Human Cytomegalovirus Proteins pp65 and Immediate Early Protein 1 Are Common Targets for CD8$^+$ T Cell Responses in Children with Congenital or Postnatal Human Cytomegalovirus Infection[1]

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Recombinant modified vaccinia Ankara- and peptide-based IFN-γ ELISPOT assays were used to detect and measure human CMV (HCMV)-specific CD8$^+$ T cell responses to the pp65 (UL83) and immediate early protein 1 (IE1; UL123) gene products in 16 HCMV-infected infants and children. Age at study ranged from birth to 2 years. HCMV-specific CD8$^+$ T cells were detected in 14 (88%) of 16 children at frequencies ranging from 60 to >2000 spots/million PBMC. Responses were detected as early as 1 day of age in infants with documented congenital infection. Nine children responded to both pp65 and IE1, whereas responses to pp65 or IE1 alone were detected in three and two children, respectively. Regardless of the specificity of initial responses, IE1-specific responses predominated by 1 year of age. Changes in HCMV epitopes targeted by the CD8$^+$ T cell responses were observed over time; epitopes commonly recognized by HLA-A2$^+$ adults with latent HCMV infection did not fully account for responses detected in early childhood. Finally, the detection of HCMV-specific CD8$^+$ T cell responses was temporally associated with a decrease in peripheral blood HCMV load. Taken altogether, these data demonstrate that the fetus and young infant can generate virus-specific CD8$^+$ T cell responses. Changes observed in the protein and epitope-specificity of HCMV-specific CD8$^+$ T cells over time are consistent with those observed after other primary viral infections. The temporal association between the detection of HCMV-specific CD8$^+$ T cell responses and the reduction in blood HCMV load supports the importance of CD8$^+$ T cells in controlling primary HCMV viremia. ***The Journal of Immunology,*** **2004, 172: 2256–2264.**

Human CMV (HCMV)[3] is the most common congenital infection worldwide, occurring in 1–3% of live births, or 40,000 infants/year in the United States. Intrauterine HCMV infection usually occurs during primary maternal infection, in which the mean rate of transmission from mother to fetus is 40%. Congenital HCMV infection is associated with significant long term morbidity, including hearing loss and mental retardation. A recent Institute of Medicine report (1) places the development of an HCMV vaccine in the highest priority category based on its potential to decrease childhood morbidity and mortality as well as health care costs due to HCMV infection.

Prior studies have shown that HCMV-specific CD8$^+$ T cells are important for control of viremia and protection against severe disease in both the mouse (2–4) and human (5–9) models. Characterization of HCMV-specific CD8$^+$ T cell responses has focused primarily on healthy adults with latent HCMV infection. Some studies have examined responses to a limited number of HCMV targets in subjects with specific HLA types (10–16), whereas others have examined a wider variety of HCMV targets and HLA types (17–21). An increasing number of HCMV CD8$^+$ T cell epitopes are being defined in healthy adults with remote HCMV infection (22, 23).

However, important aspects of HCMV-specific CD8$^+$ T cell responses remain to be defined. First, conflicting data exist on the relative importance of specific HCMV gene products as targets of CD8$^+$ T cell responses. pp65 (18, 20, 24, 25) and immediate early protein 1 (IE1) (13, 26–28) have each been identified as the predominant target of these responses. Second, few studies have examined changes in the frequency and specificity of HCMV-specific CD8$^+$ T cell responses during primary infection. Crucial to an effective HCMV vaccine strategy is the characterization of HCMV-specific T cell responses that confer protection against primary infection as well as reactivation of latent virus. Gamadia et al. (29) characterized both CD4$^+$ and CD8$^+$ T cells in adult renal transplant patients with primary HCMV infection and correlated these responses with clinical outcome. Jin et al. (30) described the

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[3] Abbreviations used in this paper: HCMV, human CMV; BLCL, B lymphoblastoid cell line; CI, congenital infection; GE, genome equivalent; IE1, immediate early protein 1; PI, postnatal infection; rMVA, recombinant modified vaccinia Ankara; SFC, spot-forming cell.

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predominance of HCMV pp65-specific CD8$^+$ T cell responses in an HLA-A*0201-positive adult with concurrent acute HCMV, EBV, and HIV-1 infections.

As HCMV can be acquired from gestation through adulthood, this virus is a useful model to evaluate potential age-related differences in the development of antiviral cell-mediated immunity during primary infection. A deficit in the ability of human neonatal lymphocytes to proliferate and produce IFN-γ in response to specific Ag stimulation has been described in vitro (31, 32), possibly due to insufficient costimulatory molecules on neonatal APCs (33). Other studies describe differential levels of IFN-γ secretion between adults and neonates (34). Previous reports (35, 36) have demonstrated weak HCMV-specific cell-mediated lymphoproliferative responses in congenitally infected infants.

Few studies have characterized virus-specific CD8$^+$ T cell responses in early life. HIV-1 specific CD8$^+$ T cells have been infrequently detected in young infants (37, 38), possibly due to HIV-1-induced CD4$^+$ T cell dysfunction (39) or viral sequence variation (40). In a recent study of four neonates with asymptomatic congenital HCMV infection, Marchant et al. (41) detected HCMV-specific CD8$^+$ T cell responses to a limited number of MHC class I-restricted viral epitopes commonly detected in adults with latent HCMV infection.

