Persistent and Selective Deficiency of CD4^{+} T Cell Immunity to Cytomegalovirus in Immunocompetent Young Children[1]

Wenwei Tu,* Sharon Chen,* Margaret Sharp,* Corry Dekker,* Anne Marie Manganello,† Eileen C. Tongson,* Holden T. Maecker,‡ Tyson H. Holmes,§ Zhaoti Wang,¶ George Kemble,¶ Stuart Adler,† Ann Arvin,* and David B. Lewis[2]*

Healthy young children who acquire CMV have prolonged viral shedding into the urine and saliva, but whether this is attributable to limitations in viral-specific immune responses has not been explored. In this study, we found that otherwise immunocompetent young children after recent primary CMV infection accumulated markedly fewer CMV-specific CD4^{+} T cells that produced IFN-γ than did adults. These differences in CD4^{+} T cell function persisted for more than 1 year after viral acquisition, and did not apply to CMV-specific IFN-γ production by CD8^{+} T cells. The IFN-γ-producing CD4^{+} T cells of children or adults that were reactive with CMV Ags were mainly the CCR7^{low} cell subset of memory (CD45R0^{high}CD45RA^{low}) cells. The decreased IFN-γ response to CMV in children was selective, because their CCR7^{low} memory CD4^{+} T cells and those of adults produced similar levels of this cytokine after stimulation with staphylococcal enterotoxin B superantigen. CD4^{+} T cells from children also had reduced CMV-specific IL-2 and CD154 (CD40 ligand) expression, suggesting an early blockade in the differentiation of viral-specific CD4^{+} T cells. Following CMV acquisition, children, but not adults, persistently shed virus in urine, and this was observable for at least 29 mo postinfection. Thus, CD4^{+} T cell-mediated immunity to CMV in humans is generated in an age-dependent manner, and may have a substantial role in controlling renal viral replication and urinary shedding. ***The Journal of Immunology,* 2004, 172: 3260–3267.**

Human CMV is a prevalent β-herpesvirus that establishes persistent lifelong infection (1, 2). Primary CMV infection is defined as the first acquisition of any strain of the virus. Approximately 99% of these primary infections are acquired at birth or during childhood or adulthood as a result of mucosal contact with CMV contained in bodily secretions, such as breast milk, cervical fluid, semen, saliva, or urine. CMV infection acquired at birth or during childhood or adulthood is usually asymptomatic in immunocompetent individuals (2–4). However, postnatally acquired CMV infection may be severe in the immunocompromised host, particularly in the face of T cell deficiency (5), such as due to primary immunogenetic defects or acquired immunodeficiency states (6), or following premature birth (7). Although only ~1% of primary CMV infections in the U.S. are acquired in utero (1, 7), congenital infection may be severe and followed by permanent sequelae, such as sensorineural deafness and mental retardation. These sequelae are particularly frequent if the virus is transmitted as a result of a primary maternal infection early in pregnancy (1, 7).

Because congenital CMV infection is an important cause of deafness and other birth defects (1, 7), an understanding of the epidemiology of CMV spread and its control by adaptive immunity is of great interest. Young children, particularly those in group day care settings, are at a high risk to acquire CMV from other children as well as transmit CMV to caregivers, such as parents or day care workers (2–4, 7). Primary CMV infection of neonates and infants usually results in continuous or frequent viral shedding into the urine and saliva for up to several years (3, 4). In contrast, limited studies have indicated that adults with primary CMV infection stop continual viral shedding by 9–12 mo after acquisition, and have only infrequent recurrences of shedding thereafter (8, 9).

The reason for age-related differences in the duration of CMV shedding after primary viral acquisition is unknown. T cell immunity appears to be important to maintain CMV in a latent state (1, 5). Studies of murine CMV indicate that CD4^{+} T cells producing IFN-γ, which are hereafter interchangeably referred to as Th1 cells, are important for the control of CMV replication and shedding from epithelial sites, such as the salivary glands (10–12).

We hypothesized that differences in the duration of CMV shedding in young children compared with adults were related to quantitative or qualitative differences in the T cell-mediated immune response. To test this, we compared CMV-specific CD4^{+} and CD8^{+} T cell responses between asymptomatic young children and adults, and found a strikingly persistent deficiency of CMV-specific CD4^{+} T cell production of IFN-γ in asymptomatic young children. Thus, CD4^{+} T cell-mediated immunity to CMV after primary infection is generated in an age-dependent manner, most likely reflecting a novel age-related increased susceptibility of the immune system in early childhood to viral-mediated immunoevasion or immunomodulation. The persistence of such age-related differences in an antiviral T cell response following postnatal infection is, to the best of our knowledge, unprecedented, and has important implications for strategies to control CMV infection.

*Department of Pediatrics, Stanford University School of Medicine, Stanford, CA 94305; †Department of Pediatrics, Virginia Commonwealth University/Medical College of Virginia, Richmond, VA 23298; ‡BD Immunocytometry Systems, San Jose, CA 95131; §Biostatistics Division, Department of Health Research Policy, Stanford University, Stanford, CA 94305; and ¶MedImmune, Mountain View, CA 94043

Received for publication August 20, 2003. Accepted for publication December 22, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by National Institutes of Health Grants P01 AI-48212 and P01 AI-50153 (to A.A. and D.B.L.) and a Jeffrey Modell Foundation for Primary Immunodeficiency Grant (to D.B.L.).

[2] Address correspondence and reprint requests to Dr. David B. Lewis, Division of Immunology/Transplantation Biology, Department of Pediatrics, Center for Clinical Sciences Research Building Room 2115b, Stanford University School of Medicine, 269 Campus Drive, Stanford, CA 94305. E-mail address: dblewis@stanford.edu

Copyright © 2004 by The American Association of Immunologists, Inc. 0022-1767/04/$02.00

Materials and Methods

Study population

Eighteen adults and 13 children were involved in this study, which was approved by the Stanford University Committee for the Protection of Human Subjects and the Institutional Review Board of the Virginia Commonwealth University/Medical College of Virginia. These subjects were divided into four groups (Table I) based on whether they entered the study as CMV Ab seropositive, with the duration of the infection unknown, or were Ab seronegative at study entry and subsequently became infected, which allowed an estimate of the duration of infection. Group 1 subjects, labeled as adult in the figures, entered the study as CMV Ab seropositive and had infection of an unknown duration. They consisted of healthy and asymptomatic 14 adults, 3 men and 11 women, with a mean average age of 36 years. On epidemiological grounds, ~99% of these adults were likely to have acquired CMV peri- or postnatally, and to have been infected for years to decades (1, 4, 7). The estimated time since acquisition of primary CMV infection for the other subjects who were initially uninfected at study entry was determined by screening assays, which are described below. Group 2 subjects, for whom the results are labeled as adult 1^0 in the figures, consisted of 4 women who were CMV Ab seronegative at study entry. They were asymptomatic and ranged between 44 and 52 years of age, with a mean average age of 48 years, and an estimated duration of CMV infection of 12–15 mo. All children evaluated immunologically were uninfected at study entry. Group 3 subjects, for whom the results are labeled as child in the figures, consisted of 13 children, 5 girls and 8 boys, with an age range of 1.5–4 years, and a mean average age of 22 mo. All attended group day care, and had an estimated duration of CMV infection of 8–29 mo. Group 4 subjects, for whom the results are labeled as child 1^0 in the figures, were a subset of the group 3 children who had been infected for 12 mo or longer, so that their duration of CMV infection matched or exceeded that of the adult 1^0 group. Group 4 children had an estimated duration of infection of 12–29 mo and ranged between 18 and 42 mo of age, with a mean average age of 27 mo.

