Longitudinal Assessment of Human Immunodeficiency Virus Type 1 (HIV-1)-Specific Gamma Interferon Responses during the First Year of Life in HIV-1-Infected Infants

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Human immunodeficiency virus type 1 (HIV-1) infection results in different patterns of viral replication in pediatric compared to adult populations. The role of early HIV-1-specific responses in viral control has not been well defined, because most studies of HIV-1-infected infants have been retrospective or cross-sectional. We evaluated the association between HIV-1-specific gamma interferon (IFN-γ) release from the cells of infants of 1 to 3 months of age and peak viral loads and mortality in the first year of life among 61 Kenyan HIV-1-infected infants. At 1 month, responses were detected in 7/12 (58%) and 6/21 (29%) of infants infected in utero and peripartum, respectively ($P = 0.09$), and in ~50% of infants thereafter. Peaks of HIV-specific spot-forming units (SFU) increased significantly with age in all infants, from $251/10^6$ peripheral blood mononuclear cells (PBMC) at 1 month of age to $501/10^6$ PBMC at 12 months of age ($P = 0.03$), although when limited to infants who survived to 1 year, the increase in peak HIV-specific SFU was no longer significant ($P = 0.18$). Over the first year of life, infants with IFN-γ responses at 1 month had peak plasma viral loads, rates of decline of viral load, and mortality risk similar to those of infants who lacked responses at 1 month. The strength and breadth of IFN-γ responses at 1 month were not significantly associated with viral containment or mortality. These results suggest that, in contrast to HIV-1-infected adults, in whom strong cytotoxic T lymphocyte responses in primary infection are associated with reductions in viremia, HIV-1-infected neonates generate HIV-1-specific CD8+-T-cell responses early in life that are not clearly associated with improved clinical outcomes.

CD8+ cytotoxic T lymphocytes (CTL) are responsible for clearing acute viral infections such as cytomegalovirus (CMV) and measles virus and play variable roles in chronic viral infections, depending on the site and degree of ongoing viral replication (reviewed in references 38 and 64). The CD8+ CTL response to human immunodeficiency virus (HIV) has been extensively studied in humans and in the rhesus macaque-simian immunodeficiency virus (SIV) model for association with virus levels and disease progression. HIV-1- and SIV-specific CD8+-T lymphocyte numbers rise during acute infection, and the peak number of CD8+-T cells coincides with the decline in plasma viremia (30, 31, 48). In the SIV model, the depletion of CD8+-T lymphocytes in either acute or chronic infection leads to an increase in viral replication which is curtailed by the regeneration of CD8+-T cells (26, 56). The conclusion that HIV-1-specific CTL are an important component of the host immune response to infection is supported by several notable findings. First, antiviral CTL, frequently gag-specific, are associated with control of HIV-1 viral replication in adults and children of ~10 years old (10, 13, 19, 39, 40, 45).

Second, levels of circulating HIV-1-specific CTL are maintained in long-term nonprogressors (24, 51). Finally, in both acute and chronic HIV-1 infections, HIV isolates have evolved mutations allowing escape from CTL recognition, indicating immune pressure on viral replication (6, 22, 47).

The study of HIV-1-specific CD8+-T-cell responses in vertically infected infants is complicated by several factors absent in horizontal HIV-1 transmission. The patterns of HIV-1 peak and set-point plasma viral loads are very different in adults and infants (49). In infants, the levels of HIV-1 plasma viremia are persistently high, with declines not seen until the second year of life (17, 18, 37). In the absence of antiretroviral therapy, vertically infected infants have a bimodal distribution of disease progression, with approximately 25% progressing to AIDS within 1 year of life (reviewed in reference 35). Factors that may influence the levels of viral replication and disease progression in infants include the phenotype of the transmitted virus, the high number of target cells available for HIV-1 infection, and an immature immune system. Infants are likely infected with a viral variant modified by maternal immune pressure due to the half-match in major histocompatibility complex alleles (21, 55). In addition, infants have high levels of thymic output, and their immune systems are predominantly naïve (8, 15, 57), although the role of the thymus in the disease

progression of HIV-1-infected infants is not well understood (7). The ability of the neonate to respond effectively to infection is thought to be limited by the number of circulating mature T and antigen-processing cells (50, 54). Cellular immune responses in HIV-1-infected infants have been inconsistently detected in infants younger than 6 months (33, 34, 36, 46, 61). The paucity of CTL responses in infants less than 1 year old has been suggested (i) to be due to diminished Th1 responses, in particular a deficiency in gamma interferon (IFN-γ) secretion (58, 62, 63) or (ii) to be influenced by age, CD4 counts, and antigen processing (53). The interpretation of the earlier reports is limited by the lack of longitudinal data and the imprecise detection of the timing of infection in the infants.

We had the opportunity, with a prospective observational cohort of infants born to HIV-1-infected women, to identify infants infected before 1 month of life and to measure HIV-1-specific CD8$^+$-T-cell responses together with viral loads over the first year of life. We hypothesized that sustained high HIV-1 viral loads observed in perinatal transmission were consistent with a deficiency in virus-specific CD8$^+$-T-cell responses. We examined the HIV-1-specific IFN-γ release from CD8$^+$-T cells at one to five time points during the first year of life of 61 Kenyan infants diagnosed with HIV-1 infection at or before the first month of life and investigated the relationship between the timing and presence of early anti-HIV-1 CD8$^+$-T-cell responses and peak viral loads and mortality in the infants.

MATERIALS AND METHODS

Patient cohort. As part of a larger cohort of infants born to HIV-1-infected women between 1999 and 2003, 61 infants with HIV-1 RNA or DNA detected in blood obtained during the first month of life were included in this cohort. Details of the larger cohort of infants born to HIV-1-infected women have been presented elsewhere (20, 41). Written informed consent was obtained from all mothers on behalf of themselves and their infants. Mothers were recruited during pregnancy and were provided with zidovudine beginning between the 34th and 36th weeks of gestation for the prevention of infant HIV-1 infection (59). Infants were breast or formula fed as per maternal preference. Infants were examined by clinicians, and 1 to 3 ml of peripheral blood was obtained at birth and at months 1, 3, 6, 9, and 12. Seven infants born to HIV-1-seronegative women were included as controls and were bled at 3 months of life. This research complied with all U.S. and Kenyan guidelines and policies.