We have previously reported HCMV-specific CD8$^+$ T cell responses detected by ELISPOT assay in a small cohort of HIV-1- and HCMV-coinfected infants (38), suggesting that young infants are capable of making virus-specific responses. We undertook the present study to better define the timing of detection, magnitude, and specificities of HCMV-specific CD8$^+$ T cell responses during primary infection in infants and young children with congenital or postnatal HCMV infection and to correlate these responses with viral load. Recombinant modified vaccinia Ankara (rMVA) expressing the HCMV gene product pp65 or IE1 was used in ELISPOT assays to detect and characterize HCMV protein-specific CD8$^+$ T cell responses. Peptide-based ELISPOT assays as well as MHC class I tetramers were used to detect and quantify HCMV epitope-specific CD8$^+$ T cell responses.

Materials and Methods

Study population

Sixteen infants with documented HCMV infection were studied prospectively over the first 24 mo of life. Characteristics of the study population are detailed in Table I. Congenital infection was defined by detectable HCMV DNA in the peripheral blood and/or virus isolation from the urine within 3 wk of age (42). Fifteen infants had asymptomatic HCMV infection, whereas one congenitally infected infant (P103) was born with intrauterine growth retardation, petechiae, hepatosplenomegaly, microcephaly, and intracranial calcification.

Five infants were diagnosed with congenital HCMV infection at the Policlinico San Matteo (Pavia, Italy) by detection of HCMV DNA in the peripheral blood and/or isolation of the virus from urine within 3 wk of age. These infants were born to HIV-1-seronegative women. Eleven infants were born to HIV-1-seropositive women followed in the Women and Infants Transmission Study or the Western New England Pediatric HIV Consortium sites in Worcester, Lowell, Lawrence, and Springfield, MA. These infants had undetectable HIV-1 provirus in serial PBMC samples through 6 mo of age, normal T lymphocyte counts (total, CD4$^+$, and CD8$^+$), and normal CD4$^+$/CD8$^+$ T lymphocyte ratios for age. HCMV infection was documented in these children by the presence of IgG Abs to HCMV in repository plasma specimens obtained at 18–24 mo of age. Urine specimens during the first 3 wk of life were not available. One of these infants (W405) was diagnosed with congenital HCMV infection by detection of HCMV DNA in the peripheral blood at birth. Six HIV-1- and HCMV-uninfected infants served as control subjects.

These studies were approved by the human subjects committees at all participating institutions. Written informed consent was obtained from the parent or legal guardian of all participants. The guidelines of the U.S. Department of Heath and Human Services regarding human experimental subjects were followed.

Table I. *HCMV infection status, timing of infection, and HLA alleles of infants studied*[a]

Subject	Timing of Infection	HLA-A2
P102	CI	A*0201
P103	CI	—
P104	CI	A*0201
P105	CI	—
P106	CI	—
W405	CI	—
W102	PI	A*0201
W103	PI	A*0201
W302	PI	—
W304	PI	A*0201
W306	PI	—
W401	PI	—
W402	PI	A*0204
W403	PI	—
W404	PI	—
W406	PI	A*0201
C102	Uninfected	—
C104	Uninfected	—
C105	Uninfected	A*0201
C106	Uninfected	A*0204
C107	Uninfected	—
C108	Uninfected	A*0201

[a] HLA types other than HLA-A2 are indicated by a dash. CI, Congenital infection; PI, postnatal infection.

Detection of HCMV DNA in peripheral blood using the PCR

PCR was performed at the Policlinico San Matteo. DNA was isolated from specimens (100 μl of anticoagulated whole blood or at least 1×10^6 PBMC) using silica-based NucliSens Isolation kit (bioMerieux, Boxtel, The Netherlands). Quantitative PCR was performed on DNA isolated from specimens according to a previously described method based on internal control of amplification and external standards (43). This method allowed reproducible HCMV DNA quantification in the range of 10^1–10^4 genome equivalents (GE)/10 μl of whole blood or 10^5 PBMC. Samples with undetectable HCMV DNA, but competent for amplification (detectable internal standard products), were submitted to a second amplification step using inner primers (44). Samples with 1–10 GE detected by nested PCR were assigned an arbitrary value of 5 GE.

HCMV isolation and serology

Urine samples from infants born to women with primary HCMV infection during pregnancy were inoculated onto confluent human embryonic lung fibroblast monolayers grown in shell vials (Policlinico San Matteo). Virus was identified after 18–24 h of incubation using indirect immunofluorescence and a mAb to the major immediate early protein p72 (45). Plasma samples obtained at 18–24 mo of age from HIV-1-uninfected children born to HIV-1-infected women were used to detect anti-HCMV IgG by enzyme immunoassay (bioMerieux, Marcy l'Etoile, France; University of Massachusetts Serology Laboratory, Worcester, MA).

Isolation of PBMC and generation of B lymphoblastoid cell lines (BLCL)

Serial PBMC samples were isolated from whole blood using Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) density centrifugation (46) and were viably cryopreserved in RPMI 1640 medium with 10% DMSO. Before their use in assays, cells were thawed and washed twice in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, and 10 mg/liter gentamicin (R10). The cells were then counted, and their viability was checked using trypan blue exclusion. Autologous BLCL were generated for each study participant by transformation with EBV and were maintained in R10 medium. BLCL ($0.5–1 \times 10^6$ cells) were infected with each rMVA at a multiplicity of infection of 5 and incubated for 16–20 h overnight at 37°C. Before use in the ELISPOT assay, infected BLCL were washed twice with R10 medium and counted. HLA typing was performed

on BLCL using a PCR-based method (University of Massachusetts Tissue Typing Laboratory, Worcester, MA).

Recombinant MVA

Recombinant MVA expressing either HCMV pp65 or IE1 were used to study HCMV-specific $CD8^+$ T cell responses to these gene products. These constructs were produced as follows.