Screening for primary CMV acquisition

Urine CMV culture and the detection of CMV serum Ab were used to determine the approximate time of acquisition of primary infection in children and adults. Children were screened for CMV acquisition by periodic sampling of urine for CMV every 3 mo based on growth of virus in tissue culture using MRC-5 fibroblasts (13). Three adults of group 2 were screened for CMV acquisition based on screening every 6–12 mo for CMV Ab seropositivity using a commercial latex agglutination assay (CMVScan; BD Biosciences, San Jose, CA) that detects both CMV-specific IgG and IgM. One individual of this group was screened based on urine CMV cultures obtained every 3 mo. The time of initial infection was estimated to be the midpoint between the last negative and the first positive assay. Previous studies of uninfected adult volunteers inoculated with an unattenuated CMV viral strain found that CMV viruria and CMV-specific Ab were detectable by ~5 and 6 wk after viral inoculation, respectively (14). Therefore, screening for CMV acquisition based on either culture-proven viruria or seropositivity for CMV Ab should result in a very similar estimate of the duration of infection. The estimated duration of CMV infection for the subjects is summarized in Table I.

T cell stimulation assays

Stimulation was performed using either heparin-anticoagulated whole blood or PBMC isolated from heparin-anticoagulated blood by Ficoll-Hypaque gradient centrifugation. PBMC were resuspended in RPMI 1640 medium with 10% heat-inactivated human AB serum. Whole blood or PBMC was incubated with a lysate of fibroblasts infected with the CMV AD-169 strain (indicated as CMV in the figures), which was obtained from BioWhittaker (Walkersville, MD), or pp65 peptide mixture (indicated as pp65 in the figures). The pp65 mixture consisted of 138 peptides of 15 aa residues in length that completely span the pp65 UL83 protein sequence of the AD-169 strain, with an overlap of 11 aa residues. This peptide mixture is effective in activating CD4 and CD8 T cells in this assay (15, 16). Although the restriction of particular peptides in this mixture for particular class I and II HLA alleles is not known, this mixture routinely induces detectable CD4 and CD8 T cell responses in all healthy adult donors who are CMV Ab seropositive (15, 16, and our unpublished results for 40 adult donors that we have tested to date). Each peptide was present in the stock mixture at a concentration of 400 μg/ml DMSO and was used at final concentration of 1.0 μg/ml. DMSO at this concentration (0.25% v/v) had no effect on assay results in pilot experiments. An equivalent volume of PBS, uninfected fibroblast cell lysates, or a peptide pool for carcinoembryonic Ag, a self protein, were routinely used as negative controls. The carcinoembryonic Ag protein pool consisted of 173 peptides that had the same length (15 mer) and overlap (11 aa) as the pp65 peptide pool. The bacterial superantigen staphylococcal enterotoxin B (SEB)[3] (Toxin Technologies, Sarasota, FL) was used as positive control in all experiments at a final concentration of 10 μg/ml. To enhance the detection of Ag-specific responses, CD28 and CD49d mAbs (BD Biosciences) were added to each sample at a final concentration of 3 μg/ml, as previously described (16). The rationale for the addition of these costimulatory mAbs is that they increase the detection of Ag-specific immune responses without significantly increasing the background frequency of these cells in the absence of specific Ag (17). After 2 h of incubation at 37°C, 5% CO_2, brefeldin A (Sigma-Aldrich, St. Louis, MO) was added to final concentration of 10 μg/ml, and tubes were incubated an additional 4 h.

Cell surface markers and intracellular cytokine staining

Following 6 h of activation, cells were treated with 2 mM of EDTA for 15 min to deaggregate any cells that were clumped or that were adherent to plastic. For whole blood stimulation assays, RBC were lysed by the addition of FACS lysing solution (BD Biosciences). The fixed cells were washed, incubated with FACS permeabilization solution (BD Biosciences) for 10 min, and stained in the dark for 30 min with a combination of fluorochrome-conjugated mAbs reactive with cell surface markers and cytokine. All mAbs were purchased from BD Biosciences, unless otherwise indicated. For whole blood assays of $CD4^+$ T cells, the mAb mixture consisted of IFN-γ-FITC/CD69-PE/CD4-PE-Cy5 (Caltag Laboratories, Burlingame, CA) and allophycocyanin (AC)-conjugated CD8-α, CD14, and CD19 mAbs. Whole blood assay of $CD8^+$ T cells was similar, except that CD8-α-PE-Cy5 mAb (Caltag Laboratories) was substituted for the CD4-PE-Cy5 mAb and CD4-AC mAb was substituted for CD8-α-AC mAb. Gates were set to exclude AC-positive cells to eliminate monocytes, B cells, and either $CD8^+$ or $CD4^+$ T cells from the analysis. For simultaneous analysis of CCR7 expression and intracellular IFN-γ accumulation, PBMC were used, because this yielded superior staining results compared with whole blood assays. PBMC were stained with purified mouse anti-human CCR7 mAb for 20 min and washed, followed by incubation with secondary Ab (biotinylated rat anti-mouse IgM) for 20 min. After additional washing, cells were surface stained using a combination of mAbs (CD4-PE-Cy5 and CD45RA-AC) and streptavidin-PE for 20 min. After surface marker staining, cells were washed and then fixed and permeabilized with FACS lysis and permeabilization solution (BD Biosciences), respectively, for 10 min. Cells were then stained with cytokine-reactive mAb (IFN-γ-FITC, IL-2-FITC, IL-4-FITC, IL-10-FITC, or IL-13-FITC) for 30 min. Analysis of CCR7 and CD154 expression was performed similarly, except that CD154-FITC was included in the initial surface-staining step and the permeabilization and intracellular steps were omitted. Following staining, cells were washed with PBS, and resuspended in PBS plus 1% paraformaldehyde. Isotype-matched control Abs of irrelevant specificity were included in all experiments.

Flow cytometric analysis

A FACSCalibur flow cytometer (BD Biosciences) was used to collect 2–5 × 10^5 events for each sample by gating on small lymphocytes (using forward vs side scatter). Logical gates were set to analyze $CD4^+CD8^-CD19^-CD14^-$ ($CD4^+$ T cells) or $CD8^+CD4^-CD19^-CD14^-$ ($CD8^+$ T cells) in whole blood stimulation assay, or $CD4^+$ T cells (using CD4 vs side scatter) in instances in which PBMC were analyzed. Data were analyzed by use of FLOW JO software (Tree Star, San Carlos, CA).