Blood collection. Freshly collected EDTA-anticoagulated blood was centrifuged for 10 min at 1,800 rpm to separate plasma, which was aliquoted and stored at $-80°$C. The remaining blood was then diluted with an equal volume of RPMI 1640 (Gibco-BRL), layered on a Ficoll gradient (Lymphocyte Separation Medium; Organon Teknika, West Chester, PA), and centrifuged for 30 min at 2,000 rpm. Peripheral blood mononuclear cells (PBMC) at the interface of the gradient were isolated, washed in RPMI 1640, and used directly in the enzyme-linked immunospot (ELISPOT) assay. The mean numbers $\pm$ standard errors of the means of PBMC recovered at months 1, 3, 6, 9, and 12 were (13.6 $\pm$ 1.0) $\times$ 10^6, (17.7 $\pm$ 1.1) $\times$ 10^6, (18.6 $\pm$ 1.7) $\times$ 10^6, (16.8 $\pm$ 1.6) $\times$ 10^6, and (15.5 $\pm$ 1.5) $\times$ 10^6, respectively.

HIV-1 diagnosis and determination of timing of infection. Infant HIV-1 infection status was determined by PCR amplification of HIV-1 *gag* DNA sequences from dried blood spotted on filter paper (44) or by quantitative analysis of infant plasma HIV-1 RNA using a transcription-mediated amplification method sensitive for the detection of multiple HIV-1 subtypes (Gen-Probe HIV-1 viral load assay) (43). Results relative to HIV RNA copies/ml plasma were considered positive if there were $\geq$100 copies per ml or, in cases where less than 500 μl was available for testing, if there were $\geq$50 RNA copies per reaction. The timing of an infection was categorized as in utero if the specimen collected within the first 48 h of life was positive for either HIV-1 DNA or HIV-1 RNA. The timing of infection was defined as peripartum if plasma HIV-1 RNA was

undetectable within the first 48 h of life and positive at 1 month of age. Quantitative plasma viral loads were determined using the Gen-Probe assay.

HLA typing. Molecular HLA typing was performed on DNA extracted from 5 $\times$ 10^6 infant PBMC using amplification refractory mutation system PCR with sequence-specific primers designed for East African populations (9).

Peptides. Sixty-eight peptides spanning five regions of HIV-1 were synthesized by Fmoc (9-fluorenylmethoxy carbonyl) chemistry at the Peptide Core Facility at the Weatherall Institute of Molecular Medicine (Tao Dong, Oxford University). The peptides were chosen based on predefined CTL epitopes of the prevalent HIV-1 clades in Kenya (A and D). Twenty-seven peptides were from *gag*, 18 were from *pol*, 19 were from *env*, 13 were from *nef*, and 1 was from *rev* (Table 1). Epitopes were chosen based on responses previously reported in HIV-1 infection and included those present in acute infection, long-term nonprogressors, and those associated with viral control (29). These peptides bind 29 common HLA class 1 alleles (12 HLA-A, 15 HLA-B, and 2 HLA-C) representative of East African populations. The identities of peptides tested for each individual were based on the infant's HLA type. In the event of a limited number of cells, peptides were prioritized to test a complete panel for each HLA allele.

IFN-γ ELISPOT assay. An ELISPOT assay was used to detect HIV-1-specific IFN-γ release from PBMC following overnight incubation with peptides. Briefly, 96-well Millipore plates (MAIP45; Millipore SA, Molsheim, France) were coated with 7.5 μg monoclonal antibody to IFN-γ (1-DIK; Mabtech Ab, Nacka, Sweden) for 2 h at 37°C. Excess antibody was removed by washing six times with RPMI 1640 and blocked with RPMI 1640 containing L-glutamine and supplemented with 10% fetal calf serum (all from Gibco-BRL), designated R10, for 30 min at room temperature before cells were added. Duplicate wells containing 2 $\times$ 10^5 PBMC/well were stimulated with 20 μg/ml peptide, 10 μg/ml phytohemagglutinin (PHA) (positive control) (Murex Biotech Limited, Dartford, United Kingdom), or R10 media alone (negative control). The mean numbers $\pm$ standard errors of the means of PBMC used per assay at months 1, 3, 6, 9, and 12 were (6.5 $\pm$ 0.5) $\times$ 10^6, (6.4 $\pm$ 0.4) $\times$ 10^6, (6.9 $\pm$ 0.4) $\times$ 10^6, (6.7 $\pm$ 0.5) $\times$ 10^6, and (6.8 $\pm$ 0.4) $\times$ 10^6, respectively. After overnight stimulation in a humidified incubator at 37°C and with 5% CO$_2$, cells were removed from the plates by washing with phosphate-buffered saline containing 0.05% Tween 20 (Sigma, St. Louis, MO), followed in sequence by the application of biotinylated anti-IFN-γ antibody (1:1,000; 7-B6-1 biotin; Mabtech) for 3 h at room temperature, washing, and the application of streptavidin alkaline phosphatase (1:1,000; Mabtech) for 1.5 h at room temperature. After the final washing, spot-forming units (SFU) were visualized by the addition of alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA) for approximately 10 min or until an intense blue reaction was visible in the wells stimulated with PHA. Additional color development was prevented by washing the plates under running water. Plates were allowed to dry overnight before reading. Plates were read visually until January 2001, after which an automated ELISPOT reader was used (Autoimmun Diagnostika, Strassberg, Germany). The number of HIV-specific SFU per 10^6 cells was defined as the average number of SFU per 10^6 cells from peptide-stimulated wells minus the average number of SFU per 10^6 cells in wells containing R10 (background control). The following criteria were used to determine a positive assay: (i) a response to PHA of $\geq$100 SFU after the subtraction of the background, (ii) the number of HIV-specific SFU per 10^6 cells being greater than or equal to 50, and (iii) the number of SFU in peptide-stimulated wells being greater than or equal to two times the number of background control SFU (4). The qualities of the ELISPOT responses were compared in two ways: relative to the mean of positive responses and relative to the peak of positive responses.

Detection of antigen-specific CD8$^+$-T cells. PBMC from an infant expressing HLA-A2 and -B8 were stained with phycoerythrin-conjugated-HLA class I tetrameric complexes refolded with either HIV-1 nef-FLKEKGGL (B8-nef) or CMV pp65-NLVPMVATV (A2-CMV) (Tao Dong, Oxford University). Briefly, for each stain, 150 μl of whole blood was incubated with phycoerythrin-conjugated tetramer for 15 min at 37°C, followed by the addition of peridin chlorophyll protein-labeled anti-CD8 and fluorescein isothiocyanate-labeled anti-CD45R0 (both from Becton Dickenson, San Diego, CA) for an additional 15 min. Three milliliters of FACSLysis solution (Becton Dickenson) was added per tube, and the tubes were left for 5 min at room temperature, after which time the tubes were centrifuged for 5 min at 1,500 rpm. The cell pellet was washed once in phosphate-buffered saline containing 0.5% fetal calf serum and 0.5 mM EDTA, suspended in 170 μl of FACSfix (Becton Dickenson), and stored overnight in the dark at 4°C prior to analysis with CellQuest software (Becton Dickenson).

