MAJOR ARTICLE

Functional Exhaustion Limits CD4$^+$ and CD8$^+$ T-Cell Responses to Congenital Cytomegalovirus Infection

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Background. Cytomegalovirus (CMV) infection during fetal life causes severe symptoms and is associated with prolonged viral excretion. Previous studies reported low CD4$^+$ T-cell responses to CMV infection in early life, contrasting with large responses of effector CD8$^+$ T cells. The mechanisms underlying the defective CD4$^+$ T-cell responses and the possible dissociation with CD8$^+$ T-cell responses have not been clarified.

Methods. The magnitude and the quality of the fetal CD8$^+$ and CD4$^+$ T-cell responses to CMV infection were compared to those of adults with primary or chronic infection.

Results. In utero CMV infection induced oligoclonal expansions of fetal CD4$^+$ and CD8$^+$ T lymphocytes expressing a T-helper type 1 or Tc1 effector phenotype similar to that of adult CMV-specific cells. However, the effector cytokine responses and the polyfunctionality of newborn CD4$^+$ and CD8$^+$ T cells were markedly lower than those of adult cells. This reduced functionality was associated with a higher expression of the programmed death 1 inhibitory receptor, and blockade of this receptor increased newborn T-cell responses.

Conclusions. Functional exhaustion limits effector CD4$^+$ and CD8$^+$ T-lymphocyte responses to CMV during fetal life.

Keywords. cytomegalovirus; congenital infection; fetus; CD4 T cell; CD8 T cell; exhaustion.

Cytomegalovirus (CMV) is a member of the *Betaherpesvirinae* subfamily and establishes lifelong persistence following primary infection. Human CMV is the most common cause of congenital infection, affecting 0.2%–2% of all live births [1]. CMV infection is usually asymptomatic in immunocompetent adults, but about 20% of infected newborns develop symptoms either in utero or during the first years of life [1, 2]. In addition, both symptomatic and asymptomatic children excrete the virus for several years after birth, whereas viral excretion is usually controlled within several months in adults [3, 4]. This reduced control of CMV replication suggests a limitation in cell-mediated immune responses in early human life [5].

In adults, CMV infection induces large oligoclonal expansions of CD4$^+$ and CD8$^+$ T cells that express a late-differentiation phenotype characterized by the loss of expression of the costimulatory molecules CD27 and CD28 and that produce multiple antiviral cytokines [6–10]. Historical studies suggested that congenitally infected newborns have defective T-cell responses to CMV [3, 11, 12]. More-recent studies reported low or undetectable CD4$^+$ T-cell responses to CMV antigens in infants infected in utero or after birth [11, 13, 14]. In contrast, several reports demonstrated large CD8$^+$ T-cell responses to congenital or postnatal CMV infection [15–20]. These responses involved large expansions of effector cells expressing a late-differentiation phenotype, similarly to the adult responses [15]. As a similar dissociation between the presence of detectable CD8$^+$ T-cell responses and very low CD4$^+$ T-cell responses has also been observed in infants infected with

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human immunodeficiency virus (HIV), it was recently proposed that the immune system in early life may have a higher capacity to develop effector $CD8^+$ T-cell responses rather than $CD4^+$ T-cell responses to viral pathogens [21, 22]. However, longitudinal studies of children infected in utero or soon after birth indicate that the frequency of CMV-specific $CD8^+$ T cells producing interferon γ (IFN-γ) increases during the first year of life [18, 20]. As CMV-specific $CD8^+$ T cells were identified in these studies on the basis of their production of cytokines, it is unclear whether the increased frequencies were related to cell multiplication or to an increased capacity of the cells to produce cytokines. Similarly, it is unclear whether the defective $CD4^+$ T-cell responses observed following CMV or HIV infection in early life are related to a defective expansion of virus-specific cells or to a reduced capacity to produce cytokines.

In this study, we demonstrate that congenital CMV infection induces expansions of effector $CD4^+$ and $CD8^+$ fetal T lymphocytes. However, both $CD4^+$ and $CD8^+$ CMV-specific T cells had a reduced capacity to produce cytokines as compared to adult cells and expressed higher levels of the inhibitory receptor programmed death 1 (PD-1). These results provide the first evidence that functional exhaustion can limit T-cell responses to a viral infection in early life.