Quantitative real-time PCR for CMV DNA

DNA was extracted from urine in a biological safety cabinet to prevent environmental contamination of samples using a MasterPure DNA Purification Kit (Epicenter Technologies, Madison, WI), following the manufacturer's instructions. Real-time PCR was performed using an ABI PRISM 7700 sequence detection system and TaqMan Universal PCR Master Mix (both from Applied Biosystems, Foster City, CA). The oligonucleotide primers and probe were used to detect the coding region of the CMV UL54 DNA polymerase gene, which is highly conserved among strains. The forward primer was 5′-CCGAGGTGGGTTACTACAACG-3′; the reverse primer was 5′-GGAAGGGTAGAGGCTGGCA-3′; and the fluorogenic internal probe was 5′-FAM-CCCCGTGGCCGTGTTCGACT TAMRA-3′. PCR was performed for 40 cycles (50°C/2 min; 95°C/15 s; 60°C/1 min). CMV genome concentration was estimated by analysis of the

[3] Abbreviations used in this paper: SEB, staphylococcal enterotoxin B; AC, allophycocyanin.

Table I. *Characteristics of child and adult groups*

Group	Labeled in Figures	*n*	Age (years)	Estimated Duration of CMV Infection	CMV Detection in Urine by Viral Culture or Quantitative PCR
1	Adult	14	26–45	Chronic	NA
2	Adult 1^0	4	44–52	12–15 mo	Undetectable in all subjects
3	Child	13	1.5–4	8–29 mo	Positive in all subjects (4.7×10^2-1.3×10^5 copies/ml)
4[a]	Child 1^0	7	1.5–4	12–29 mo	Positive in all subjects (4.7×10^2-1.3×10^5 copies/ml)

[a] Group 4 was a subset of group 3 children, as indicated in *Materials and Methods.*

signal obtained from various dilutions of a CMV UL54 plasmid or purified CMV genomic DNA.

Results

Decreased frequency of CMV-specific CD4$^+$ T cells, but not CD8$^+$ T cells in young children

The frequencies of CMV-specific T cells were first compared between 14 asymptomatic adults (group 1) who were CMV Ab seropositive, and presumably chronically infected, and 13 asymptomatic young children with primary CMV infection of ~8- to 29-mo duration (group 3). In agreement with previous results (15, 18, 19), adults had discrete subsets of CD4$^+$ and CD8$^+$ T cells that coexpressed IFN-γ and the activation-dependent protein CD69 in response to either whole CMV Ag (CMV) or a mixture CMV pp65 (UL83) peptides (pp65) (Fig. 1*A*). Although the level of CD69 expression by the child sample shown in Fig. 1*A* appeared to be greater than for the adult sample, this was not a consistent and statistically significant finding when a larger number of children and adults was compared (data not shown). A control Ag (mock-infected and lysed fibroblasts) and a control peptide pool derived from the carcinoembryonic Ag, a self protein, resulted in a minimal response (<0.07% IFN-γ^+ cells) similar to incubation of cells without additive.

In contrast, young children had a markedly and significantly lower frequency of circulating CMV-specific CD4$^+$ T cells that produced IFN-γ compared with adults (Fig. 1, *A* and *B*). Children also had a markedly reduced frequency of CD4$^+$ T cells that produced IFN-γ in response to the pp65 peptide mixture (Fig. 1, *A* and *B*). Because activation of pp65-reactive CD4$^+$ or CD8$^+$ T cells by the peptide mixture does not require Ag uptake and processing by APCs, but only MHC surface expression (15, 16), these results indicated that the decreased CMV-specific CD4$^+$ T cell response of children was not due to impaired APC function during ex vivo stimulation. However, adults and children had comparable levels of CMV- and pp65-specific CD8$^+$ T cells producing IFN-γ (Fig. 1*A*, and our unpublished data), indicating that this decreased T cell immunity was selective for the CD4$^+$ subset.

CMV-specific CD4$^+$ T cells producing IFN-γ have been detected as early as 30 days following viral exposure in adults with primary CMV infection as a result of renal transplantation, and who receive potent immunosuppressive drugs to limit transplant rejection (20, 21). However, the time required for healthy immunocompetent adults to develop a Th1 immune response after primary CMV infection from mucosal exposure to the virus is unknown, but could conceivably require months to years. If so, the reduced IFN-γ response by CD4$^+$ T cells of healthy CMV-infected young children would merely reflect their shorter duration of CMV infection compared with the healthy adult group with presumed chronic infection. To exclude this possibility, the CMV-specific Th1 immune responses of four healthy adults with primary CMV infection (group 2) of an estimated 12–15 mo of duration were then examined. This immune response was compared with a subset of the children (group 4) for whom the estimated period of CMV infection (12–29 mo) was equal to or greater than that of the adults (Fig. 1*C*). The CMV- and pp65-specific Th1 immune responses of adults with recent primary infection were similar to

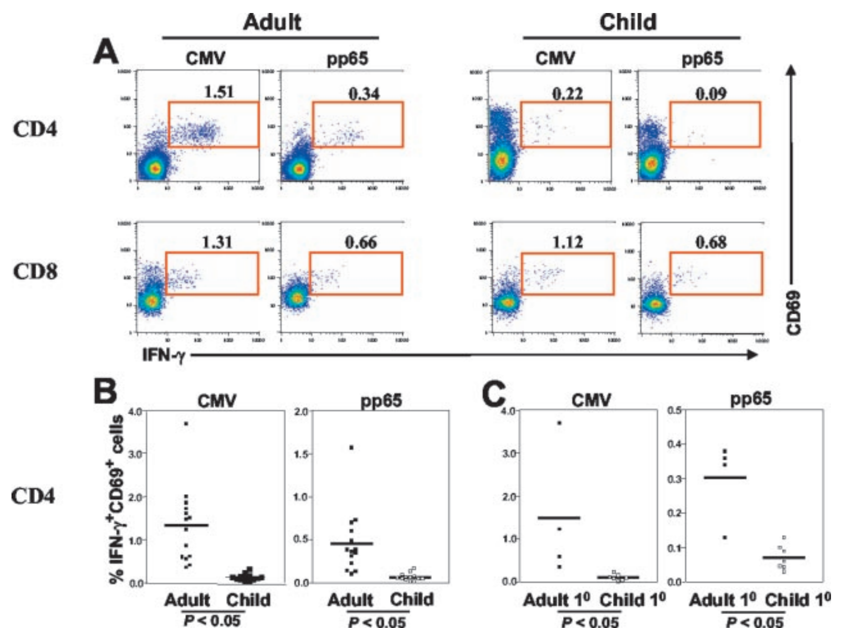

FIGURE 1. Flow cytometric analysis demonstrating that children have decreased CMV-specific CD4$^+$ T cell responses compared with adults. *A*, Representative results of CMV-specific IFN-γ and CD69 expression by CD4$^+$ and CD8$^+$ T cells of a CMV Ab-seropositive adult with presumed chronic infection and a young child. The numbers in each histogram represent the percentage of CMV-specific or pp65-specific CD4$^+$ or CD8$^+$ T cells, as assessed by cells positive for both IFN-γ and CD69, following a published approach (16). *B*, Frequency of Ag-specific CD4$^+$ T cells after CMV or pp65 stimulation for a cohort of children with 8- to 29-mo duration of primary infection (child) and a group of adults with presumed chronic CMV infection (adult). *C*, Frequency of CMV-specific and pp65-specific CD4$^+$ T cells for children and adults with similar duration (child 1^0 vs adult 1^0). Horizontal bars in (*B*) and (*C*) indicate group means. Values of *p* for comparison of means were based on two-tailed, unpaired Student's *t* tests. To conserve power, testing here and throughout was not adjusted for multiplicity of type I error: any $p < 0.05$ was interpreted to indicate statistical significance.

those of the adult chronic group and, again, were significantly greater than those of the children with primary infection of a similar duration (Fig. 1*C*). These results demonstrated that healthy young children had a markedly limited ability to mount a CMV-specific Th1 immune response for 1 year or more following viral acquisition, while this lag in antiviral immunity was not observed in adults.