Statistical analysis. To compare differences in the IFN-γ responses of infants infected with HIV in utero to the responses of those infected peripartum, differences in the distributions of background SFU, numbers of mean and peak positive HIV-specific SFU, numbers of peptides tested, and numbers of peptides

TABLE 1. Panel of HIV-1 peptides, HLA restriction, and frequency of positive individuals[a]

HIV gene product	Peptide[b]	No. of positive individuals/no. of individuals tested (%)	HIV gene product	Peptide[b]	No. of positive individuals/no. of individuals tested (%)
gag	**B8-D/EIYKRWII**	2/2 (100)		A2-VIYQYMDDL	3/12 (25)
	B35-PPIPVGDIY	2/3 (67)		A3/A11/A33-A/SIFQSSMTK	1/4 (25)
	B57-I/LSPRTLNAWL	2/4 (50)		A6802-DTVLEEMNL	2/9 (22)
	B8-GGKKKYR/KL	1/2 (50)		A30-KLNWASQIY	2/13 (15)
	B57/B5801-KAFSPEVIPMF	3/7 (43)		B57/B5801-IVLPEKDSW	1/7 (14)
	A3-KIRLRPGGK	1/3 (33)		A2-ILKE/DPVHGV	1/13 (8)
	B14/Cw8-RAEQAS/TQEV	1/3 (33)		A30-KQNPDIVIYQY	1/15 (7)
	B14/Cw8-DRFF/YKTLRA	1/3 (33)		A3/A11/A31/A33-DLEIGQHRTK	0/5 (0)
	A2-TLNAWVKVI/V	4/13 (31)		A6802-DVTLEDINL	0/9 (0)
	Cw4-QASQEVKNW	2/7 (29)			
	B57/B5801-TSTLQEQIG/AW	2/7 (29)	env	**B14-ERYLKDQQL**	1/1 (100)
	B7/B42-GPGHKARVL	3/12 (25)		**A29-FNCGGEFFY**	5/8 (63)
	B18/B49-FRDYVDRFY/FK	1/5 (20)		**Cw8-NCSFNISTSI**	1/2 (50)
	B53-QATQEVKNW	1/5 (20)		B35-TA/NPWNA/SSW	1/3 (33)
	B7/B8101-ATPQDLNTM	1/6 (17)		A24/B8-YLR/KDQQLL	2/7 (29)
	A2-SLF/YNTVATL	2/13 (15)		A3/A31-RLRDLLLIVTR	1/4 (25)
	B53-TPQDLNM/TML	2/13 (15)		B15/Cw4-SFNCGGEFF	3/17 (18)
	B53-AS/TQEVKNWM	0/5 (0)		A30-IVNRVRQGY	1/16 (6)
	B53 VKNWMTETLL	0/5 (0)		A3-TVYYGVPVWK	0/3 (0)
	A24/B44-RDYVDRFY/FKTL	0/9 (0)		A30-KYCWNLLQY	0/16 (0)
	Cw4-KYRLKHLVW	0/9 (0)		B7-IPRRIRQGL	0/4 (0)
	A26-YVDRFFKTL	0/1 (0)			
	B7-SPRTLNAWV	0/4 (0)	nef	**B35-VPLRPMTY**	3/3 (100)
	A1-GSEELRSLY	0/5 (0)		**Cw8-KAAVDLSMFL**	2/2 (100)
	A30-RSLYNTVATLY	0/16 (0)		**A3-QVPLRPMTYK**	2/3 (67)
	B14/Cw8-DLNM/TMLNI/TV	0/3 (0)		**B42-TPQVPLRPM**	5/10 (50)
	A25/B53-D/ETINEEAAEW	0/6 (0)		**B7/B42/B8101-TPGPGI/VRYPL**	2/4 (50)
				B18/B49-YPLTFGWCY/F	2/5 (40)
pol	**B35-H/NPDIVIYQY**	2/3 (67)		A2-PLTFGWCYKL	3/11 (27)
	Cw8-VTDSQYALGI	1/2 (50)		A3-DLSHFLKEK	1/5 (20)
	B8-GPKVKQWPL	1/2 (50)		B8-FLKEKGGL	2/11 (18)
	A6802/A74-ITLWQRPLV	6/13 (46)		A2-VLEWRFDSRL	1/11 (9)
	B45-GAETFYVDGA	3/7 (43)		A2-ALKHRAYEL	1/12 (8)
	B35-EPIVGAETFY	2/5 (40)		B7-FPVTPQVPLR	0/4 (0)
	A6802/A74-ETFYVDGAAN	5/13 (38)		A24-DSRLAFHHM	0/5 (0)
	B57-KITTESIVIW	1/3 (33)			
	A6802-ETAYFILKL	3/9 (33)	rev	A1-ISTERILSTY	1/5 (25)

[a] Expressed as the number of babies with a positive peptide response over the number of infants tested with that peptide, followed by the percent. Assays were conducted at multiple ages per infant, and thus responses to a given peptide were counted once per infant if there was ever detection of a response with a particular peptide. Peptides that stimulated positive responses in ≥50% of infants are indicated in boldface.
[b] HLA restriction of each peptide precedes the sequence. Clade variants are indicated by a slash within the amino acid sequence.

recognized were determined using Mann-Whitney U tests. Pearson's chi-square statistic was used for the comparison of the proportion of infants with positive HIV-1-specific ELISPOT results who were infected in utero to that of those who were infected peripartum. Data was log transformed for analysis of viral loads and changes in the numbers of HIV-specific SFU over time. Differences between the mean and peak $\log_{10}$s of the HIV-1 RNA plasma viral loads of (i) infants infected with HIV-1 in utero and those of the infants infected peripartum or (ii) between infants who had and those who lacked HIV-1-specific ELISPOT responses were assessed using t tests for independent samples. To model the changes in peak numbers of HIV-specific SFU over the first year of life of infected infants, a linear mixed-effect model was used. For determination of the change in viral load over time, a linear mixed-effect model with a compound symmetric covariance structure was used with the $\log_{10}$ of the HIV RNA viral load between month 1 and month 12 as the outcome variable. Linear regression analysis was used to compare peak plasma HIV-1 RNA loads in infants who had or lacked HIV-1-specific IFN-γ responses at 1 month of age, controlling for viral load at 1 month of age after testing for and excluding nonsignificant effect modification. Cox regression analysis was used to model the association between mortality and (i) the presence of either HIV-1-specific IFN-γ responses at 1 month of age or (ii) weak HIV-specific IFN-γ responses (<500 HIV-specific SFU), controlling for viral load at 1 month of age and in utero infection.

