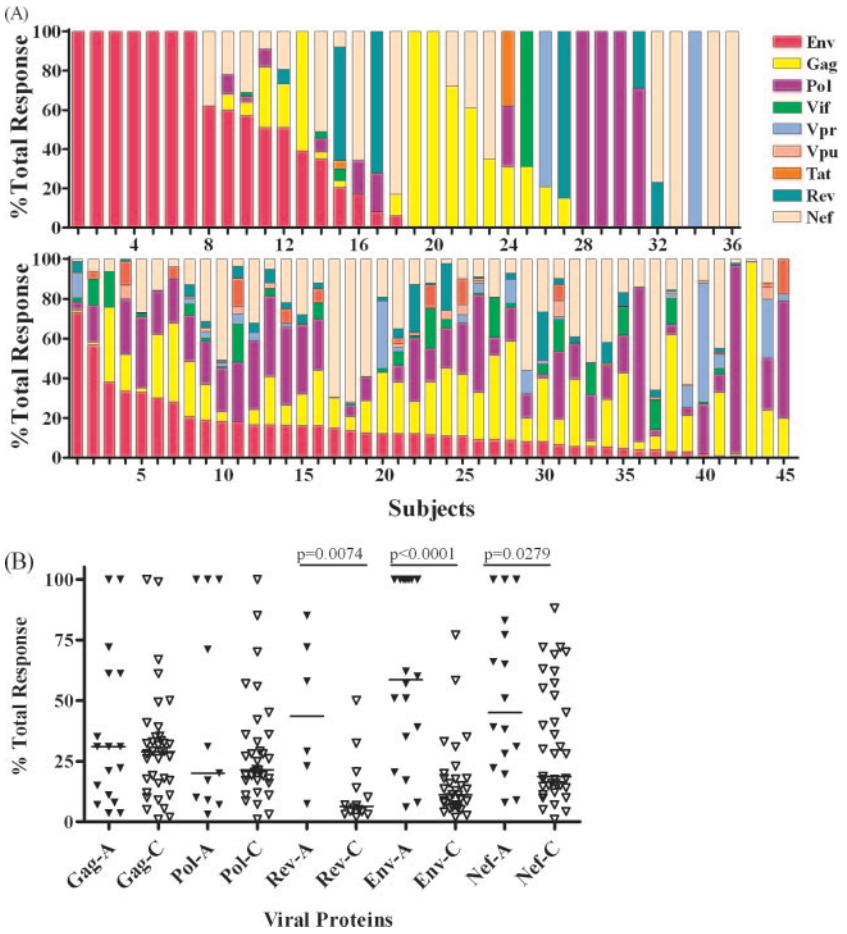

FIG. 3. (A) Relative contribution of protein response to overall response in acute and chronic infection. A reanalysis of the same data shown in Fig. 2 is used to show the contribution of each protein to the total HIV-specific response in the acutely infected infants (top panel) and chronically infected children (bottom panel) who had significant responses on the first ELISPOT assay. (B) Comparison of the contributions of responses to different proteins in acutely (A) and chronically (C) HIV-infected children with detectable responses.

trate the initially high contribution to the total HIV-specific response made by Env-specific $CD8^+$ T cells in early pediatric infection. 447-AC required HAART after 14 months of life. 349-AC initially had dominant Env responses, which decreased over the first 12 months and were later replaced by Gag- and Pol-specific responses; development of the latter coincided with a significant decrease in viral load (186,000 RNA copies/ml at 14 months versus 2,280 RNA copies/ml at 24 months) and an increase in CD4 counts from 30% at 14 months to 41% at 24 months without HAART (Fig. 4A and B).

133-AC, who achieved successful control of viremia without the need for antiretroviral therapy (to 494 copies/ml and a CD4 percentage of 29 at 31 months), showed a strongly dominant $CD8^+$ Gag-specific response that persisted. Similarly, 517-AC initially had dominant Rev-specific responses that were soon replaced by dominant Gag-specific responses (Fig. 4C and D). Although viremia has remained high in this child ($>1 \times 10^6$ copies/ml), the CD4 percentage has also remained at high levels of >30%. However, these four anecdotal cases are insufficient to allow an analysis of specificity of the $CD8^+$ response in infants and subsequent immunological and virological control of HIV infection.