MATERIALS AND METHODS

Study Design

This study was approved by the ethics committees of Hôpital Erasme and Hôpital Saint-Pierre, Brussels, and Hôpital Tivoli, La Louvière. Pregnant women with primary CMV infection and their newborns were enrolled after mothers provided written informed consent. Diagnosis of primary maternal infection was performed as previously described [23]. No anti-CMV therapy was given to the mothers. Diagnosis of congenital infection was based on the detection of CMV genome by polymerase chain reaction or of CMV virus by viral culture in amniotic fluid or in newborn urine specimens collected during the first week of life. Outcome of pregnancies and clinical information on the fetuses and newborns are presented in Supplementary Table 1. Maternal blood was collected within the first 3 days after delivery. The study included 28 mothers with primary CMV infection, 26 newborns with congenital infection, and 5 uninfected newborns. Diagnosis of primary maternal infection was made at a mean gestational age (±standard deviation [SD]) of 15 ± 8 weeks. In addition, samples from 14 pregnant women with primary CMV infection and 10 infected newborns participating in the ongoing GlaxoSmithKline Biologicals sponsored study (clinical trials registration NCT01251744) were analyzed in agreement with the study protocol and consent form. Diagnosis of primary maternal infection in this group was made at a mean gestational age (±SD) of 22 ± 6 weeks. Twenty-four healthy subjects chronically infected with CMV were recruited as controls by ImmuneHealth, Gosselies. Adult peripheral blood mononuclear cells (PBMCs) and cord blood mononuclear cells (CBMCs) were isolated by gradient centrifugation and were analyzed immediately or, more commonly, after storage in liquid nitrogen. Erythrocytes were depleted from CBMCs with purified anti-human CD235ab antibody (Imtec) and Dynabeads Pan Mouse immunoglobulin G (Invitrogen) according to the instructions of the manufacturer.

T-Cell Repertoire and Phenotype

The analysis of the Vβ repertoire was performed by flow cytometry using the IOTest Beta Mark TCR V Kit (Beckman Coulter) according to the instructions of the manufacturer. Cells were phenotyped with the antibodies listed in Supplementary Table 2.

T-Cell Cytokine Production and Proliferation

PBMCs and CBMCs (2×10^6 cells/mL) were cultured in Roswell Park Memorial Institute 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (PAA) and stimulated for 6 hours with pools of 15–amino acid peptides overlapping by 11 amino acids and covering the total sequence of CMV proteins (1.5 μg/mL per peptide; JPT, Germany). Brefeldin A (BFA; 2 μg/mL; Sigma-Aldrich) was added after 1 hour of stimulation. $CD8^+$ T cells were stained with HLA-A2 or HLA-B7 dextramer-loaded CMV pp65 peptides (NLVPMVATV or TPRVTGGGAM, respectively; Immunodex) for 15 minutes at 37°C before stimulation with the cognate peptides (Eurogentec) for 5 hours in the presence of BFA (2 μg/mL). The analysis of cytokine production by flow cytometry was performed using the antibodies listed in Supplementary Table 2. For T-cell proliferation analyses, CBMCs were cultured in Iscove's modified Dulbecco's medium containing 5% human serum and glutamine (Lonza) and were stimulated with a lysate of CMV-infected fibroblasts (1 μg/mL; Virusys) for 7 days and a PD-1 blocking antibody or an isotype control antibody (Imtec) at 5 μg/mL. Cells were pulsed with BrdU for the last 18 hours of stimulation and were stained with the reagents listed in Supplementary Table 2. The secretion of cytokines (IFN-γ and macrophage inflammatory protein 1β [MIP-1β]) was measured in supernatants obtained on day 7, using commercially available enzyme-linked immunosorbent assays (eBiosciences).

Flow Cytometry Analysis

Interexperiment standardization of mean fluorescence intensities was performed using SPHERO Rainbow Beads (BD Biosciences, Erembodegem, Belgium). Data were obtained on a Cyan ADP LX9 cytometer (DakoCytomation) and analyzed using FlowJo 9.6 software (TreeStar, Ashland, Oregon). For functional analyses, background responses were subtracted using the software Pestle (courtesy of Mario Roederer, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes

of Health [NIH], Bethesda, Maryland) and samples with a viability <70% (Violet Live/Dead kit, Invitrogen, Molecular Probes) were excluded. Detectable responses were defined as production of at least one of the 4 cytokines by ≥0.05% or ≥0.1% of the total $CD4^+$ T-cell or $CD8^+$ T-cell populations, respectively. Polyfunctionality was analyzed with SPICE (version 5.1; courtesy of Mario Roederer and Joshua Nozzi, NIAID, NIH) and FUNKY CELLS Data Miner as previously described [24, 25].