HCMV IE1 cDNA was reverse transcribed using mRNA derived from HCMV (strain AD169)-infected MRC-5 cells using AMV reverse transcriptase (Promega, Madison, WI). The entire HCMV IE1 open reading frame was amplified from the cDNA sample using 5′ primer GCAGT CACCGTCGACGACACGATGGAG and 3′ primer GTGACGTGGGAT CCATAACAGTA. The IE1 PCR product (1.5 kb) was digested with *Sal*I and *Bam*HI and gel-purified. The *Sal*I and *Bam*HI-digested IE1 PCR gene product was cloned into pBluescript II SK+ (Stratagene, La Jolla, CA) and then subcloned into pNEB193 (New England Biolabs, Beverly, MA) to yield the IE1-pNEB193 plasmid. The IE1 gene was excised from IE-pNEB193 with *Pme*I and *Asc*I and then cloned into pLW22 (provided by Drs. L. Wyatt and B. Moss, National Institute of Allergy and Infectious Diseases, National Institutes of Health) (47) under the control of the efficient synthetic early-late vaccinia virus promoter ($P_{E/L}$) to yield the IE1-pLW22 plasmid (48).

The HCMV pp65 gene was similarly subcloned into pLW22. Briefly, the HCMV pp65 gene (1.6 kb) was cloned into the *Sma*I site of pBluescript II SK^+ by blunt end ligation and subcloned into pNEB193 (10), then pLW22 as described above to yield the pp65-pLW22 plasmid. All plasmid constructs were verified and confirmed by restriction enzyme digestion and DNA sequencing using IRD-800 labeled primers (Li-cor, Lincoln, NE).

Recombinant MVA viruses expressing HCMV pp65 protein (pp65-MVA) or HCMV IE1 protein (IE1-MVA) were generated by transfecting the pp65-pLW22 or IE1-pLW22 plasmid into 5×10^5 BHK-21 cells previously infected with MVA (multiplicity of infection of 0.01) in six-well plates. The pp65-MVA and IE1-MVA were selected by β-galactosidase screening in the presence of Bluo-gal substrate (49) (Sigma-Aldrich, St. Louis, MO). The protein expression levels of pp65 and IE1 in MVA-infected BHK-21 cells were evaluated by Western blot (Amersham Pharmacia Biotech, Piscataway, NJ) analysis (data not shown). After eight to 10 rounds of plaque purification, pp65-MVA and IE1-MVA viruses were grown and amplified by serially infecting greater numbers of plates of BHK-21 cells. The rMVAs were harvested after appearance of cytopathic effect. The virus/cell pellet was resuspended in MEM (Mediatech, Herndon, VA) containing 2% FCS and subjected to three freeze-thaw and sonication cycles. The virus stocks, typically between 10^9–10^{10} PFU/ml, were aliquoted and stored at −80°C. Inquiries concerning these viruses should be directed to Dr. Diamond.

Expression of the rMVA vectors was evaluated by chromium release assay. The pp65- or IE1-specific $CD8^+$ T cell clones exhibited strong specific killing of pp65- or IE-MVA-infected BLCL, respectively, in an HLA-restricted fashion (data not shown).

Peptides

The nonamer peptides aa 495–503 NLVPMVATV (NV) (10) and aa 316–324 VLEETSVML (VL) (13) representing defined HLA-A*0201-restricted HCMV pp65 and IE1 epitopes, respectively (Genemed Synthesis, San Francisco, CA), were used in ELISPOT assays or to generate MHC class I tetramers.

ELISPOT assays

A modified IFN-γ ELISPOT assay (38, 50) was used to detect HCMV Ag-specific IFN-γ release by $CD8^+$ T cells. A 96-well, flat-bottom plate (MAIPN1450; Millipore, Bedford, MA) was coated with 1 mg/ml anti-human IFN-γ mAb (Mabtech, Nacka, Sweden) and incubated overnight at 4°C. The plate was then washed with cold PBS, and 200 μl/well R10 was added for 2 h at 37°C to block nonspecific Ab binding. PBMC from each time point (5×10^4 cells/vector well or 10^5 cells/peptide well in duplicate) were incubated overnight at 37°C either with rMVA-infected BLCL (10^4 cells/vector well in duplicate for an E:T cell ratio of 5:1) or with HCMV peptide (final concentration, 10 μg/ml) in a total volume of 100 μl/well. After 16- to 20-h incubation, the plate was washed with cold PBS then incubated with 0.5 mg/ml of a secondary, biotinylated mAb specific for bound IFN-γ (Mabtech) for 3 h at room temperature. The plate was washed again then incubated with 0.5 mg/ml streptavidin-alkaline phosphatase (Mabtech) for 2 h at room temperature. After alkaline phosphatase color development (Bio-Rad, Richmond, CA), spots representing IFN-γ secreted by a single cell were visualized and counted using a sterioscope at ×20 magnification (American Optical, Buffalo, NY).

The average number of spots per well was used to express each experimental value as spot-forming cells (SFC) per 10^6 PBMC. Designation as a significant response required 1) a minimum of 3 SFC/well (60 SFC/10^6 PBMC), and 2) an experimental value at least 2 SD above the value for the negative control. All assays included positive (PMA-ionomycin) and negative (wild-type MVA or PBMC alone) controls. Prior studies from our laboratory (38) and others (50, 51) have shown that the responses detected are predominantly mediated by $CD8^+$ T cells.