Weak $CD4^+$ Gag-specific T-cell activity in early pediatric infection. Since $CD8^+$ T cells require functional $CD4^+$ T-cell help to sustain their effector activity (7), we examined Gag-specific $CD8^+$ and $CD4^+$ T-cell activity in infants with early infection. We focused on Gag-specific responses since Gag is the dominant target for HIV-specific T-helper activity (30) and limitations on cell numbers precluded analysis of $CD4^+$ T-cell responses to non-Gag proteins. In addition, Gag-specific $CD4^+$ T-cell responses have been detected in acute HIV infection of adults (39). Gag-specific responses were measured at 2, 4, and 6 months of age only, as 50 and 75% of the infants required HAART by 6 and 12 months of age, respectively. The infants with acute infection had detectable $CD8^+$ T-cell responses at all three time points (median Gag-specific $CD8^+$ T-cell responses were 0.095, 0.155, and 0.14% at 2, 4, and 6 months, respectively). These responses were lower than but did not differ significantly from those measured in chronically infected children (median, 0.31%) (Fig. 5A). In contrast, infants with acute infection had significantly lower $CD4^+$ T-cell responses at all time points than the chronically infected children, with median Gag-specific $CD4^+$ T-cell responses being 0.01%, 0.01%, and 0.02% CD4 at 2, 4, and 6

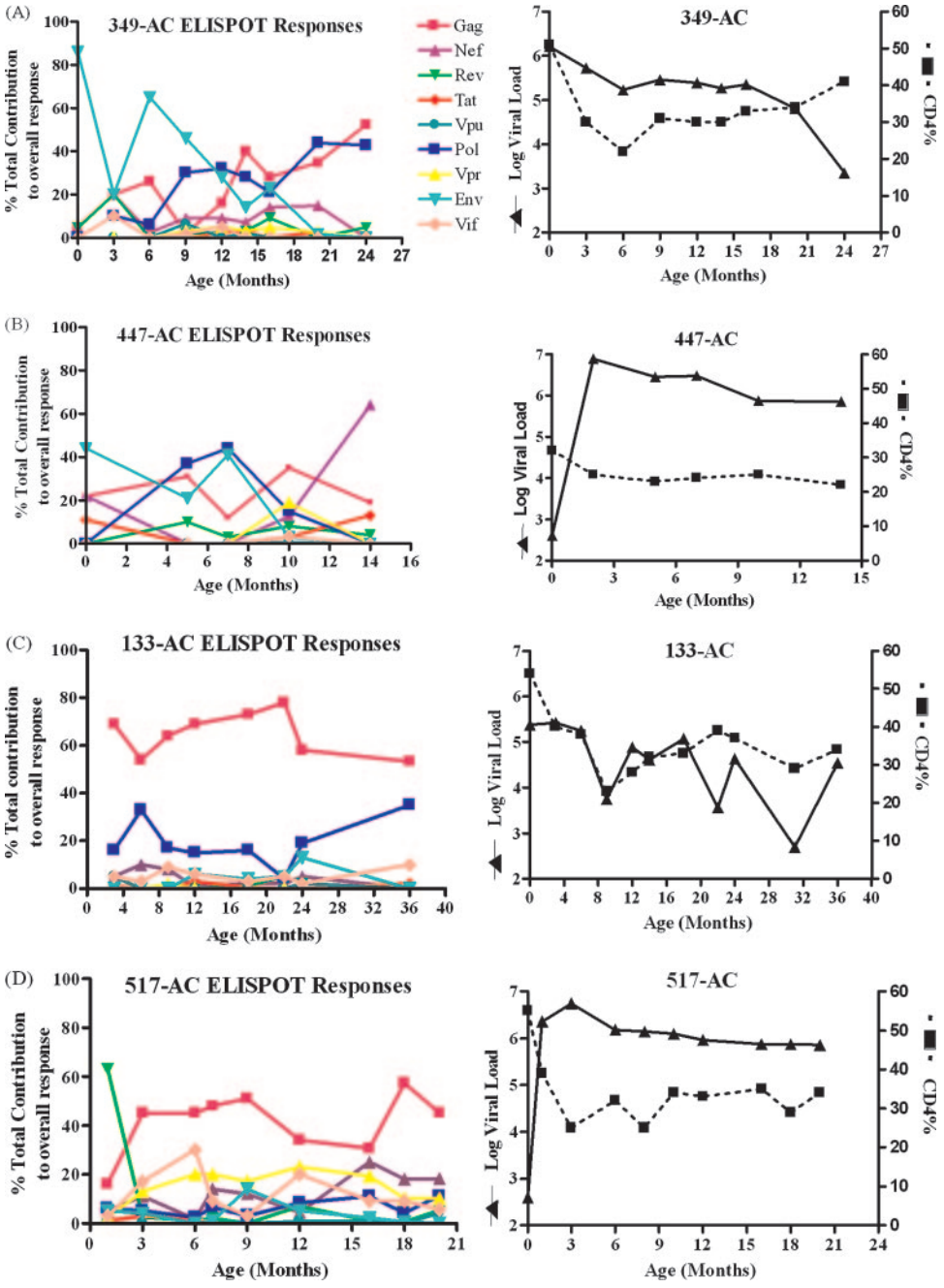

FIG. 4. Longitudinal measurement of $CD8^+$ T-cell responses, CD4, and viral load in therapy-naïve subjects A-349 (A), A-447 (B), A-133 (C), and A-517 (D). The CD4 counts and viral load measurements correspond to the time points at which $CD8^+$ T-cell responses were determined.

months, respectively, compared to 0.06% for chronically infected children ($P < 0.0001$, $P < 0.0001$, and $P < 0.0008$, respectively) (Fig. 5B).