The CCR7^{low} subset accounts for most IFN-γ produced by CD4$^+$ memory T cells

To assess whether the reduced CMV-specific Th1 response of young children reflected a generally limited ability to mount Th1 immune responses to Ags, we first determined the phenotype of CMV-specific CD4$^+$ T cells that accounted for most IFN-γ production in adults and children. It has been reported that IFN-γ production by adult CD4$^+$ T cells in response to protein Ag (22–24), including CMV (18) or SEB (25), is largely confined to CD45RA^{low}CD45R0^{high} memory/effector subset. We verified that this applied to CMV-specific and SEB-induced IFN-γ production by CD4$^+$ T cells from adults with presumed chronic CMV infection or adults and children with documented recent primary infection (data not shown). Because memory CD4$^+$ T cells expressing low levels of CCR7 have been reported to be highly enriched in IFN-γ production after engagement of CD3 and CD28 (25, 26), we next determined whether this restriction of Th1 function to CCR7^{low} memory cells also applied to CMV Ag-specific responses. Among adult and child memory CD4$^+$ T cells, >90% of cells that produced IFN-γ in response to either stimulation with CMV Ag, the pp65 peptide mixture, or SEB were CCR7^{low} (Fig. 2). Similar results were also observed for CD4$^+$ T cells of adults with documented recent primary CMV infection (our unpublished data). These results suggested that Th1 responses generated postnatally to specific Ag, or at least to CMV-derived Ags, are largely restricted to a CCR7^{low} memory CD4$^+$ T cell subset.

Children have a lower fraction of CCR7^{low} memory CD4$^+$ T cells than adults

Because the CMV-specific Th1 response was limited to the CCR7^{low} memory CD4$^+$ T cell subset, it was plausible that reduced CMV-specific Th1 responses of children reflected a decreased accumulation of CCR7^{low} cells during memory CD4$^+$ T cell generation. In fact, the fraction of memory CD4$^+$ T cells that were CCR7^{low} was modestly, but significantly lower in children with documented recent primary CMV infection compared with adults with chronic CMV infection (Fig. 3). A similar trend was also observed in a comparison of children and adults with primary CMV infection of similar duration (groups 2 and 4), although this did not reach statistical significance (Fig. 3). These results suggested that a tendency to accumulate a lower fraction of CCR7^{low} cells as part of memory CD4$^+$ T cell responses may contribute, in part, to the decreased CMV-specific Th1 responses of young children.

CMV-specific Th1 responses by CCR7^{low} memory CD4$^+$ T cells are selectively decreased in children

The ability of CCR7^{low} memory CD4$^+$ T cells to produce IFN-γ was further compared between children and adults with documented primary CMV infection of similar duration. The CCR7^{low} subset of memory CD4$^+$ T cells of children had a markedly and significantly lower frequency of IFN-γ-producing cells in response to whole CMV Ag or the pp65 peptide mixture than the analogous adult cell population (Fig. 4). This was in agreement with the earlier results analyzing unfractionated or memory CD4$^+$ T cells. In contrast, the child and adult CCR7^{low} subsets of memory CD4$^+$ T cells contained a similar frequency of cells producing IFN-γ in response to SEB stimulation (Fig. 4). In addition, SEB stimulation induced similar amounts of IFN-γ per cell, based on mean fluorescent intensity

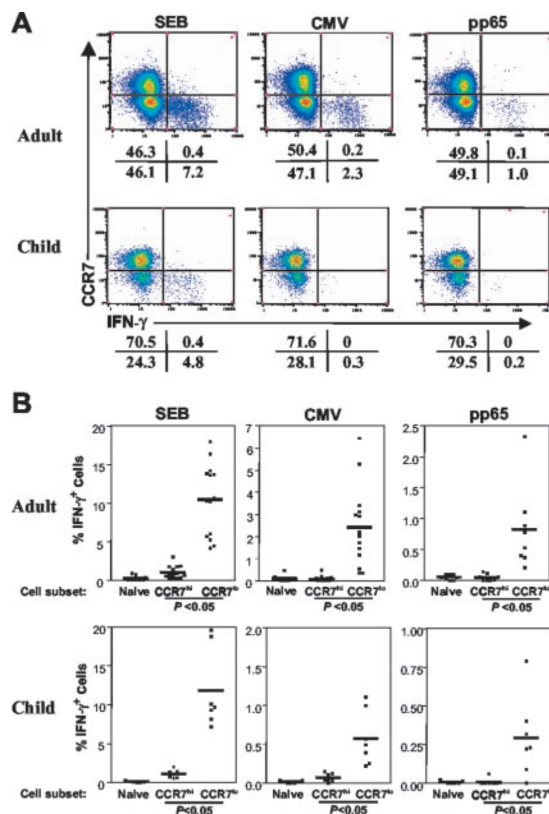

FIGURE 2. CMV-specific CD4$^+$ T cells producing IFN-γ are mainly CCR7^{low} memory cells for both young children and adults. *A*, Representative flow cytometric analysis of memory CD4$^+$ T cells for expression of CCR7 vs IFN-γ using PBMC from an adult with presumed chronic CMV infection and a child with documented recent primary infection. Events shown are gated for memory CD4$^+$ T cells, based on CD4 expression, lack of CD45RA expression, and forward scatter and side scatter properties of lymphocytes, with the percentage of cells in each quadrant indicated. *B*, Frequency of IFN-γ-producing CD4$^+$ T cells among naive, CCR7^{high} memory, and CCR7^{low} memory cell subsets of adults and children. Horizontal bars indicate group means. Values of *p* are reported for comparisons of means based on two-tailed, unpaired Student's *t* tests.

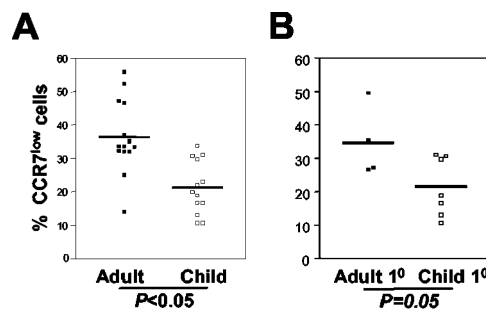

FIGURE 3. Children have a lower fraction of CCR7^{low} memory CD4$^+$ T cells than adults. *A*, Percentage of CCR7^{low} memory CD4$^+$ T cells for the cohort of young children with primary CMV infection of 8- to 29-mo duration (child) and the adult group with presumed chronic CMV infection (adult). *B*, Percentage of CCR7^{low} memory CD4$^+$ T cells for children and adults with similar duration of primary CMV infection (child 1^0 vs adult 1^0). Horizontal bars in *A* and *B* indicate groups means. Values of *p* are comparisons of means based on two-tailed, unpaired Student's *t* tests.