Statistical Analysis

Data are presented as individual values, median values and interquartile ranges, or mean values and standard errors of the mean. Multiple parameter comparisons were performed with 2-way analysis of variance. When significant differences were observed, data were compared using the Mann–Whitney *U* test. Statistical significance was defined at *P* values of <.05. GraphPad Prism 5 was used to perform the analyses.

RESULTS

Congenital CMV Infection Induces Oligoclonal Expansions of Fetal T-Helper Type 1 (Th1) and Tc1 Lymphocytes

The response of fetal $CD4^+$ and $CD8^+$ T lymphocytes to CMV infection was studied by comparing newborns infected in utero (CB^i), uninfected newborns (CB^{ni}), their mothers who had developed primary CMV infection during pregnancy (MB), and healthy adults with chronic CMV infection (chronic). The impact of CMV infection on the differentiation of fetal $CD4^+$ and $CD8^+$ T lymphocytes was first analyzed by measuring the presence of cells expressing the late-differentiation phenotype ($CD27^-CD28^-$) characteristic of CMV-specific T cells (Figure 1*A*). As expected, $CD4^+$ and $CD8^+$ T lymphocytes from uninfected newborns expressed the CD27 and the CD28 molecules, in agreement with their naive phenotype. In contrast, high frequencies of differentiated $CD27^-CD28^-$ $CD4^+$ T cells were detected in newborns with congenital CMV infection. These frequencies ranged from 16% to <0.05% and were significantly lower than those measured in adults with primary or chronic infection. $CD4^+$ T cells expressing an intermediate differentiation phenotype ($CD27^-CD28^+$) were undetectable in most infected newborns (Figure 1*A*). As previously described, high frequencies of $CD8^+$ T cells expressing a late ($CD27^-CD28^-$) or intermediate ($CD27^+CD28^-$) differentiation phenotype were also detected in congenitally infected newborns, and these frequencies were comparable to those for adults with primary or chronic infection (Figure 1*A*) [15, 19]. In agreement with their late-differentiation phenotype, $CD27^-CD28^-$ $CD4^+$ and $CD27^-CD28^-$ $CD8^+$ T lymphocytes from infected newborns expressed an effector phenotype characterized by decreased expression of CCR7 and IL-7R and increased expression of CD57 as compared to naive T cells (Figure 1*B*). In addition, newborn differentiated $CD4^+$ and $CD8^+$ T cells expressed high levels of the transcription factor T-bet and the chemokine receptor CCR5, indicating Th1 and Tc1 phenotypes, respectively (Figure 1*B*). Th1 $CD4^+$ and Tc1 $CD8^+$ cells are T-cell subsets producing antiviral cytokines and specialized in the control of intracellular pathogens. The phenotype of the differentiated newborn T cells was identical to that observed in adults with primary CMV infection (Figure 1*B*). The impact of congenital CMV infection on the repertoire of fetal T cells was assessed by measuring the frequencies of T-cell receptor (TCR) Vβ families within the $CD4^+$ and $CD8^+$ T-cell subsets (Figure 2). As expected, similar frequencies of the different Vβ families were detected in naive T-cell populations. In contrast, expansions at specific Vβ families were detected within differentiated $CD4^+$ and $CD8^+$ T-cell lymphocytes and involved different Vβ families in $CD4^+$ and $CD8^+$ T-cell subsets. Together, these results indicate that congenital CMV infection induces oligoclonal expansions of fetal Th1 and Tc1 effector T lymphocytes. Oligoclonal expansions are referred to as expansions of a limited set of T-cell clones, suggesting an antigen-specific response, rather than a nonspecific polyclonal T-cell activation.