We also compared Gag-specific $CD8^+$ (Fig. 5C) and $CD4^+$ (Fig. 5D) IFN-γ, IL-2, and TNF-α production in the same of group of infants at 6 months of life. IL-2 and TNF-α responses were significantly less frequently detected than IFN-γ. In contrast, adults can generate substantial HIV-specific $CD4^+$ IFN-γ and IL-2 responses during acute HIV infection (39), while chronically infected children on therapy show an increase in the frequency of IL-2-secreting $CD4^+$ T cells (6).

DISCUSSION

These studies describe the early HIV-specific T-cell responses in pediatric infection. $CD8^+$ T-cell responses are detectable from the first days of life in the majority (70%) of IU-infected infants. Responses were broadly directed, although Env-specific $CD8^+$ T-cell activity in particular contrib-

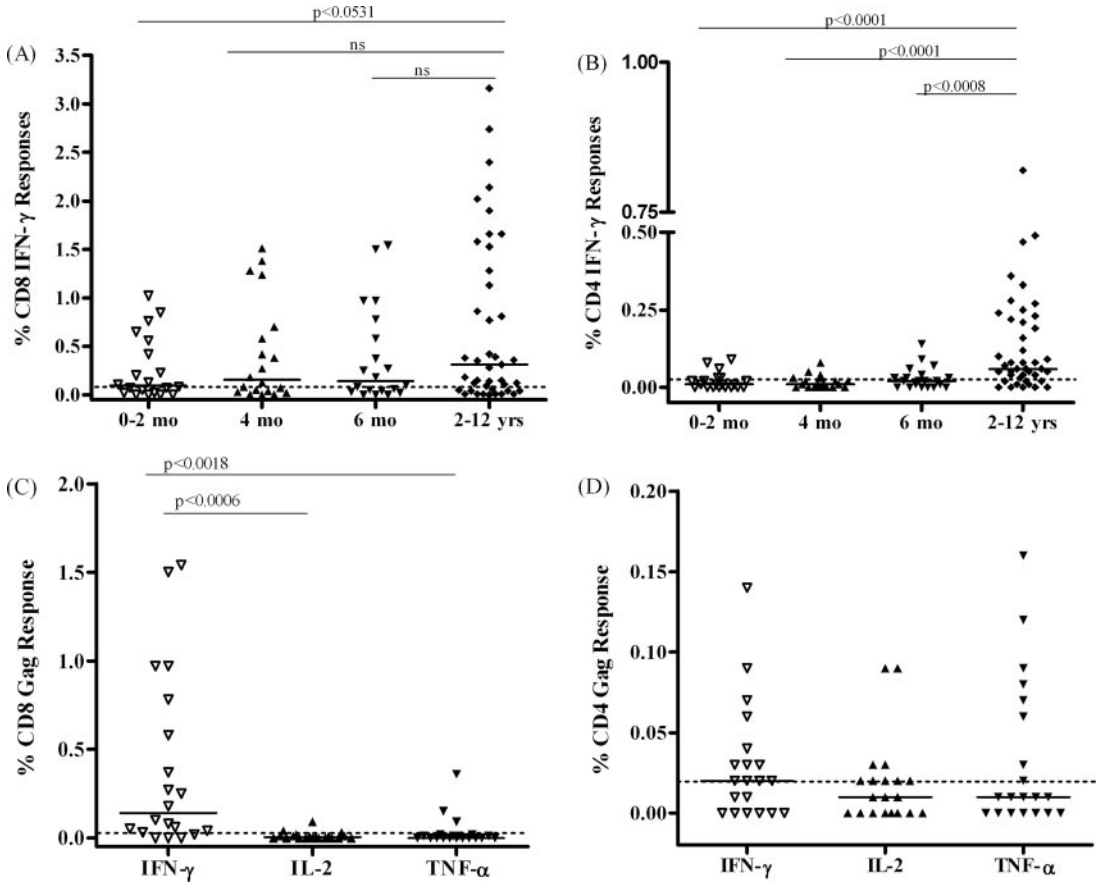

FIG. 5. (A and B) Gag-specific $CD8^+$ and $CD4^+$ T-cell responses in acutely and chronically infected children by intracellular cytokine staining. (C and D) Gag-specific $CD8^+$ and $CD4^+$ IFN-γ, IL-2, and TNF-α at 6 months of life in acutely infected infants. All infants studied were antiretroviral therapy naïve at time of analysis. Dashed lines indicate the upper limit of responses detected from the same assays undertaken with 23 HIV-uninfected control infants (see Materials and Methods). Horizontal bars indicate median responses in HIV-infected study subjects.

uted significantly more to the total HIV-specific response in acutely infected infants than to that in chronically infected children ($P < 0.0001$). Gag-specific $CD4^+$ T-cell responses were weak or undetectable in the first 6 months of life, contrasting with chronic pediatric or acute adult infection.