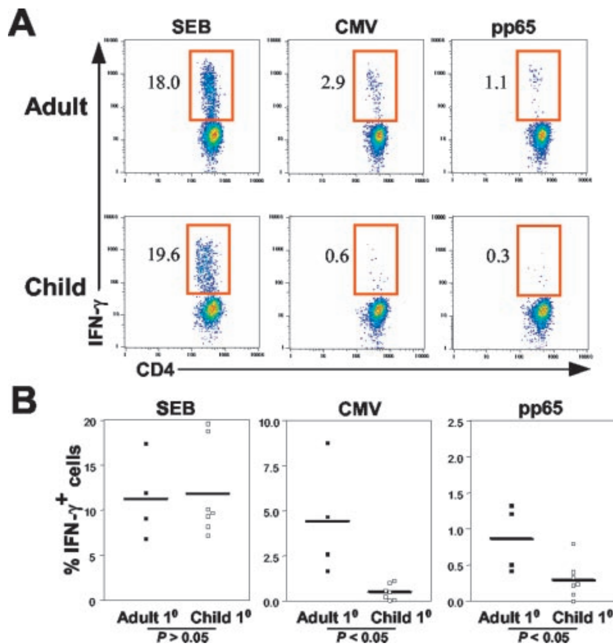

FIGURE 4. Young children have reduced IFN-γ production by CCR7^{low} memory CD4$^+$ T cells in response to CMV Ags, but not SEB. *A*, Representative flow cytometric analysis of IFN-γ expression by CCR7^{low} memory CD4$^+$ T cells of adults and children after stimulation with CMV whole cell lysate, pp65 peptide pool, or SEB. The events shown are CCR7^{low} memory CD4$^+$ T cells based on positive gating for CD4 and negative gating for CD45RA and CCR7. Reported values are percentages of IFN-γ-producing cells. *B*, Frequency of IFN-γ-producing cells within the CCR7^{low} subset of memory CD4$^+$ T cells of the adult 1^0 and child 1^0 groups. Horizontal bars indicate group means. Values of p are reported for comparisons of means based on two-tailed, unpaired Student's t tests.

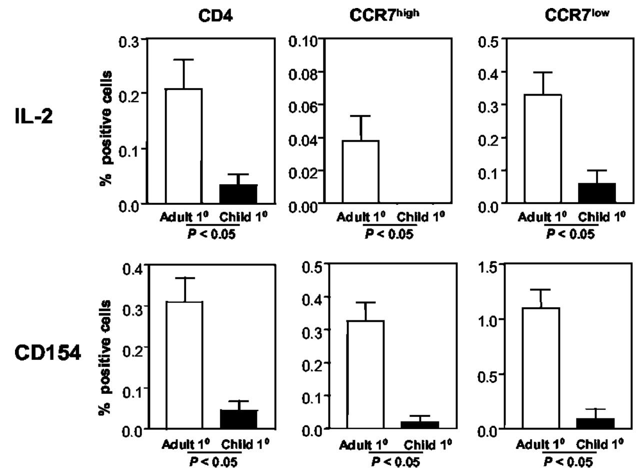

FIGURE 5. Young children with recent primary CMV infection have reduced CMV-specific IL-2 and CD154 expression by CD4$^+$ T cells. Frequency of adult and child CD4$^+$ T cells and the CCR7^{high} and CCR7^{low} subsets of memory CD4$^+$ T cells expressing IL-2 (*top panels*) or CD154 (*bottom panels*) after activation with CMV cell lysate Ag for 6 h. The CCR7^{high} and CCR7^{low} cells were analyzed by gating on CD4$^+$CD45RA$^-$ (memory) T cells. Results represent samples from four adults and four children of the 1^0 groups presented as mean ± SEM. Values of p are reported for comparisons of means based on two-tailed, unpaired, Student's t tests.

measurements (Fig. 4*A* and data not shown). Together, these results demonstrated that the decreased CMV-specific Th1 response by CCR7^{low} cells in children was relatively selective in that it did not apply to all Th1 immune responses.

Decreased CMV-specific IL-2 and CD154 expression by CD4$^+$ T cells of children

We next determined whether reduced CMV-specific CD4$^+$ T cell effector function of young children extended to other cytokines, such as IL-2, and to CD154 (CD40 ligand), a surface molecule that is key for the generation of memory CD4$^+$ T cells with Th1 function (27). As for IFN-γ, the frequency of CMV-specific CD4$^+$ T cells expressing IL-2 or CD154 was substantially lower for children compared with adults with similar duration of primary CMV infection (Fig. 5). However, unlike IFN-γ expression, IL-2 and CD154 were expressed by a small, but detectable fraction of adult CCR7^{high} memory CD4$^+$ T cells in response to CMV Ag, although a greater fraction was found in the CCR7^{low} subset. In contrast, IL-2 and CD154 expression by CD4$^+$ T cells of children was essentially undetectable for both the CCR7^{low} and CCR7^{high} subsets (Fig. 5). Thus, children had decreased IL-2 and CD154 expression by unfractionated CD4$^+$ T cells as well as by the CCR7^{high} or CCR7^{low} subsets of memory CD4$^+$ T cells. We also analyzed CD4$^+$ T cells from children and adults for CMV-specific production of IL-4, IL-10, and IL-13 using intracellular cytokine staining, but were not able to detect cells expressing these cytokines in any samples (data not shown).

Relationship between viral shedding and the lack of a CMV-specific Th1 response

Previous studies have documented prolonged urinary and salivary shedding of CMV in young children acquiring this infection in the day care setting (3, 28). We confirmed that this applied to the young children in our study by measuring CMV DNA levels in urine using a real-time PCR assay and by viral culture of urine. All children were positive by both assays, indicating that viral shedding in the urine persisted for at least 12–29 mo after acquisition of CMV (Table I). In contrast, CMV was not detected by either assay for urine from any adult with recent primary infection. To determine whether this result was typical for adults with primary CMV infection, we also retrospectively analyzed data on a group of 30 healthy women with documented primary CMV infection and found that 90% of these subjects ceased urinary shedding by 6 mo after acquisition of virus (Table II). Together, these findings demonstrate a striking inverse correlation between viral shedding and the CMV-specific Th1 response.

Discussion

In this study, we found that healthy young children with primary CMV infection accumulated markedly fewer CMV-specific CD4$^+$

Table II. *Duration of CMV shedding in urine of 30 women following primary infection*[a]

Duration of Viral Shedding after Acquisition of CMV	No. of Cases
6 mo or less	27
7 mo	1
14 mo	1
15 mo	1

[a] All women were initially CMV Ab seronegative and were not shedding CMV into the urine or saliva when they entered into the study. All had young children who had recently been infected with CMV, and who were the likely source of infection. The blood and urine samples were taken at enrollment and then every 2–3 mo until resolution of shedding.

T cells that produced IFN-γ than did adults. These striking differences in $CD4^+$ T cell function persisted for more than 1 year after viral acquisition, while, in contrast, the CMV-specific $CD8^+$ T cell response of these young children was comparable to that of adults.

These significant decreases of CMV-specific Th1 cells for young children were likely to apply to the absolute number of these cells. Because total lymphocyte counts per microliter were not determined as part of this study, to assess this we reanalyzed the data by assuming that the young children had on average a 2.0-fold greater absolute number of $CD4^+$ T cells than the adults. In various studies, this value has been variously reported as 1.4 (29), 1.6 (30), 1.7 (31), and 2.0 (32), so that this modification of the data was assuming that the maximal differences in circulating numbers of $CD4^+$ T cells applied to our subjects. When the values of the children for frequency were multiplied by 2.0 and compared with the adult values,[4] all of the differences that were significant for CMV-specific and pp65-specific $CD4^+$ T cell frequency remained significant ($p < 0.05$ by the Mann-Whitney U test) after this modification (data not shown).