MHC class I peptide tetramers

HLA-A2 tetramers expressing the HCMV pp65 peptide NLVPMVATV were used to detect and quantify epitope-specific $CD8^+$ T cells in selected HLA-$A2^+$ infants. Whole blood (100 μl) or PBMC ($0.5–1 \times 10^6$) was incubated with allophycocyanin-labeled tetramer, followed by incubation with conjugated mAbs anti-CD3-PerCP, anti-CD8-FITC, and anti-CD45RO-PE (BD Biosciences, San Diego, CA). RBC were lysed (FACS Lysing Solution; BD Biosciences), and the sample was washed with PBS supplemented with 1% FCS. To stain for intracellular molecules, cells were permeabilized (FACS Permeabilizing Solution; BD Biosciences), then incubated with anti-perforin-PE, anti-granzyme-A-PE, or isotype controls IgG2b or IgG1, respectively (BD Biosciences). The percentage of $CD8^+$ T lymphocytes costaining with tetramer and other markers was determined by flow cytometry.

Results

HCMV-specific T cell responses are commonly detected in early infancy

HCMV-specific $CD8^+$ T cell responses were evaluated in 16 infected infants and six uninfected infants (Table I). Six children had congenital infection (CI) defined by detectable peripheral blood HCMV DNA and/or virus isolation from the urine within 3 wk of age (42). Timing of infection was unknown in the remaining 10 children, and they were presumed to have perinatal or postnatal infection (PI).

To detect and quantify HCMV-specific $CD8^+$ T cell responses, autologous BLCL infected with rMVA vectors expressing pp65 and IE1 were incubated with PBMC in an IFN-γ ELISPOT assay. For each infant, specimens from all time points were studied in a single assay. HCMV-specific $CD8^+$ T cell responses against one or both HCMV gene products were detected in 14 (88%) of 16 infants (Fig. 1). Five (83%) of six infants with CI had detectable responses (Fig. 1, *top panel*), including infant P103 with symptomatic infection. These responses were first detected at 1 day of age in two infants and between 1–3 mo of age in the remaining three infants. Nine (90%) of 10 infants with PI had detectable responses (Fig. 1, *middle* and *bottom panels*). These responses were first detected between 2–12 mo of age. Background responses to wild-type MVA were low (Fig. 1). HCMV-specific $CD8^+$ T cell responses were not detected in six age-matched HCMV-uninfected infants (data not shown).

Protein specificities of HCMV-specific $CD8^+$ T cell responses

Of the 14 children with detectable HCMV-specific $CD8^+$ T cell responses, nine (64%) responded to both pp65 and IE1 during the course of HCMV infection (Figs. 1 and 2). Responses were directed at pp65 alone in three (22%) infants and at IE1 alone in two (14%) infants (Fig. 2). Overall, the frequencies of responses ranged from 60 to >2000 SFC/million PBMC (Fig. 1). The frequencies of pp65-specific responses ranged from 60–720 SFC/million PBMC, whereas the frequencies of IE1-specific responses ranged from 80 to >2000 SFC/million PBMC. There was no apparent difference between the frequencies of responses detected in CI and PI infants.

First detectable HCMV-specific $CD8^+$ T cell responses were directed at both pp65 and IE1 in six (43%) of 14 children (Fig. 1). First detectable responses were directed at pp65 alone in four (29%) infants and IE1 alone in another four (29%) infants. Of the