RESULTS

Concordance between peptide-stimulated IFN-γ secretion and detection of antigen-specific CD8^{+}-T cells. The presence of antigen-specific CD8^{+}-T cells and the relationship between the detection of these cells by major histocompatibility complex-tetrameric complexes and detection by IFN-γ ELISPOT assay were confirmed in three individuals. Figure 1 illustrates representative results obtained from one HIV-1-infected infant at 9 and 12 months of age, demonstrating the presence of both A2-CMV and B8-nef-specific CD8^{+}-T cells in this infant together with peptide-specific ELISPOT assay results. At 9 months of age, we detected tetramer-positive A2-CMV-specific and B8-nef-specific CD8^{+}-T cells, comprising 0.68% and 0.27%, respectively, of total lymphocytes. In the concordant ELISPOT assay, the frequency of IFN-γ-producing cells responding to A2-CMV peptide was 1,497 per 10^6 PBMC, and the frequency of cells responding to B8-nef-peptide was 303
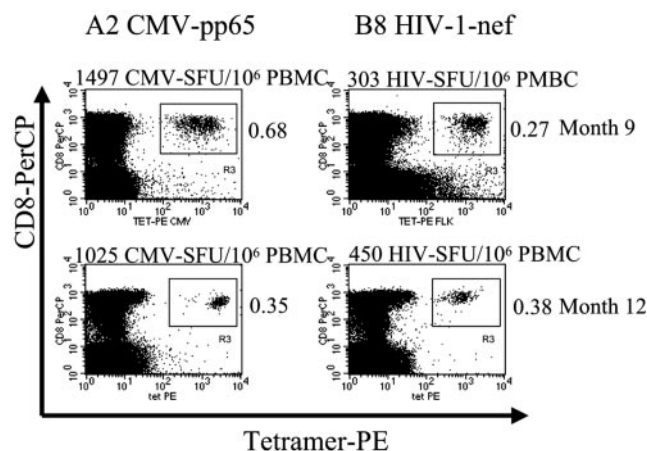
FIG. 1. Assays of CMV- and HIV-specific tetramer staining and IFN-γ ELISPOT responses. Responses detected from an HIV-1-infected infant at 9 (upper panels) and 12 (lower panels) months of age. PBMC were stained with anti-CD8 monoclonal antibody (*y* axes) and with the relevant HLA class I/peptide tetramer (*x* axes) or stimulated with peptides in an overnight ELISPOT assay to detect IFN-γ secretion. A2-CMVpp65-NLVPMVATV (left panels) and B8-HIV-1 nef-F LKEDGGL (right panels) responses were detected by both assays. The tetramer-positive CD8+ T cells, as percentages of total lymphocytes, are indicated adjacent to each plot. The antigen-specific IFN-γ ELISPOT assay results are shown above each plot.

TABLE 2. Prevalence of detection of HIV-1-specific IFN-γ ELISPOT responses during the first year of life of 61 HIV-1-infected infants

Age (months)	No. tested (% of cohort)	Prevalence of HIV-1-specific responses [no. of positive infants/no. of infants infected (%)]		P value[b]
		In utero[a]	Peripartum	
1	33 (54)	7/12 (58)	6/21 (29)	.09
3	45 (74)	12/18 (67)	14/27 (52)	.4
6	41 (67)	6/15 (40)	13/26 (50)	.4
9	29 (48)	6/11 (54)	8/18 (44)	.5
12	26 (43)	5/10 (50)	10/16 (63)	.6

[a] The timing of infection of infants was categorized as in utero if HIV-1 DNA or RNA was detected in dried blood spots or plasma collected within the first 48 h of life. Infants were categorized as infected *peripartum* if HIV-1 DNA or RNA was undetectable within the first 48 h. of life and detectable at 1 month of life.

[b] P values determined by Pearson's chi-square statistic.

per 10^6 PBMC. When the assays were repeated 3 months later, the frequencies of tetramer-positive CD8+-T cells were 0.35% and 0.38% of total lymphocytes specific for A2-CMV and B8-nef, respectively. The corresponding frequencies of A2-CMV and B8-nef peptide-specific IFN-γ-producing cells were 1,025 per 10^6 PBMC and 450 per 10^6 PBMC, respectively. Differences between the percentage of CD8+-T cells detected by tetramer and the corresponding functional response following cognate peptide stimulation have been observed before (3, 29a), and the detection of IFN-γ ELISPOT responses is likely representative of the presence of CD8+ antigen-specific T cells in circulation.

HIV-1-specific ELISPOT responses in infants infected in utero or peripartum. We measured HIV-1-specific IFN-γ secretions from PBMC isolated from infants infected with HIV-1 in utero or peripartum and followed those responses over the first year of life. The peptides were used either singly or paired by clade variants and are presented by decreasing frequency of response in Table 1.

Using this panel, we estimated the prevalence of the detection of HIV-1-specific IFN-γ responses during the first year of life in HIV-1-infected infants (Table 2). The number of infants tested at each time point varied because of clinic attendance and mortality. We were able to test all HLA-restricted peptides in our panel on 29/33 (88%) infants tested at month 1 and on 43/45 (96%), 40/41 (97%), 26/29 (90%), and 24/26 (92%) infants at months 3, 6, 9, and 12, respectively. At 1 month of age, there was a trend for more-likely detection of HIV-1-specific IFN-γ responses in infants infected in utero compared to infants infected peripartum. Seven of 12 (58%) infants infected in utero had detectable responses versus 6 (29%) of 21 infants infected peripartum (P = 0.09). Although the differences did not reach significance due to small sample sizes, this trend is likely explained by

the duration of virus infection. By 3 months of age, the prevalence of HIV-1-specific IFN-γ responses was approximately 50% in either group and, at this and subsequent ages, there was no significant difference in the prevalences of the detection of responses in infants with respect to the timing of infection with HIV-1.