The decreased CMV-specific Th1 response of young children was mainly attributable to a lack of CMV-reactive Th1 cells among the $CCR7^{low}$ memory $CD4^+$ T cell population. For both young children and adults, this $CCR7^{low}$ subset accounted not only for the bulk of IFN-γ in response to CMV Ags, but also to SEB stimulation (Fig. 2). These findings are in agreement with two earlier studies (25, 26) in which the $CCR7^{low}$ subset of memory $CD4^+$ T cells was observed to contain almost all IFN-γ-producing cells among $CD4^+$ T cells polyclonally activated using CD3 and CD28 mAbs. In one of these studies (26), the production of IL-4 by memory $CD4^+$ T cells was also largely restricted to the $CCR7^{low}$ rather than the $CCR7^{high}$ subset. Thus, our results indicate that Ag-specific Th1 immune responses in vivo in humans, or at least those that are involved in antiviral immunity, may be largely mediated cells that are $CCR7^{low}$. Whether pathogen-specific Th2 responses, e.g., to extracellular parasites, are similarly restricted to the $CCR7^{low}$ subset in humans remains to be determined.

The decreased Th1 response to CMV of young children was largely accounted for by the markedly reduced frequency of $CCR7^{low}$ $CD4^+$ T cells that produced IFN-γ in response to CMV proteins or peptides. Nevertheless, these $CCR7^{low}$ memory $CD4^+$ T cells were present in substantial amounts in young children and had a similar capacity to produce IFN-γ after stimulation with SEB. Because SEB activates T cells based on Vβ segment usage by the $\alpha\beta$-TCR rather than peptide antigenic specificity (33), it is likely to activate T cells that have undergone clonal expansion and Th1 differentiation in response to a diverse group of peptide Ags. Thus, these results suggested that the reduced CMV-specific $CD4^+$ T cell IFN-γ production of children was apparently selective, and not due to a general inability of their $CCR7^{low}$ memory $CD4^+$ T cells to mediate Th1 effector function. Although the pathways by which $CCR7^{low}$ Th1 cells differentiate from less mature $CD4^+$ T cells remain controversial (34, 35), our results demonstrate that the failure of young children to develop robust Th1 immunity to CMV is not due to a general blockade in this process of differentiation and cell accumulation.

In young children, the proportion of circulating memory $CD4^+$ T cells that were $CCR7^{low}$ was ~50–60% of that found in adults (Figs. 2*A* and 3, *A* and *B*). This modest reduction could reflect a general tendency of young children to produce fewer $CCR7^{low}$ cells to antigenic stimuli, although this remains to be shown. Consistent with this idea is the recent finding that circulating monocytes from healthy infants and young children have a reduced capacity to produce IL-12 (36), a cytokine that is required for the accumulation of $CCR7^{low}$ memory $CD4^+$ T cells with Th1 function (25). Although speculative, it is also plausible that the increased proportion of $CCR7^{low}$ memory $CD4^+$ T cells in adults could reflect their greater cumulative exposure to natural infections that are effective at driving Th1 immune responses compared with the young children of our study, for whom a greater proportion of antigenic stimulation may be derived from vaccines that are relatively ineffective Th1 inducers. Regardless of the mechanisms involved, the reduced accumulation of $CCR7^{low}$ memory $CD4^+$ T cells in children accounted for only a minor portion of the profound reduction in Th1 immunity to CMV that was observed.

Previous studies have shown that healthy infants and young children who acquire CMV infection after birth have prolonged viral urinary and salivary shedding compared with adults (1, 3, 4, 7, 13), but the mechanism of this prolonged viral shedding has until now remained unclear. Our results demonstrate a selective limitation in Th1 immunity in young children with postnatally acquired primary CMV infection and a striking inverse correlation between viral shedding and the CMV-specific Th1 response. The importance of Th1 responses in control of CMV shedding is strongly supported by studies of murine CMV, in which $CD4^+$ T cells producing IFN-γ, but not $CD8^+$ T cells were found to be critical for limiting CMV replication in acinar epithelial cells and consequent salivary shedding (9–11). This most likely applies to other epithelial sites of shedding, such as the kidney. In contrast, control of CMV replication at other tissue sites, such as the lung, depends critically on $CD8^+$ T cells, but not $CD4^+$ Th1 cells (11). We speculate that the limited consequence of reduced Th1 responses in these healthy children (i.e., asymptomatic, but prolonged CMV shedding) is due to the fact that children develop reduced, but detectable CMV-specific Th1 immunity and have robust $CD8^+$ T cell responses (Fig. 1*A*, and our unpublished data).

The importance of $CD4^+$ T cell-mediated immunity in the control of CMV shedding is also supported by studies of congenital CMV infection. Epidemiological analysis of children with congenital CMV infection, who also have prolonged viral shedding, indicates that termination of CMV shedding was associated with an increased proliferative response (37) indicative of $CD4^+$ T cell immunity to the virus. Moreover, a recent report found that asymptomatic newborns with congenital CMV infection had a mature $CD8^+$ T cell response (38), supporting the idea that CMV-specific $CD8^+$ T cells are not sufficient for the control of viral shedding.

The finding that healthy young children, including some as old as 3–4 years of age, have limited CMV-specific Th1 responses for more than 1 year postinfection is, to the best of our knowledge, unprecedented for a persistent viral infection. Human neonates also have a lag in the appearance of a normal Th1 immune response following primary HSV infection compared with adults (39); but this lag only lasts several weeks and not for months to years as in primary CMV infection of young children. It will be of interest to determine whether there are significantly delayed Th1 responses in young children who acquire other persistent viral infections, such as with other herpesviruses.

The underlying mechanism of the reduced CMV-specific Th1 response in young immunocompetent children is unknown. Our results demonstrated not only reduced Th1 responses, including IFN-γ and IL-2 production, but also decreased CMV-specific expression of CD154 by $CD4^+$ T cells, including cells that are either

[4] Let γ = quantity of IFN-γ^+ cells, T = the number of events that are $CD4^+$ T cells. Subscript a denotes adult values and subscript c denotes child values. Then data for children were defined as $(\gamma_c/T_c)(2T_a/\mu l)$, and data for adults were defined as $(\gamma_a/T_a)(T_a/\mu l)$. If these definitions are then divided by $T_a/\mu l$, this yields the data that were used for reanalysis.

CCR7^{low} or CCR7^{high}. This suggests an action that may apply relatively early to the generation of memory CD4$^+$ T cells from less mature precursors, as CCR7^{high} memory CD4$^+$ T cells have been proposed as the precursors of the CCR7^{low} cells with Th1 activity (26, 34, 35), although this is a controversial issue.