Previous studies using noncomprehensive screening assays and in very limited numbers of subjects have demonstrated weak HIV-specific $CD8^+$ T-cell responses in infancy (24, 25). HIV-specific cytotoxic T lymphocytes (CTL) have been detected in cord blood at birth previously (24), and human cytomegalovirus-specific CTL have been detected in cord blood IU (27), indicating that immune responses to HIV can be detected very early in life. The studies described here demonstrate not only that these early responses can arise but that they are detectable in a high proportion (70%) of infants at the earliest time point that they were tested.

Studies evaluating the specificity of the initial $CD8^+$ T-cell response in infants infected with HIV have not been systematically undertaken, and the finding that Env-specific $CD8^+$ T-cell activity contributes substantially to this initial response is of interest in relation to recent studies with adults indicating that Env-specific $CD8^+$ T-cell responses are associated with higher viral loads and Gag-specific $CD8^+$ T-cell responses with lower viral loads (4, 19, 32). More studies will be needed to determine if this is related to the lack of a rapid reduction in viral load observed in early pediatric infection compared to early adult infection, where Env makes a smaller contribution to the initial $CD8^+$ T-cell responses (22).

It was not possible here to relate the specificity of the early $CD8^+$ T-cell response and progression in this cohort, as two-thirds of the study subjects were enrolled in a study of early HAART in pediatric HIV infection. Of the 20 infants who did not receive early HAART, only 2 achieved viral loads of <100,000 in the first year. The finding of early $CD8^+$ T-cell responses combined with persistently high viral loads in the first year of life suggests that these $CD8^+$ T cells are ineffective, and additional functional studies will be needed to determine how this apparent dysfunction compares to that seen in adults (4, 8). Comprehensive phenotypic analysis of $CD8^+$ T cells in adults has demonstrated the association of polyfunctional $CD8^+$ T cells with control of viremia (3). These comprehensive analyses were not undertaken with these study infants, but the lack of IL-2 and TNF-α responses suggests that the majority of the $CD8^+$ T-cell responses detectable via the IFN-γ ELISPOT assay may be monofunctional.

Limitations imposed by cell numbers allowed only Gag-specific $CD4^+$ T-cell responses to be assessed. Gag was chosen since it is consistently the dominant target for HIV-specific

$CD4^+$ T-cell responses (30). The weak or undetectable Gag-specific $CD4^+$ T-cell activity in acutely infected infants seen in this study also contrasts with acute adult infection, where adults present with high levels of $CD4^+$ T-cell responses during acute infection (39). In addition, Gag-specific $CD4^+$ T-cell activity has been reported in chronic infection in children >5 years old who have spontaneously controlled viremia without antiretroviral therapy (9). The marked absence of HIV-specific $CD4^+$ T-cell activity even to 6 months of age suggests a fundamental reason why $CD8^+$ T cells in infected infants are ineffective (12, 21). These findings of a lack of HIV-specific $CD4^+$ T-cell activity are consistent with other studies (24), in one case showing minimal $CD4^+$ T-cell responses in HIV-infected children until 3 to 5 years of age (34). Furthermore, where detectable, HIV-specific $CD4^+$ T-cell responses have been reported to be type 2 as opposed to type 1 (37, 38) and therefore less likely to support the induction and maintenance of HIV-specific CTL activity. Of concern, and again in contrast to what is observed in acute adult infection (33), early treatment with antiretroviral therapy in infected infants did not result in increased HIV-specific $CD4^+$ T-cell responses (D. Ramduth et al., unpublished data). However, the extent to which these findings result from or cause the persistent high levels of viremia observed in early pediatric HIV infection is not known.

The encouraging aspect of these data is that IU-infected infants mount $CD8^+$ T-cell responses from the first day of life, while those infected IP have detectable responses a month after infection. Although 85% of infected infants (in this cohort) met current WHO criteria to initiate HAART within 12 months of infection, it is also clear that a small minority (2/20) of infected infants showed viral loads of <10,000 and a CD4 percentage of >30 by 24 months of age. Thus, spontaneous control of HIV is possible in pediatric HIV infection. Identification of greater numbers of "relative controller" children will facilitate further definition of what constitutes an effective immune response in early pediatric HIV infection. Moreover, the fact that the neonatal immune system can generate adaptive immune responses to HIV provides important information for the development of vaccines to prevent peripartum infection.

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