To determine if the timing of HIV-1 infection affected the quality of the cellular immune response, we compared the breadths and strengths of IFN-γ responses in infants infected in utero versus peripartum (Table 3). We chose the month 3 time point for comparisons because the greatest number of infants (45/61 [75%]) were tested at that time. To determine HIV-1 specificity, we compared the prevalence of positive assays in HIV-1-infected infants with that in HIV-1 unexposed, uninfected infants. None of seven 3-month-old HIV-1 unexposed, uninfected infants had positive ELISPOT responses, suggesting a high specificity for the assay. We observed trends for infants infected peripartum to have stronger HIV-1-specific IFN-γ responses detected at 3 months of age, although the data did not reach significance. For infants infected in utero, the median magnitudes of mean and peak responses were 151 and 155 HIV-specific SFU per 10^6 PBMC, respectively, versus 384 and 458 per 10^6 PBMC, respectively, in infants infected peripartum (P = 0.06 for both).

HIV-1-specific IFN-γ responses over the first year of life. In a subset of 18 infants, IFN-γ responses and plasma viral load measurements were conducted at every time point up to month 12 or death. These results are shown in Fig. 2a and b, respectively, and demonstrate a diverse pattern of immune recognition and viral replication. Three infants were completely lacking in detectable responses (B1-276, B1-160, and B1-005). Two infants demonstrated strengthening and broadening responses to infection over time (B1-454, B1-473). Two infants lost early responses, but the loss was not associated with mortality (B1-093, B1-259). In one infant, the loss of early responses preceded the infant's death (B1-424). To interpret the changes in HIV-1-specific IFN-γ responses over the first year of life in all the infants, we employed a model that adjusted for age, incomplete and repeated measures, and the timing of infections. Log_10-transformed peak HIV-specific SFU numbers were used to model the responses over time. We found a significant change in peak HIV-1-specific IFN-γ responses in infants dur-

TABLE 3. HIV-1-specific IFN-γ responses in HIV-1 unexposed, uninfected infants and HIV-1-infected infants measured at 3 months of age

Parameter	Infant			P value[c]
	Unexposed[a]	Exposed-infected[b]		
		In utero	Peripartum	
No. of individuals tested	7	18	27	
No. of peptides tested (range of median)	5 (1–9)	11 (6–18)	9 (1–19)	.2
Background SFU/10^6 PBMC median (25th–75th quartile)	57 (20–90)	34 (19–64)	20 (14–40)	.08
No. with positive responses (%)	0	12 (67)	14 (52)	.4
No. of positive peptides (range of median)	0	2 (1–6)	2 (1–6)	.3
Mean positive HIV SFU/10^6 PBMC median (25th–75th quartile)	n.a.[d]	151 (83–396)	384 (216–522)	.06
Peak positive HIV SFU/10^6 PBMC median (25th–75th quartile)	n.a.	155 (103–693)	458 (216–953)	.06

[a] Unexposed infants were born to HIV-1-seronegative women. The infection status of an infant was confirmed by testing for HIV-1 DNA in dried blood spots collected at the time of the visit, as described in Materials and Methods.

[b] Infants were categorized as infected in utero if HIV-1 DNA or RNA was detected in dried blood spots or plasma collected within the first 48 h of life. Infants were categorized as infected peripartum if HIV-1 DNA or RNA was undetectable within the first 48 h of life and detectable at 1 month of life.

[c] The P values for differences in responses between infants infected in utero and peripartum was determined by nonparametric test for differences in medians from independent samples, except for comparisons of numbers of individuals with positive responses, in which the P values were calculated from the chi-square statistic.

[d] n.a., not applicable.

ing the first year of life, independent of the timing of infection with HIV-1 ($P = 0.03$) (Fig. 3). Peak HIV-specific SFU increased twofold with increasing age, from a mean of $251/10^6$ PBMC (2.4 $\log_{10}$) at 1 month of age to mean of $501/10^6$ PBMC (2.7 $\log_{10}$) at 12 months of age. To address the potential bias of survivor effect, we also limited the analysis to 30 infants who survived to month 12. We found a similar increase in peak IFN-γ responses with age, but the change was no longer statistically significant ($P = 0.18$). The strength of the peak HIV-specific SFU increased 1.5-fold with increasing age in the infants who survived to month 12, from a mean of $343/10^6$ PBMC (2.53 $\log_{10}$) at 1 month of age to a mean of $540/10^6$ PBMC (2.73 $\log_{10}$) at 12 months of age.

Early HIV-1-specific IFN-γ responses and HIV-1 replication kinetics. The emergence of HIV- and SIV-specific CD8+-T-cell immune responses in primary infection has been shown to correlate with declines in levels of viral replication. Therefore, we sought to determine the relationship between early HIV-1-specific IFN-γ responses and outcomes of HIV-1 infection in infants, as measured by peak viral loads, rates of decline of viral replication, and risk of mortality. The patterns of HIV-1 RNA plasma viral replication over time in infants with or without HIV-1-specific IFN-γ responses at 1 month of age, depicted by timing of infection, are shown in Fig. 4.

Peak HIV-1 plasma load is an important marker of disease progression in HIV-1-infected infants (52). Peak viral load was defined as the highest viral load detected within 6 months postinfection. We found no significant difference in mean $\log_{10}$ peak viral loads for infants infected in utero versus those infected peripartum. The mean peak viral load for 23 infants with defined in utero infection was 6.59 $\log_{10} \pm 0.84$ versus 6.86 $\log_{10} \pm 0.67$ among 37 infants infected peripartum ($P = 0.2$). Thirty-two infants had concurrent HIV-1-specific IFN-γ ELISPOT assays and HIV-1 RNA plasma viral loads measured at 1 month of age. As the timing of HIV-1 infection was shown not to significantly affect peak viral load, all 32 infants were considered as one group for the subsequent analyses. There was not a significant difference in mean $\log_{10}$ peak viral loads for infants with or without detectable HIV-1-specific IFN-γ responses at 1 month of life, after controlling for the baseline month 1 viral load. The mean peak viral load for 12 infants

with detectable HIV-1-specific IFN-γ responses at 1 month of age was 6.82 $\log_{10} \pm 0.18$ versus 6.62 $\log_{10} \pm 0.18$ for 20 infants who lacked detectable responses at 1 month of age ($P = 0.5$). There were no significant differences in peak viral loads for infants with broad (>2 peptides) or strong (>500 HIV-1 SFU) IFN-γ responses at 1 month compared to those with narrower, weaker, or negative responses (data not shown).