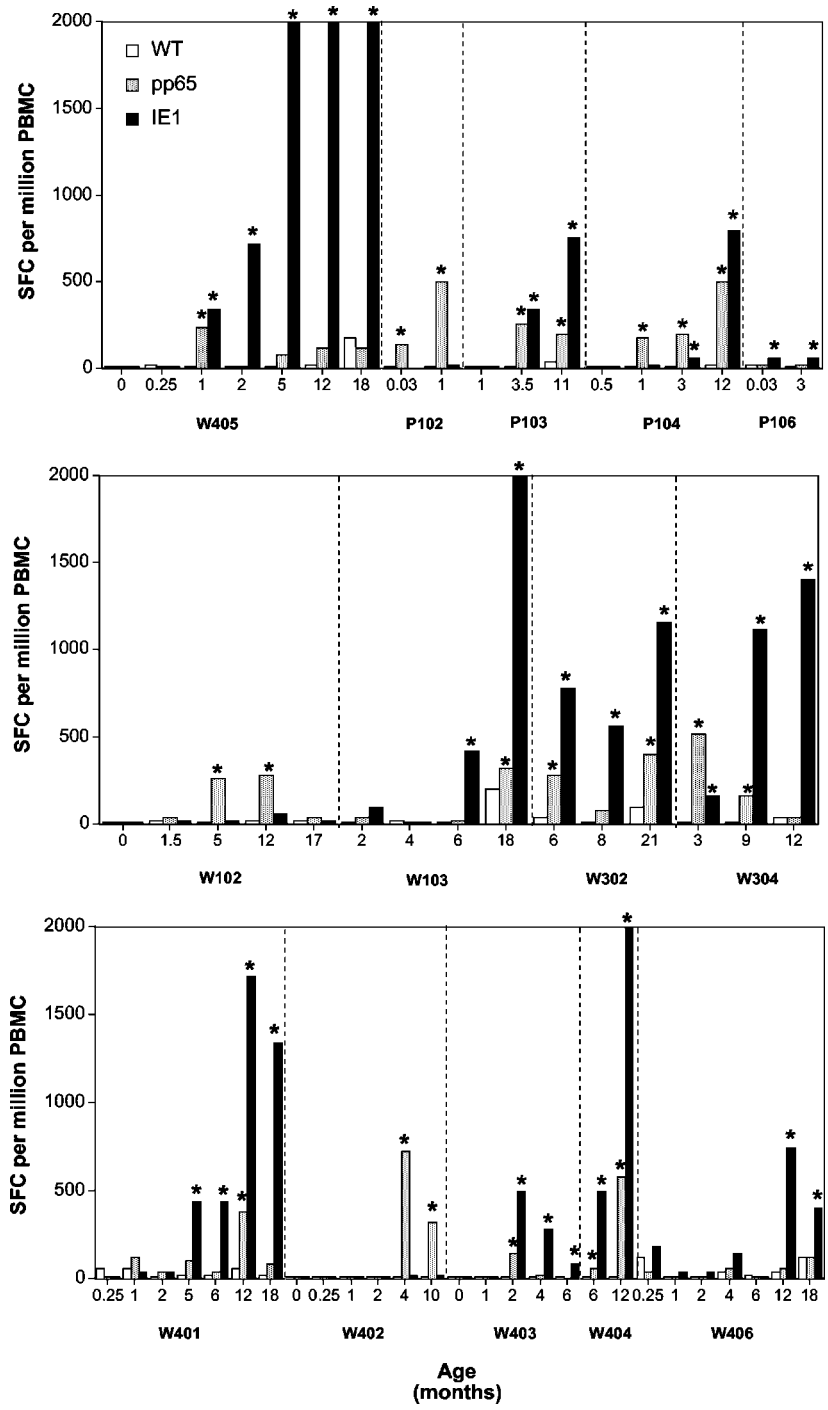

FIGURE 1. HCMV-specific $CD8^+$ T cell responses against pp65 and IE1 over time in 14 HCMV-infected children. Data from two infants without detectable responses are not shown. Results are expressed as SFC per million PBMC. Maximum level bars indicate response frequencies greater than 2000 SFC per million PBMC. Asterisks indicate significant responses (see *Materials and Methods*). WT, Wild-type MVA.

six infants with initial responses to both proteins, three had higher frequencies of responses to IE1 than to pp65, one infant had a higher frequency of response to pp65 than to IE1, and the remaining two children showed approximately equal responses to both proteins. Regardless of initial HCMV protein specificity, IE1-specific responses predominated over pp65-specific responses by 1 year of age in all infants with responses to both proteins (Fig. 1).

Epitope specificities of HCMV-specific $CD8^+$ T cell responses

Differential $CD8^+$ T cell recognition of viral protein epitopes has been described from acute through chronic EBV (52) and HIV-1 (53) infection. We therefore began to study the pp65 and IE1 epitope specificities of HCMV-specific $CD8^+$ T cell responses in children with congenital or postnatal HCMV infection. Several HCMV-specific $CD8^+$ T cell epitopes have been defined in healthy adults with latent infection (22, 23), especially in HLA-$A2^+$ individuals. To compare HCMV protein- and epitope-specific $CD8^+$ T cell responses in young children, the rMVA- and peptide-based ELISPOT assay and MHC class I tetramer staining were used to study seven HLA-$A2^+$ infants (Fig. 3). Six of seven infants were HLA-A*0201, whereas one was HLA-A*0204 (Table I). HCMV-specific $CD8^+$ T cell responses were detected in all seven HLA-$A2^+$ infants. Three infants had responses to pp65 alone, one to IE1 alone, and three to both gene products (Fig. 2).

In five of six HLA-$A2^+$ infants with detectable pp65-specific $CD8^+$ T cell responses, the frequency of initial responses to the whole protein exceeded the frequency of responses to a pp65 epitope (peptide NV, aa 495–503) reported as immunodominant in

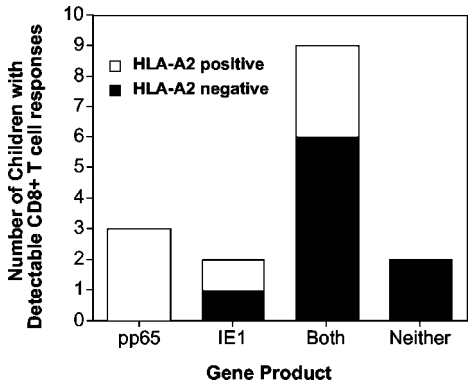

FIGURE 2. HCMV gene product specificity of HCMV-specific $CD8^+$ T cell responses in 16 infants. □, Responses in children with the HLA-A2 allele; ■, responses in children without the HLA-A2 allele.

$HLA\text{-}A2^+$ adults with latent HCMV infection (Fig. 3). Infant P102 showed pp65 protein-specific responses that exceeded pp65 epitope-specific responses by ELISPOT assay at birth and 1 mo of age. Similarly, infant W304 had higher pp65 protein-specific responses than pp65 epitope-specific responses at 3 mo of age. These responses became equivalent at 9 mo of age, then undetectable at 12 mo of age.

In two of four $HLA\text{-}A2^+$ infants with detectable IE1-specific $CD8^+$ T cell responses, the frequency of responses to the whole protein exceeded that of the responses to an IE1 epitope (peptide VL, aa 316–324) commonly recognized by $HLA\text{-}A2^+$ adults with latent infection (Fig. 3). The frequencies of $CD8^+$ T cell responses directed at IE1 whole protein were equivalent to those directed at VL peptide in the remaining two infants. Altogether these data demonstrate that early HCMV-specific $CD8^+$ T cell responses appeared to target different epitopes than those targeted later in infection and those commonly targeted in adults with chronic infection.

Comparison of tetramer staining and ELISPOT assays

We and others have previously shown phenotypic and functional heterogeneity of virus-specific $CD8^+$ T cell populations over the course of viral infections (52, 54). Tetramer staining is a method that quantifies epitope-specific T cells without regard to functional activity; thus, virus-specific T cell frequencies detected by tetramer staining are commonly higher than those detected by other methods. Sufficient cells were available for MHC class I-NV peptide tetramer staining in two infants (Fig. 4). At each time point, the frequency of tetramer staining cells was greater than the frequency of responses measured using the protein- or peptide-based ELISPOT assay. Costaining with MHC class I-NV peptide tetramer and perforin or granzyme A was also performed at all time points for infant W304 (Fig. 5). Although the frequency of tetramer-staining cells decreased over time (Figs. 4 and 5), >85% of tetramer-positive cells costained with granzyme A or perforin at each time point.