The CMV-specific CD4$^+$ T cell immune response of young children or adults was also not characterized by detectable flow cytometric expression of Th2 cytokines, such as IL-4 and IL-13. Thus, although Th2 cytokines have been suggested to inhibit the development of Th1 responses in an Ag-specific fashion by a phenomenon known as cross-regulation (40), this does not appear to be a major mechanism for the reduced Th1 responses that we observed in young children. IL-10 is another attractive candidate cytokine to block Th1 responses (41) in this context, and decreases class II MHC expression by APCs and their ability to activate Th1 cells (42, 43). IL-10 also directly inhibits CD4$^+$ Th1 cell differentiation and from less mature precursors and, instead, favors the generation of CD4$^+$ T cells with anergic properties (44). We did not observe increased production of IL-10 by CMV-specific CD4$^+$ T cells of children compared with adults, but we cannot rule out that increased secretion of IL-10 by other cellular sources, such as regulatory T cell populations or APCs, might participate in limiting CMV-specific Th1 immunity in young children.

CMV is known to encode several gene products that could potentially inhibit the generation of CD4$^+$ T cell immune responses in vivo in young children (45). These include the US2 and US3, which have been shown to directly interfere with class II MHC Ag presentation (46, 47), and a viral orthologue of IL-10 that has similar immunosuppressive properties as human IL-10 (31, 45). CMV can also directly infect human myeloid dendritic cells (48, 49), which are key APCs for the initiation of the adaptive immune response and in directing CD4$^+$ T cell differentiation toward Th1 effector function or other fates (50). Such infection may interfere with the ability of dendritic cells to present Ags to CD4$^+$ T cells (48) and produce IL-12 (49), a key cytokine for the generation of Th1 cells in humans (25). These and other potential mechanisms, such as blockade by CMV infection of IFN-γ-mediated intracellular signaling and downstream up-regulation of molecules involved in Ag presentation (51), could inhibit the generation of Th1 responses in vivo. However, it remains unclear why such mechanisms might be more effective in young children compared with adults following primary infection.

Our results suggest that CMV may immunosuppress the human host in a striking developmentally regulated manner. This implies that CD4$^+$ T cells or other cells required for T cell activation, such as dendritic cells, may undergo an age-dependent susceptibility to viral immunoevasion and/or viral-mediated immunosuppression that is more evident in young children. Considerable evolutionary pressure may have favored human CMV's development and maintenance of the capacity to avoid inducing Th1 responses to spread efficiently via secretions during early life. Regardless of the precise mechanism, this developmental limitation in adaptive immunity will need to be considered in immunotherapeutic strategies to control CMV, such as vaccination. CMV has presumably evolved to be transmitted at a young age, and does so in much of the world today (1, 4, 7), suggesting that the failure to induce a strong CD4$^+$ T cell immune response in early childhood is a key part of the pathobiology of this virus.

Acknowledgments

We thank E. Mocarski, Stanford University, for helpful discussions and suggestions, and P. Stepick-Biek for excellent technical assistance.

References

1. Pass, R. 2001. Cytomegalovirus. In *Fields Virology*. D. M. Knipe and P. M. Howley, eds. Lippincott Williams & Wilkins, Philadelphia, p. 2675.
2. Mocarski, E. S., and C. T. Courcelle. 2001. Cytomegaloviruses and their replication. In *Fields Virology*. D. M. Knipe and P. M. Howley, eds. Lippincott Williams & Wilkins, Philadelphia, p. 2629.
3. Adler, S. P. 1992. Cytomegalovirus and child day care. *Adv. Pediatr. Infect. Dis. 7:109*.
4. Stagno, S., and G. A. Cloud. 1994. Working parents: the impact of day care and breast-feeding on cytomegalovirus infections in offspring. *Proc. Natl. Acad. Sci. USA 91:2384*.
5. Greenberg, P. D., and S. Riddell. 2000. T-cell therapy of cytomegalovirus and human immunodeficiency infection. *J. Antimicrob. Chemother. 45(Suppl. T3):35*.
6. Komanduri, K. V., M. N. Viswanathan, E. D. Wieder, D. K. Schmidt, B. M. Bredt, M. A. Jacobson, and J. M. McCune. 1998. Restoration of cytomegalovirus-specific CD4$^+$ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1. *Nat. Med. 4:953*.
7. Stagno, S. 2001. Cytomegalovirus. In *Infectious Diseases of the Fetus and Newborn Infant*. J. S. Remington and J. O. Klein, eds. W. B. Saunders, Philadelphia, p. 389.
8. Revello, M. G., M. Zavattoni, A. Sarasini, E. Percivalle, L. Simoncini, and G. Gerna. 1998. Human cytomegalovirus in blood of immunocompetent persons during primary infection: prognostic implications for pregnancy. *J. Infect. Dis. 177:1170*.
9. Jonjic, S., W. Mutter, F. Weiland, M. J. Reddehase, and U. H. Koszinowski. 1989. Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4$^+$ T lymphocytes. *J. Exp. Med. 169:1199*.
10. Lucin, P., I. Pavic, B. Polic, S. Jonjic, and U. H. Koszinowski. 1992. γ Interferon-dependent clearance of cytomegalovirus infection in salivary glands. *J. Virol. 66:1977*.
11. Polic, B., H. Hengel, A. Krmpotic, J. Trgovcich, I. Pavic, P. Luccaronin, S. Jonjic, and U. H. Koszinowski. 1998. Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection. *J. Exp. Med. 188:1047*.
12. Zanghellini, F., S. B. Boppana, V. C. Emery, P. D. Griffiths, and R. F. Pass. 1999. Asymptomatic primary cytomegalovirus infection: virologic and immunologic features. *J. Infect. Dis. 180:702*.
13. Adler, S. P. 1985. The molecular epidemiology of cytomegalovirus transmission among children attending a cay-care center. *J. Infect. Dis. 152:760*.
14. Plotkin, S. J., S. E. Starr, H. M. Friedman, E. Gonczol, and R. E. Weibel. 1989. Protective effects of Towne cytomegalovirus vaccine against low-passage cytomegalovirus administered as a challenge. *J. Infect. Dis. 159:860*.
15. Maecker, H. T., H. S. Dunn, M. A. Suni, E. Khatamzas, C. J. Pitcher, T. Bunde, N. Persaud, W. Trigona, T. M. Fu, E. Sinclair, et al. 2001. Use of overlapping peptide mixtures as antigens for flow cytometry *J. Immunol. Methods 255:27*.
16. Kern, F., T. Bunde, N. Faulhaber, F. Kiecker, E. Khatamzas, I. M. Rudawski, A. Pruss, J. W. Gratama, R. Volkmer-Engert, R. Ewert, et al. 2002. Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T-cell repertoire in CMV-exposed individuals. *J. Infect. Dis. 185:1709*.
17. Waldrop, S. L., K. A. Davis, V. C. Maino, and L. J. Picker. 1998. Normal human CD4$^+$ memory T cells display broad heterogeneity in their activation threshold for cytokine synthesis. *J. Immunol. 161:5284*.
18. Waldrop, S. L., C. J. Pitcher, D. M. Peterson, V. C. Maino, and L. J. Picker. 1997. Determination of antigen-specific memory/effector CD4$^+$ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. *J. Clin. Invest. 99:1739*.
19. Dunn, H. S., D. J. Haney, S. A. Ghanekar, P. Stepick-Biek, D. B. Lewis, and H. T. Maecker. 2002. Dynamics of CD4 and CD8 T cell responses to cytomegalovirus in healthy human donors. *J. Infect. Dis. 186:15*.
20. Rentenaar, R. J., L. E. Gamadia, N. ven Dehoek, F. N. van Diepen, R. Boom, J. F. Weel, P. M. Wertheim-van Dillen, R. A. van Lier, and I. J. ten Berge. 2000. Development of virus-specific CD4$^+$ T cells during primary cytomegalovirus infection. *J. Clin. Invest. 105:541*.
21. Gamadia, L. E., E. B. Remmerswaal, J. F. Weel, F. Bemelman, R. A. van Lier, and I. J. ten Berge. 2003. Primary immune responses to human CMV: a critical role for IFN-γ-producing CD4$^+$ T cells in protection against CMV disease. *Blood 101:2686*.
22. Young, J. L., J. M. Ramage, J. S. Gaston, and P. C. Beverly. 1997. In vitro responses of human CD45R0^{bright}RA$^-$ and CD45R0$^-$RA^{bright} T cell subsets and their relationship to memory and naive T cells. *Eur. J. Immunol. 27:2383*.
23. Picker, L. J., M. K. Singh, Z. Zdraveski, J. R. Treer, S. L. Waldrop, P. R. Bergstresser, and V. C. Maino. 1995. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood 86:1408*.
24. Sanders, M. E., M. W. Makgoba, S. O. Sharrow, D. Stephany, T. A. Springer, H. A. Young, and S. Shaw. 1988. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-γ production. *J. Immunol. 140:1401*.
25. Cleary, A. M., W. Tu, A. Enright, T. Giffon, R. de Waal-Malefyt, K. Gutierrez, and D. B. Lewis. 2003. Impaired accumulation and function of memory CD4 T cells in human IL-12 receptor β1 deficiency. *J. Immunol. 170:597*.
26. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature 401:708*.