Having seen no difference in the peak viral loads, we next investigated the relationship between the presence of HIV-1-specific IFN-γ responses at 1 month of age and the change in viral load over time. The presence of detectable HIV-1-specific IFN-γ responses at 1 month of life was not associated with a difference in the rate of change of viral load over the first year of life compared to the rate for infants who lacked such responses at 1 month (decline of 0.04 versus 0.02 $\log_{10}$ HIV-1 RNA copies/ml plasma/month, respectively; $P = 0.2$).

We lastly investigated the relationship between the presence and strength of HIV-1-specific IFN-γ responses at 1 month of age and infant mortality during the first year of life. There was a trend correlating the presence of HIV-1-specific IFN-γ responses at 1 month of life and increased mortality found with univariate analysis (hazard ratio [HR] = 2.72; 95% confidence interval [CI], 0.89 to 8.36; $P = 0.08$). There was also a trend for higher mortality in those infants with IFN-γ responses of <500 HIV-1 SFU (HR = 3.77; 95% CI, 0.94 to 15.13; $P = 0.06$). After controlling for the viral load at month 1 and the timing of infection in multivariate analysis, there were no statistically significant relationships either between mortality and the presence of month 1 HIV-1-specific IFN-γ responses (HR, 2.20; 95% CI, 0.62 to 7.87; $P = 0.2$) or between mortality and IFN-γ responses of <500 HIV-1 SFU (HR, 2.08; 95% CI, 0.70 to 6.17; $P = 0.2$). Thus, the presence or strength of HIV-1-specific IFN-γ responses in the first months of life had no effect on peak viral load, on the rate of decline in HIV-1 plasma viral load, or on survival in this cohort of HIV-1-infected infants.

DISCUSSION

We addressed a mechanism hypothesized to contribute to the sustained high viral loads measured in perinatal HIV-1
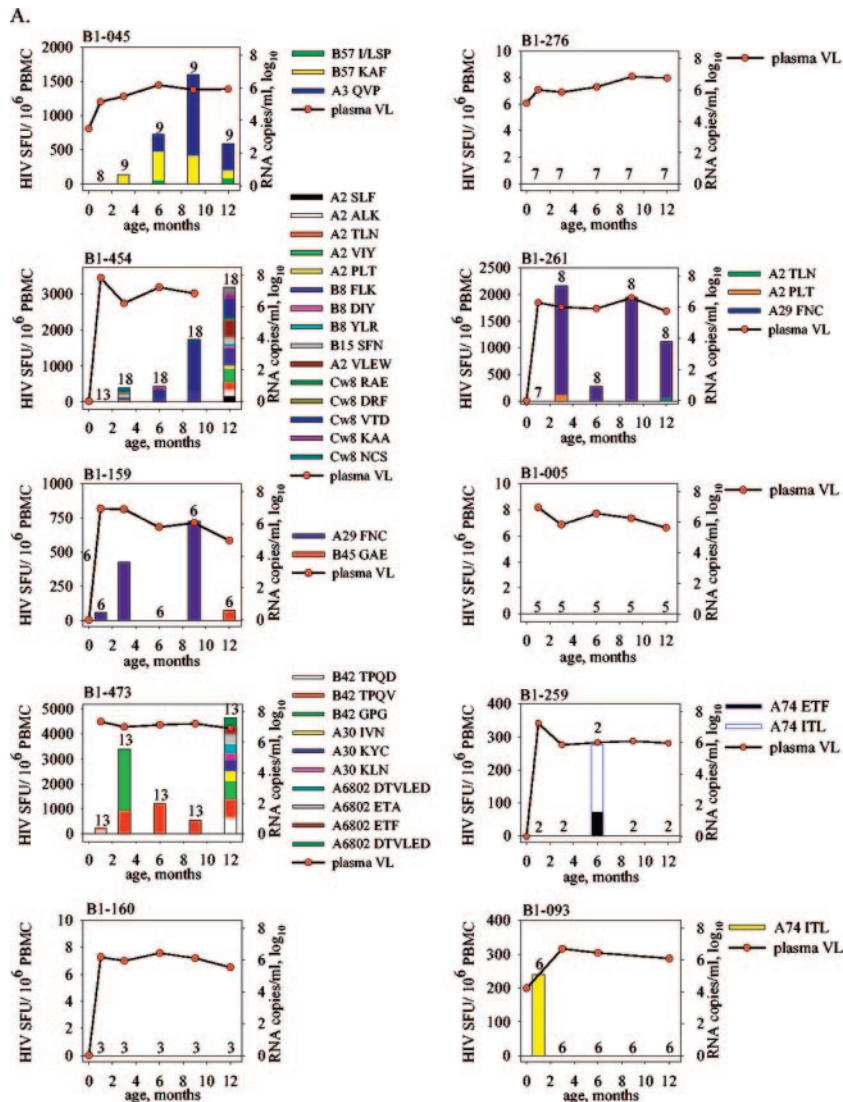
FIG. 2. Spectrum of HIV-1-specific peptide responses in HIV-1-infected infants with measurements at every time point to 1 year (A) or death (B). Individual graphs present HIV-1-specific SFU/10^6 PBMC (stacked colored bars) and HIV-1 RNA copies/ml plasma (closed circles) as functions of age. The numbers above each bar or above the horizontal axes indicate the numbers of peptides tested at each time point, while the heights of each of the colored sections of the bars indicate the strengths of the peptide-specific responses. The number of peptides was the lowest at month 1, due to limitations in cell numbers, and remained constant from month 3 to 1 year or death. The notation of death indicates the infant died 1 to 3 months after the last measurement.

infection, the adequacy of the CD8$^+$-T-cell immune response. We were able to establish the timing of infection in the infants as occurring either in utero or peripartum and to compare the development of CD8$^+$-T-cell effector function and viral loads. Infants infected peripartum had a tendency to generate stronger but not broader responses to HIV-1 peptides than did infants infected in utero. Both groups had similar increases in the magnitudes of HIV-1-specific IFN-γ responses over their first year of life, an indication that infants infected in utero are not more immunosuppressed than infants infected peripartum and that both groups possess the capacity for continuing immune maturation. Infants infected in utero did not demonstrate higher peak viral loads than infants infected peripartum, as has also been described in the Abidjan ANRS 049 Ditrame Study (52). A primary report describing the bimodal disease

progression in perinatally infected infants suggests that in utero infection may interfere with the maturation of the immune system and increase the rate of development of immunosuppression (5), something we do not show with our data. Despite a rapid and relatively robust immune response to infection, induction of these early responses did not appear beneficial to infants during the first year of life, regardless of the timing of infection. Specifically, there was no relationship between the detection of these responses and a reduction in peak viral load, rate of decline of viral replication, or risk of mortality (independent of viral load).