Fetal $CD4^+$ and $CD8^+$ T Lymphocytes Have Low and Paucifunctional Cytokine Responses to CMV Antigens

The functional response of fetal $CD4^+$ and $CD8^+$ T cells was measured by intracellular staining of cytokines (IFN-γ, MIP-1β, tumor necrosis factor α [TNF-α], and interleukin 2 [IL-2]) following stimulation with a panel of pools of overlapping peptides derived from 10 immunodominant and subdominant CMV proteins [26]. The proportion of antigens eliciting a detectable response to individual antigens was calculated for each subject (Figure 3*A*). Most newborns had no detectable $CD4^+$ T-cell response to CMV antigens (Figure 3*A*). Importantly, very low or undetectable responses were also observed in newborns with the highest frequencies of differentiated $CD27^-CD28^-$ $CD4^+$ T cells and cytokine-producing cells consistently expressed a late-differentiation phenotype characterized by the downregulation of CD28 (Figure 3*B*). In contrast, high frequencies of $CD4^+$ T cells responding to at least 1 CMV antigen were detected in adults with primary or chronic CMV infection (Figure 3*A*). Of note, $CD4^+$ T cells from adults with primary infection responded to fewer antigens and had lower frequencies of cells responding to pp65, IE1, and UL82 antigens than adults with chronic infection (Figure 3*A*). Newborn $CD8^+$ T cells also responded to fewer antigens than adult cells, and the frequencies of cells responding to IE1 or UL55 were lower than in adults with primary CMV infection (Figure 3*A*). On the other hand, lower frequencies of $CD8^+$ T cells responding to UL32 or UL55 antigens were detected during primary infection as compared to chronic infection (Figure 3*A*). These results indicate that the breadth of CMV antigens inducing detectable $CD4^+$ and $CD8^+$ T-cell responses was lower in CMV-infected newborns than in adults and that