Viral load and timing of detection of HCMV-specific $CD8^+$ T cell responses

The relationship between HCMV-specific $CD8^+$ T cell responses and HCMV viral load was examined in CI infants. Sufficient samples were available in four infants (Fig. 6). HCMV DNA was consistently detected and was highest during the first 4 mo of life. Copy numbers ranged from 5–3000 copies/10^5 PBMC. A decrease in viral load was associated with an increase in the frequency of HCMV-specific $CD8^+$ T cell responses over time. Concurrent viral load and urine culture results were available for three infants.

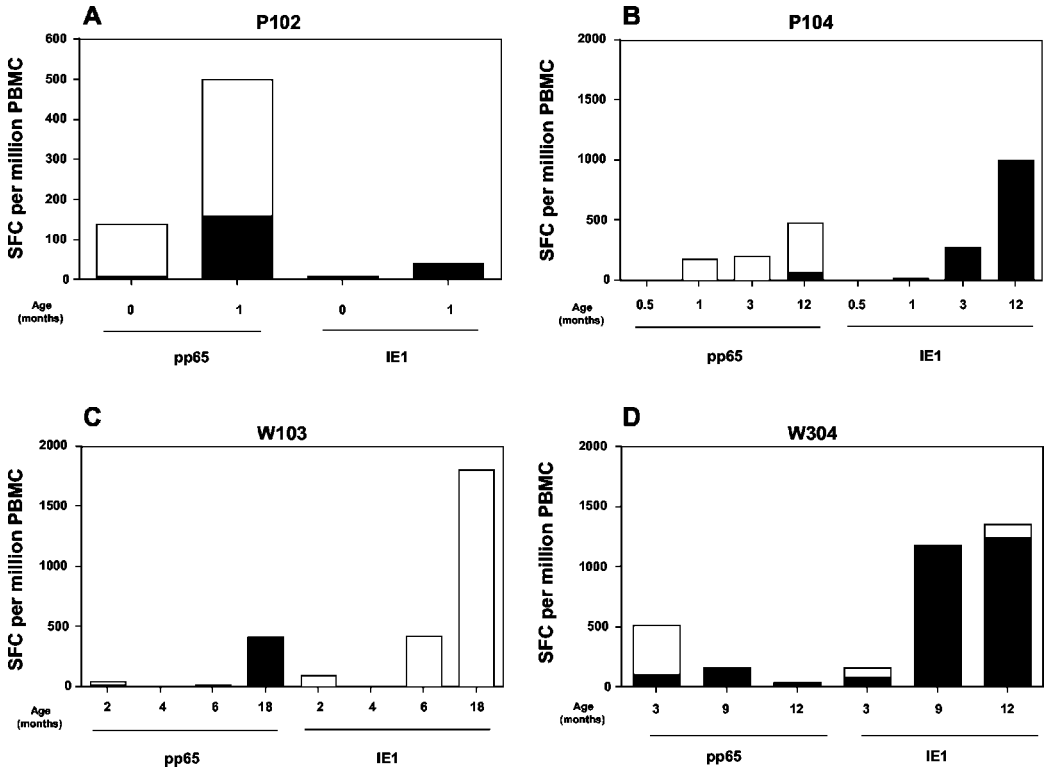

FIGURE 3. HCMV protein- or peptide-specific $CD8^+$ T cell responses in four $HLA\text{-}A*0201^+$ infants. Responses were measured by IFN-γ secretion in rMVA- and peptide-based ELISPOT assays. □, Responses to HCMV whole protein pp65 or IE1; ■, responses to pp65 NV peptide or IE1 VL peptide. Each bar shows peptide-specific responses as a fraction of whole protein-specific responses.

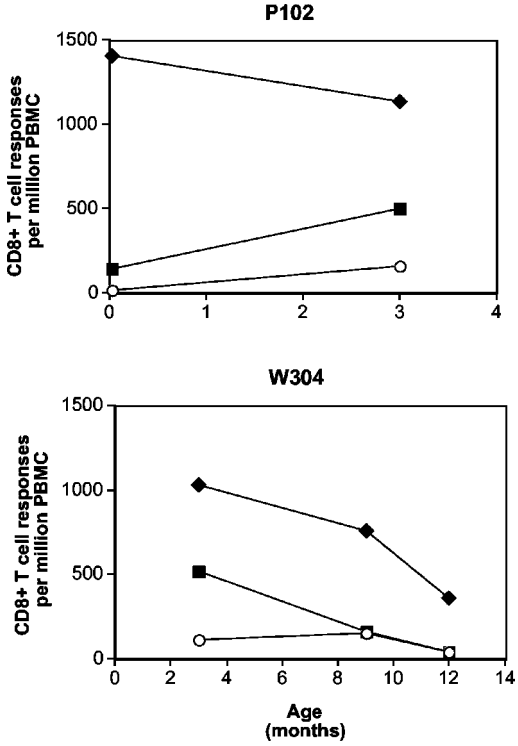

FIGURE 4. HCMV protein- and epitope-specific $CD8^+$ T cell responses in two HLA-A*0201 children over time. ELISPOT data are expressed as SFC per million PBMC in response to pp65 whole protein (■) or to pp65 NV peptide (○). Tetramer data are expressed as the number of pp65 NV peptide tetramer-staining cells per million PBMC (◆).