Previous studies have relied on cross-sectional studies in older children, where there may be bias towards those who survived. The approach here, using a longitudinal analysis of a cohort of HIV-1-infected infants with well-defined timing of
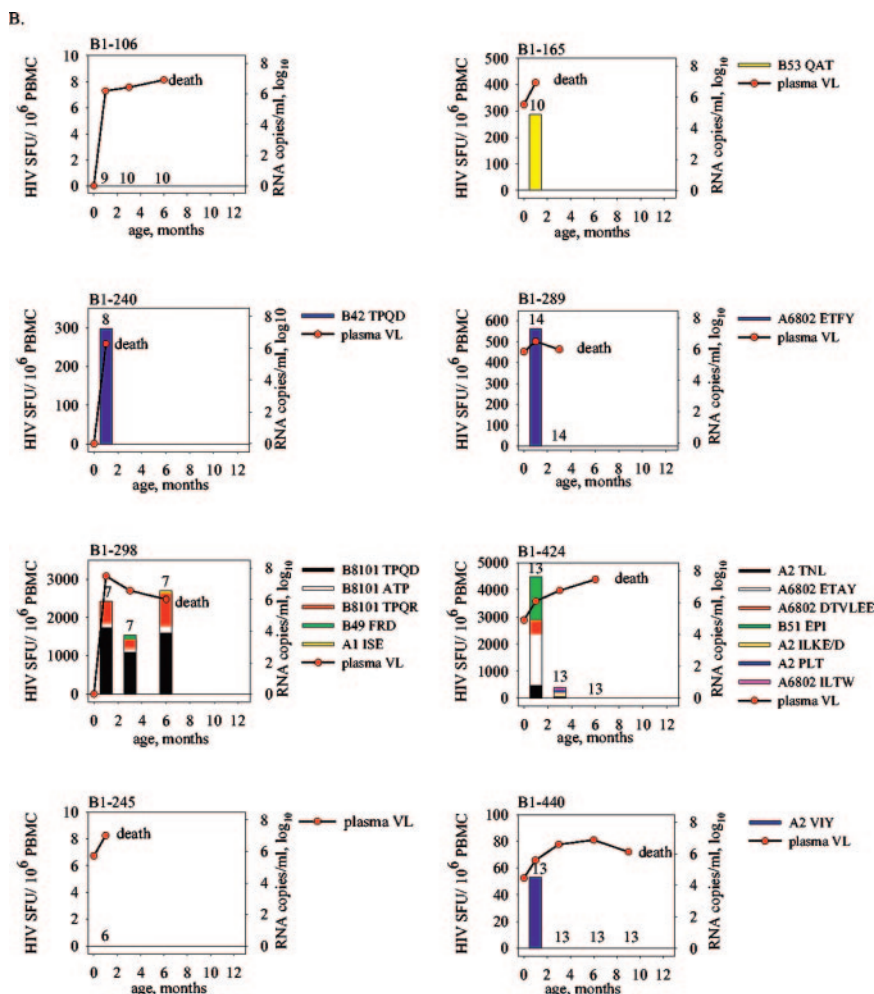
FIG. 2—*Continued.*

infection, has allowed us to address survivor bias and enabled prospective characterization of HIV-1-specific immunity over the first year of life. In our study, results from serial assays of the same infants revealed a significant twofold increase in HIV-1-specific IFN-γ responses over the first year of life, which became a trend for increasing responses when limited to those infants who survived to 1 year. Thus, older infants had stronger responses, which may be due in part to a survivor effect and in part to age-related maturation.

We observed that neonates with in utero or peripartum HIV-1 infection were able to generate IFN-γ responses of breadth and strength similar to those reported for adults with primary HIV-1 infection, albeit weaker than responses reported for adults with chronic infection (1). Two detailed studies with adults of CD8+-T-cell responses to primary HIV-1 infection both show a narrow response to approximately two epitopes within the first year of infection and a lack of correlation between the frequency of virus-specific IFN-γ responses and viral containment (11, 14). In our study, we find very similar kinetics in the induction of HIV-1-specific responses, with 52% of infants having detectable responses by 3 months of age to a median of two peptides at a mean magnitude of 384 HIV-1-specific SFU. There are few studies that describe virus-

specific IFN-γ responses in perinatally infected infants. The results presented here indicate that neonatal CD8+ IFN-γ responses to HIV-1 infection are more prevalent than previously reported. Scott and colleagues investigated IFN-γ responses in a group of 13 infants of less than 6 months of age and found that 2/13 (15%) infants had detectable HIV-1-specific responses before the initiation of antiretroviral therapy (58). Wasik et al. observed an age-related increase in responses in their cohort of children on antiretroviral therapy and described two infants under 1 year of age who generated increasing IFN-γ responses in the setting of increasing viral loads (63). Our findings based on a large number of infants in the longitudinal cohort extend these reports and support the concept that the human neonate appears quite capable of mounting appreciable CD8+-T-cell-mediated IFN-γ responses. The timing and strength of the responses suggest that the main factor in the development of HIV-1-specific IFN-γ responses is not the age at the time of infection but rather the duration of exposure to HIV-1.

We believe our report to be the first comprehensive study of immune function in HIV-1-infected neonates. Our sample size permitted multivariate analysis of factors including baseline viral loads (month 1), the presence of detectable IFN-γ re-
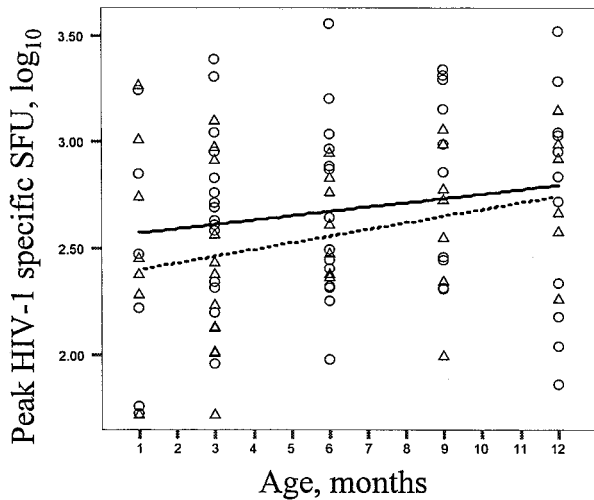
FIG. 3. HIV-1-specific IFN-γ responses increase with age in HIV-1-infected infants. Individual peak HIV-1 SFU responses are plotted on the y axis with respect to age of the infant, which is plotted on the x axis. Infants infected with HIV-1 in utero are represented by triangles; those infected peripartum are represented by circles. The linear mixed-effect regression lines are shown for changes in peak HIV-1 SFU numbers over time for infants infected in utero (dotted) and peripartum (solid) and are not significantly different from each other.