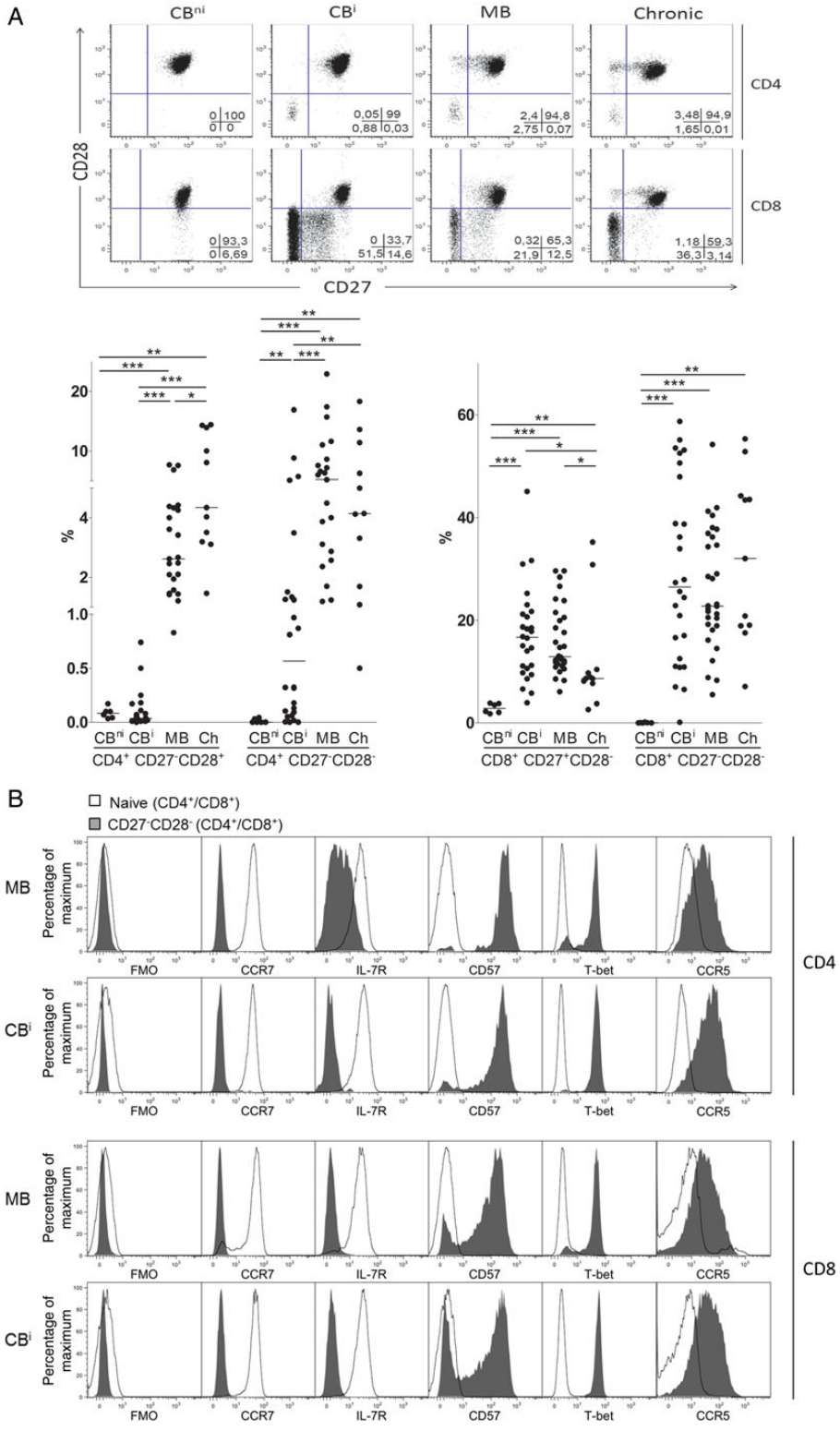

Figure 1. Congenital cytomegalovirus (CMV) infection induces the differentiation of fetal T-helper type 1 and Tc1 cells. Cord blood mononuclear cells and peripheral blood mononuclear cells were obtained from CMV-uninfected newborns (CB^{ni}) and CMV-infected newborns (CB^{i}), from their mothers with primary CMV infection (MB), and from chronically infected adults (chronic). *A*, The percentage of $CD4^+$ or $CD8^+$ T cells expressing an intermediate ($CD27^-CD28^+$ or $CD27^+CD28^-$, respectively) or late ($CD27^-CD28^-$) differentiation phenotype within the total $CD4^+$ or $CD8^+$ T-lymphocyte populations was measured by flow cytometry. A representative example is shown for each study group. Data are median values for 6 CB^{ni}, 26 CB^{i}, 23 MB, and 11 chronic subjects. *B*, The expression of CCR7, interleukin 7 receptor (IL-7R), CD57, T-bet, and CCR5 by differentiated ($CD27^-CD28^-$; gray histograms) and naive ($CD45RO^-CD27^+/CD28^+$; white histograms) $CD4^+$ and $CD8^+$ T cells from CB^{i} and MB was measured by flow cytometry. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$. Abbreviation: FMO, fluorescence minus one.