27. Jain, A., T. P. Atkinson, P. E. Lipsky, J. E. Slater, D. L. Nelson, and W. Strober. 1999. Defects of T-cell effector function and post-thymic maturation in X-linked hyper-IgM syndrome. *J. Clin. Invest. 103:1151*.
28. Adler, S. P. 1991. Molecular epidemiology of cytomegalovirus: a study of factors affecting transmission among children at three day-care centers. *Pediatr. Infect. Dis. J. 10:584*.
29. Heldrup, J., O. Kalm, and K. Prellner. 1992. Blood T and B lymphocyte subpopulations in healthy infants and children. *Acta Paediatr. 81:125*.
30. Denny, T., R. Yogev, R. Gelman, C. Skuza, J. Oleske, E. Chadwick, S.-C. Cheng, and E. Connor. 1992. Lymphocyte subsets in healthy children during the first 5 years of life. *J. Am. Med. Assoc. 267:1484*.
31. Hulstaert, F., I. Hannet, V. Deneys, V. Munhyeshuli, T. Reichert, M. De Bruyere, and K. Strauss. 1994. Age-related changes in human blood lymphocyte subpopulations. II. Varying kinetics of percentage and absolute count measurements. *Clin. Immunol. Immunopathol. 70:152*.
32. Erkeller-Yuksel, F. M., V. Deneys, B. Yuksel, I. Hannet, F. Hulstaert, C. Hamilton, H. Mackinnon, L. T. Stokes, V. Munhyeshuli, F. Vanlangendonck, et al. 1992. Age-related changes in human blood lymphocyte subpopulations. *J. Pediatr. 120:216*.
33. Marrack, P., and J. Kappler. 1990. The staphylococcal enterotoxins and their relatives. *Science 248:705*.
34. Blander, J. M., D. B. Sant-Angelo, D. Metz, S.-W. Kim, R. A. Flavell, K. Bottomly, and C. A. Janeway. 2003. A pool of central memory-like CD4 T cells contains effector memory precursors. *J. Immunol. 170:2940*.
35. Lanzavecchia, A., and F. Sallusto. 2002. Progressive differentiation and selection of the fittest in the immune response. *Nat. Rev. Immunol. 2:982*.
36. Upham, J. W., P. T. Lee, B. J. Holt, T. Heaton, S. J. Prescott, M. J. Sharp, P. D. Sly, and P. G. Holt. 2002. Development of interleukin-12-producing capacity throughout childhood. *Infect. Immun. 70:6583*.
37. Pass, R. F., S. Stagno, W. J. Britt, and C. A. Alford. 1983. Specific cell-mediated immunity and the natural history of congenital infection with cytomegalovirus. *J. Infect. Dis. 148:953*.
38. Marchant, A., V. Appay, M. Van Der Sande, N. Dulphy, C. Liesnard, M. Kidd, S. Kaye, O. Ojuola, G. M. Gillespie, A. L. Vargas Cuero, et al. 2003. Mature $CD8^+$ T lymphocyte response to viral infection during fetal life. *J. Clin. Invest. 111:1747*.
39. Burchett, S. K., L. Corey, K. M. Mohan, J. Westall, R. Ashley, and C. B. Wilson. 1992. Diminished interferon-γ and lymphocyte proliferation in neonatal and postpartum primary herpes simplex virus infection. *J. Infect. Dis. 165:813*.
40. Morel, P. A., and T. B. Oriss. 1998. Crossregulation between Th1 and Th2 cells. *Crit. Rev. Immunol. 18:275*.
41. Moore, K. W., R. de Waal-Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol. 19:683*.
42. De Waal Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med. 174:1209*.
43. Fiorentino, D. F., A. Zlotnik, P. Vieira, T. R. Mosmann, M. Howard, K. W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol. 146:3444*.
44. Groux, H., M. Bigler, J. E. de Vries, and M. G. Roncarolo. 1996. Interleukin-10 induces a long-term antigen-specific anergic state in human $CD4^+$ T cells. *J. Exp. Med. 184:19*.
45. Mocarski, E. S. 2002. Immunomodulation by cytomegaloviruses: manipulative strategies beyond evasion. *Trends Immunol. 10:332*.
46. Tomazin, R., J. Boname, N. R. Hedgde, D. M. Lewinsohn, Y. Altschuler, T. R. Jones, P. Cresswell, J. A. Nelson, S. R. Riddell, and D. C. Johnson. 1999. Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by $CD4^+$ T cells. *Nat. Med. 5:1039*.
47. Hegde, N. R., R. A. Tomazin, T. W. Wisner, C. Dunn, J. M. Boname, D. M. Lewinsohn, and D. C. Johnson. 2002. Inhibition of HLA-DR assembly, transport, and loading by human cytomegalovirus glycoprotein US3: a novel mechanism for evading major histocompatibility complex class II antigen presentation. *J. Virol. 76:10929*.
48. Raftery, M. J., M. Schwab, S. M. Eibert, Y. Samstag, H. Walczak, and G. Schonrich. 2001. Targeting the function of mature dendritic cells by human cytomegalovirus: a multilayered defense strategy. *Immunity 15:997*.
49. Moutaftsi, M., A. M. Mehl, L. K. Borysiewicz, and Z. Tabi. 2002. Human cytomegalovirus inhibits maturation and impairs function of monocyte-derived dendritic cells. *Blood 99:2913*.
50. Moser, M., and K. M. Murphy. 2000. Dendritic cell regulation of TH1-TH2 development. *Nat. Immun. 1:199*.
51. Miller, S. M., C. M. Cebulla, and D. D. Sedmak. 2002. Human cytomegalovirus inhibition of major histocompatibility complex transcription and interferon signal transduction. *Curr. Top. Microbiol. Immunol. 269:153*.