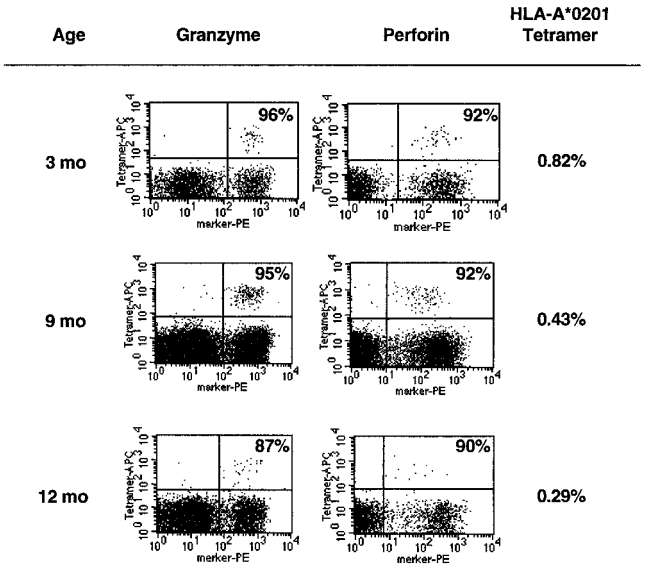

FIGURE 5. HLA-A*0201 NV peptide tetramer staining and granzyme and perforin expression over time in infant W304. Although the percentage of $CD8^+$ T cells staining with tetramer decreased over the course of HCMV infection (*right column*), the percentage of tetramer-staining cells that coexpress granzyme or perforin remained relatively constant.

Urine culture remained positive despite a decreasing viral load over the first 6–12 mo of life.

Discussion

HCMV-specific $CD8^+$ T cell responses have been characterized primarily in adults with established HCMV infection (55), with many studies focusing on a limited number of HCMV-specific $CD8^+$ T cell epitopes and restricting HLA types. Relatively little is known about HCMV-specific $CD8^+$ T cell responses over the course of primary HCMV infection, particularly in young children. Novel techniques, such as the ELISPOT assay and tetramer staining, have improved our ability to detect these responses in young infants for whom few specimens and low response frequencies may be present. Using these techniques, we have previously demonstrated HCMV-specific $CD8^+$ T cell responses in a small group of HIV-1- and HCMV-coinfected infants, suggesting that young infants are capable of making virus-specific responses (38). We therefore considered it important to study the timing, magnitude, and specificities of HCMV pp65- and IE1-specific $CD8^+$ T cell responses in infants and children with HCMV infection.

In the present study, HCMV-specific $CD8^+$ T cell responses were commonly detected in children with congenital or postnatal HCMV infection. There were no apparent differences in the observed frequencies or protein specificities of responses in CI and PI infants. Responses were detected in some infants with congenital infection as early as 1 day of age, presumably reflecting responses generated in utero. Most (64%) children made HCMV-specific $CD8^+$ T cell responses directed at both the pp65 and IE1 gene products. However, in these infants IE1-specific responses predominated by 12 mo of age regardless of the specificity of initial responses.

Prior studies have suggested that pp65 is the primary target for HCMV-specific $CD8^+$ T cell responses (10, 17, 18, 56), and that pp65 may inhibit the processing and presentation of IE1 (57). Our data suggest that infants are capable of generating IE1-specific $CD8^+$ T cell responses, and that IE1 may be more commonly targeted by $CD8^+$ T cells. The apparent prominence of IE1-specific $CD8^+$ T cell responses over time is compatible with the reported immunogenicity of IE1 in mice (58, 59) and humans (26). Moreover, culture-based methodologies used in early studies to identify targets of HCMV-specific $CD8^+$ T cell responses may have preferentially expanded pp65-specific $CD8^+$ T cells (55). ELISPOT assay and tetramer staining identify virus-specific $CD8^+$ T cells directly ex vivo and therefore may more accurately reflect $CD8^+$ T cell specificities in vivo. Finally, the protein specificity of HCMV-specific $CD8^+$ T cell responses may depend on HLA type (52, 60, 61). In our study all infants with responses to pp65 alone were HLA-$A2^+$, whereas only one-third of those with responses to both gene products were HLA-$A2^+$.

In a study of adults with latent HCMV infection, Kern et al. (27) showed that all subjects with undetectable pp65-specific $CD8^+$ T cell responses were HLA types other than A2, whereas most subjects with undetectable IE1-specific $CD8^+$ T cell responses were HLA-$A2^+$. Kern et al. (19) later showed that the mean frequency of pp65-specific responses was significantly higher in HLA-A2-positive compared with HLA-A2-negative subjects, whereas the frequencies of IE1-specific responses were significantly higher in HLA-A1-, -B7-, or -B8-positive subjects compared with those without these HLA types. These data as well as the present study suggest that the hierarchy of HCMV protein recognition by HCMV-specific $CD8^+$ T cells is strongly influenced by HLA type.