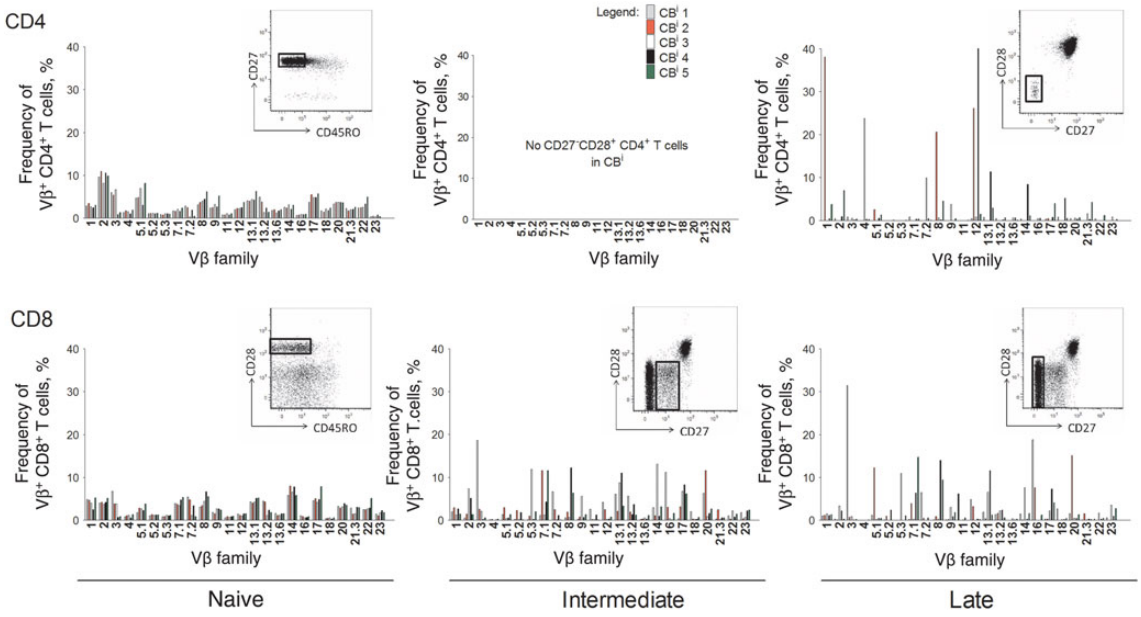

Figure 2. Congenital cytomegalovirus (CMV) infection induces the oligoclonal expansions of differentiated fetal $CD4^+$ and $CD8^+$ T lymphocytes. The T-cell receptor Vβ repertoire was studied by flow cytometry in naive ($CD45RO^-CD27^+/CD28^+$) and differentiated $CD4^+$ ($CD27^-CD28^-$) and $CD8^+$ ($CD27^+CD28^-$ and $CD27^-CD28^-$) T lymphocytes from 5 CMV-infected newborns (CB^i) (1 color per newborn).

similar differences, but of lower magnitude, were observed between adults with primary or chronic infection.

To gain insight into the functionality of fetal CMV-specific $CD4^+$ and $CD8^+$ T cells, their capacity to produce multiple cytokines (polyfunctionality) was analyzed among the detectable responses (see "Materials and Methods" section). Newborn CMV-specific $CD4^+$ and $CD8^+$ T cells included a lower proportion of cells producing multiple cytokines as compared to adults with primary or chronic infection (Figure 4*A*). Most newborn cells produced only 1 or 2 cytokines, whereas most adult cells produced 2 or 3 cytokines. MIP-1β was the most commonly produced cytokine, whereas IL-2 was the least commonly produced cytokine. Similar results were obtained with total CMV-specific $CD8^+$ T cells (Figure 4*A*). These results were further confirmed by the analysis of CMV dextramer$^+$ $CD8^+$ T cells. As shown in Figure 4*B*, newborn CMV dextramer$^+$ $CD8^+$ T cells included lower frequencies of cytokine-producing cells and were less polyfunctional than adult cells. Of note, CMV dextramer$^+$ $CD8^+$ T cells were mostly $CD28^-$ in newborns and adults, further supporting the notion that during primary infection, CMV-specific T cells express a late-differentiated phenotype in fetuses and in adults [27]. Cell polyfunctionality was then quantified using a previously described index [25]. Newborn CMV-specific $CD4^+$ and $CD8^+$ T cells had a lower polyfunctionality index than cells from adults with primary or chronic CMV infection (Figure 4*A* and 4*B*). The polyfunctionality index of total CMV-specific $CD8^+$ T cells was also lower in adults with primary infection as compared to those with chronic infection. Together, these results indicate that the magnitude and the polyfunctionality of newborn $CD4^+$ and $CD8^+$ T-cell responses to CMV are lower than in adults.

$CD4^+$ and $CD8^+$ T Lymphocytes Induced by Congenital CMV Infection Express High Levels of PD-1

The reduced polyfunctionality of newborn $CD4^+$ and $CD8^+$ T cells is analogous to the functional profile of exhausted T lymphocytes observed during persistent viral infections. Exhausted T lymphocytes have a reduced capacity to produce cytokines and express high levels of inhibitory receptors, of which PD-1 is the most characteristic [28]. Compared with naive T cells, differentiated $CD4^+$ and $CD8^+$ T cells and CMV dextramer$^+$ $CD8^+$ T cells expressed higher levels of PD-1 in the 3 study groups. Differentiated newborn $CD4^+$ and $CD8^+$ T cells expressed higher levels of PD-1 than adult T cells (Figure 5*A*–*C*). As shown in Figure 5*D* and 5*E*, CMV antigens induced only low or undetectable proliferative responses or cytokine secretion in T cells from CMV-infected newborns. Blocking PD-1 upregulated these responses, indicating that the inhibitory receptor was functional and controlled newborn T-cell responses to CMV antigens.