granzyme production (2, 23), T-cell receptor flexibility (32), and the quality of CD4$^+$ T-cell help (27), and all these factors are likely important for the control of HIV-1 replication. Additionally, the repertoire of cytokine production from human CD8$^+$-T cells stimulated by either vaccination or natural infection is broad and diverse, and limiting analyses to one cytokine reduces the ability to detect associations (3, 16). Alternatively, IFN-γ secretion may accurately reflect the CD8$^+$-T-cell responses to pediatric HIV-1 infection, but the immune pressure may promote generation of CTL escape variants. High levels of HIV-1 replication in the setting of antiviral CTL responses have been linked to the rapid emergence of CTL escape variants (6, 12, 22, 42). Additionally, viral variants adapted to the maternal CTL responses may be transmitted to the infant, limiting the capacity of the infant's CTL response to have an effect on viral replication. The appearance of viral variants not recognized by the neonatal immune system may contribute significantly to the high viral loads we observed in this cohort. Delineation of viral and host factors in the pathogenesis of pediatric HIV-1 infection will hopefully generate better options for treatment and care of this population.

The lack of detectable responses in almost half of the individuals over the course of the study may not reflect a complete absence of HIV-1-specific IFN-γ responses, because of our use of defined CD8$^+$-T-cell epitopes rather than overlapping peptides spanning the HIV genome. By its nature, the peptide panel does not fully represent the HIV-1 viral genome or recombinant variants, limiting our ability to detect responses to undefined epitopes. Also, by testing individual peptides rather than peptide pools, we maximized our chances of detecting low-level responses (4), and we were able to test peptides derived from HIV-1 subtypes A and D, previously known to elicit responses in HIV-1-infected individuals from Kenya (28), including many defined as being in a state of acute infection (14).

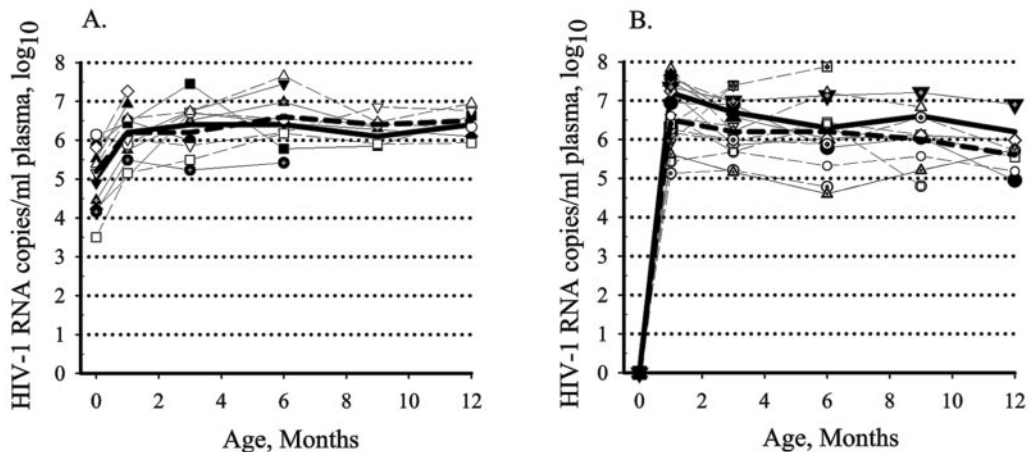We find no link between the detection of early HIV-1-spe-

sponses, and the timing of infection in the model. The presence of HIV-1-specific IFN-γ responses was not associated with the control of HIV-1 infection in neonates. One explanation for this finding is the possibility that although IFN-γ secretion is widely used as a surrogate for CTL activity, it may not predict CTL levels and may be an inadequate marker to use as a measure of HIV-1-specific immunity. The ability of cells to produce IFN-γ in response to HIV-1 peptide stimulation has been shown not to fully predict levels of functional CTL. The quality of the CD8$^+$-T-cell response is affected by perforin and

FIG. 4. HIV-1 RNA plasma viral loads during the first year of life in infants with or without HIV-1-specific IFN-γ responses detected at 1 month of age. (A) Twelve infants infected with HIV-1 in utero: 7 with month 1 HIV-1-specific IFN-γ early responses, 5 without. (B) Twenty infants infected peripartum: 6 with HIV-1-specific IFN-γ responses, 14 without. Open symbols/dashed lines represent individuals who lacked detectable HIV-1-specific IFN-γ responses at 1 month of life. Closed symbols/solid lines represent those who had month 1 HIV-1-specific IFN-γ responses. The mean log$_{10}$s of HIV-1 RNA copies per ml plasma for infants with month 1 HIV-1-specific IFN-γ responses are indicated by bold solid lines; the mean log$_{10}$s of HIV-1 RNA copies/ml plasma for infants without month 1-specific responses are represented by bold dashed lines.

cific immune responses and viral pathogenesis, but this lack of apparent protective effect in the setting of infection does not indicate vaccine-induced cellular responses will be nonprotective, as the outcome of neonatal exposure to antigen is likely determined by the antigenic dose and the timing and route of exposure. Our observation that neonates have the capacity to mount antiviral immune responses in the first months of life that are comparable to levels observed in adults with primary infection supports the concept of newborn immunization strategies. Immunization of newborn rhesus macaques results in the emergence of SIV-specific immune responses, and immunized infants demonstrated prolonged survival after challenge virus (60). In the lymphocytic choriomeningitis model, DNA immunization of newborn mice results in rapidly generated CD8$^+$-T-cell responses within the first weeks of life as well as long-lived, fully functional responses detectable 1 year postimmunization (25, 65). However, our data support a cautionary approach with vaccines designed to mimic HIV-1-specific IFN-γ responses observed in infected individuals reliant on IFN-γ secretion as the sole immune correlate. These responses may be ineffective in the control of early HIV-1 replication and may not be representative of the spectrum of immune responses generated by vaccination.

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