In most infants, HLA-A2-restricted HCMV pp65- or IE1-specific $CD8^+$ T cell responses were not directed at pp65 or IE1 epitopes reported as immunodominant in adults with latent HCMV infection (13, 18, 23). Our data demonstrate that young infants display differential epitope recognition over the course of HCMV infection, and that they may recognize epitopes other than those described in latently infected adults. Changes in $CD8^+$ T cell epitope recognition during EBV (52) and HIV-1 infection (53)

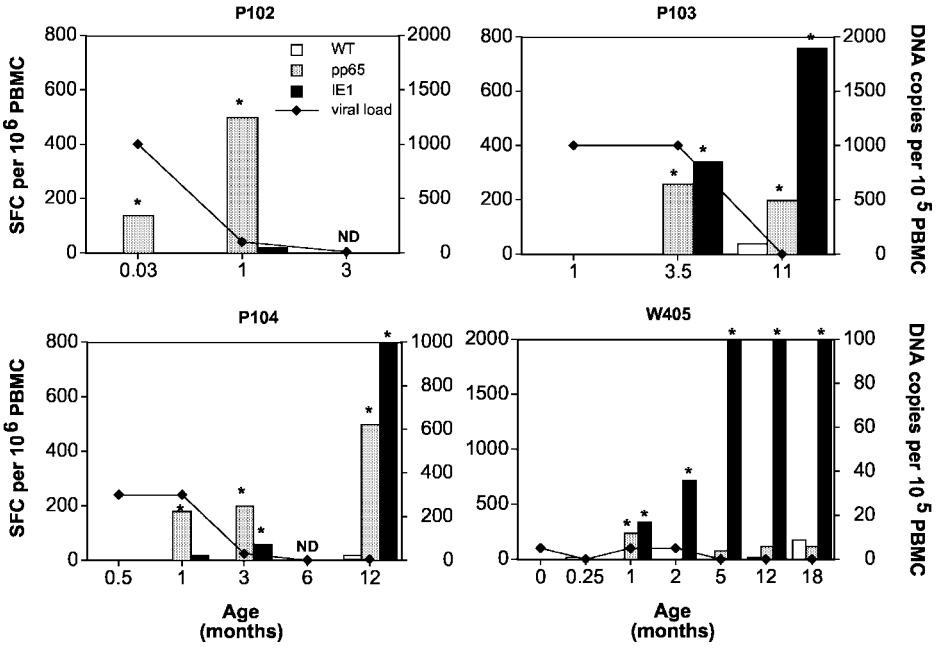

FIGURE 6. HCMV protein-specific $CD8^+$ T cell responses and peripheral blood HCMV viral load over time in four congenitally infected infants. Asterisks indicate significant responses (see *Materials and Methods*). ND, ELISPOT assay not done due to insufficient samples; WT, Wild-type MVA.

have also been reported and are probably not explained by differential viral protein expression over the course of infection (62). Instead, qualitative differences in Ag processing and presentation may be a more significant factor (33). Because HCMV-specific $CD8^+$ T cell epitope and protein specificities may change with time, the targets of these responses during latent infection are not necessarily relevant in early infection. Our data suggest that an effective HCMV vaccine may require targeting HCMV Ags processed and presented during acute infection.

Although the protein and epitope specificity of $CD8^+$ T cell responses may be important, functional properties of $CD8^+$ T cell responses may also impact the outcome of HCMV infection. Higher frequencies of NV epitope-specific $CD8^+$ T cells were detected by tetramer staining than by ELISPOT assay over time in two infants (Fig. 4). Khan et al. (13) have similarly reported that pp65 and IE1 epitope-specific $CD8^+$ T cell frequencies measured by ELISPOT assay were only 10–50% of those measured by tetramer staining for the same epitope in adults with latent HCMV infection, suggesting that discordance between these measures may persist throughout the course of HCMV infection. However, direct measurement of IFN-γ production by tetramer staining cells would better demonstrate the effector function of epitope-specific $CD8^+$ T cells. Unfortunately, sufficient PBMC were not available to complete these studies in our cohort of infants. Consistently high levels of perforin or granzyme were detected in NV peptide tetramer-staining cells of infant W304 (Fig. 5), suggesting effective cytolytic activity over time.

To compare the timing of detection of HCMV-specific $CD8^+$ T cell responses with HCMV DNA in peripheral blood, HCMV viral load was measured prospectively in infants with congenital HCMV infection. Measured viral loads were highest within 1–2 mo of birth and then decreased by 6 mo of age. This pattern of viral load is consistent with prior studies of immunocompetent adolescents with primary HCMV infection (63) and infants with congenital HCMV infection (42). In our study of CI infants, the detection of HCMV-specific $CD8^+$ T cell responses appeared to correlate temporally with a decrease in HCMV viral load. The frequencies of HCMV-specific $CD8^+$ T cell responses appeared to increase as viral load decreased over time, suggesting that $CD8^+$ T cells have antiviral activity and play a role in controlling viral replication.

In summary, HCMV-specific $CD8^+$ T cell responses were commonly detected in young infants with HCMV infection. These responses were directed at both pp65 and IE1 in most children studied, although IE1-specific responses predominated by 1 year of age. HLA-A*0201-restricted HCMV epitope-specific $CD8^+$ T cell responses were detected against pp65 and IE1 epitopes reported as immunodominant in adults with latent HCMV infection, but their frequencies did not fully account for responses to the whole proteins. The timing of detection of HCMV-specific $CD8^+$ T cell responses correlated with a decrease in HCMV viral load, suggesting that these responses have significant antiviral activity in vivo.

Our data have several important implications for the development of an HCMV vaccine. We have shown that young infants can generate functional virus-specific $CD8^+$ T cells. Because of differences in HLA types and epitope recognition over the course of infection, whole viral proteins may be more effective components of an HCMV vaccine than selected viral epitopes. The attenuated poxviruses used to detect HCMV-specific $CD8^+$ T cells have been used successfully to generate immune responses against HIV and malaria (64, 65). The appeal of these viruses is demonstrated safety and lack of virulence in vulnerable populations such as adolescents and the elderly (66, 67). Future studies using these viruses in clinical trials with adolescents or women of childbearing age may yield a strategy to prevent HCMV infection and its associated morbidity from occurring in neonates.

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