DISCUSSION

This study demonstrates that congenital CMV infection induces the differentiation and functional exhaustion of fetal effector Th1 and Tc1 cells. High frequencies of both $CD4^+$ and $CD8^+$

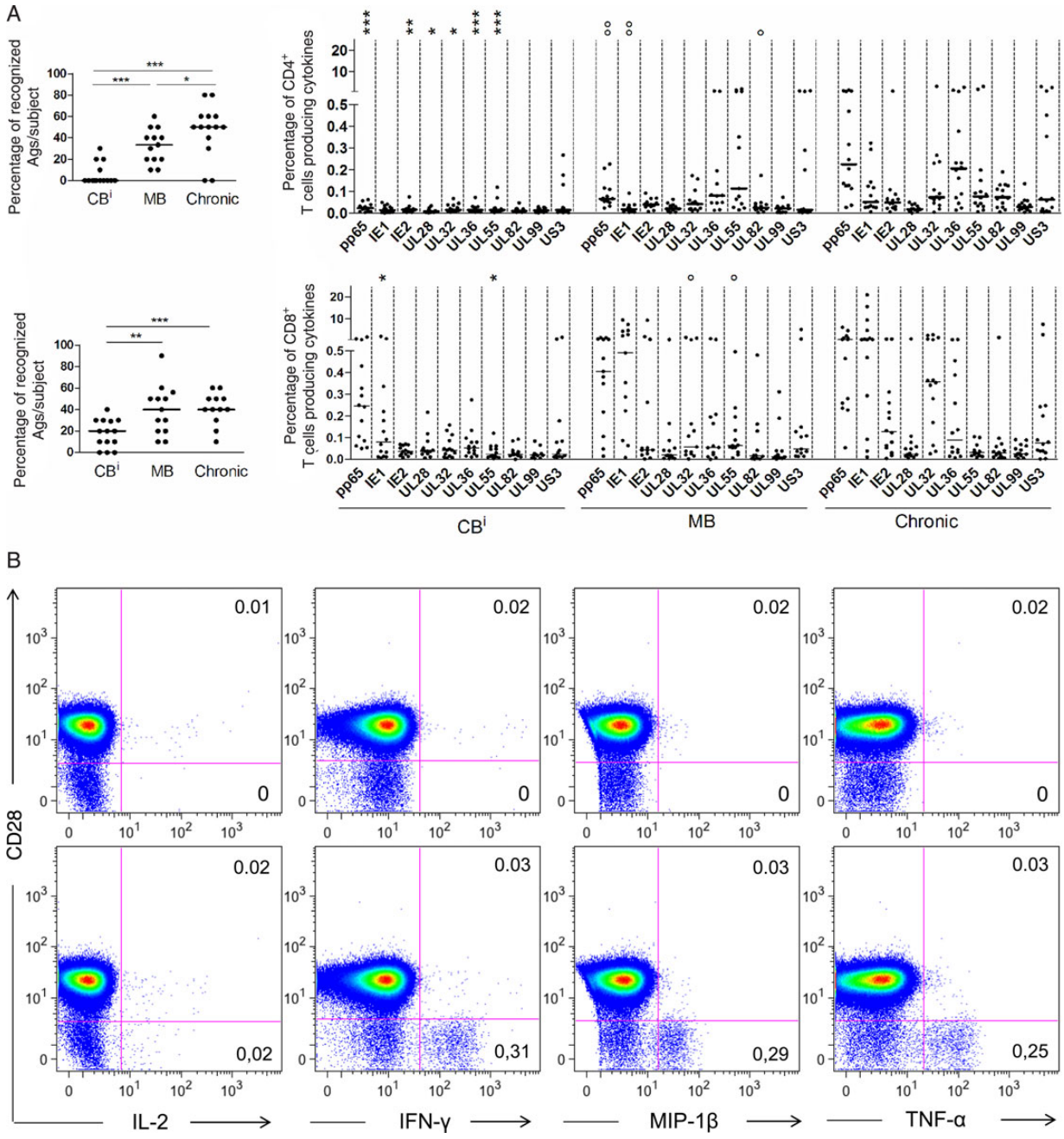

Figure 3. Newborn CD4+ and CD8+ T lymphocytes have low cytokine responses to cytomegalovirus (CMV) antigens (Ags). Cord blood mononuclear cells and peripheral blood mononuclear cells were obtained from CMV-infected newborns (CB^i; n = 13–14), from their mothers with primary CMV infection (MB; n = 12–13), and from chronically infected adults (chronic; n = 14) and were stimulated with a panel of peptide pools from immunodominant and subdominant CMV Ags. *A*, The percentage of CD4+ and CD8+ T lymphocytes producing any cytokine (interferon γ [IFN-γ], macrophage inflammatory protein 1β [MIP-1β], tumor necrosis factor α [TNF-α], and interleukin 2 [IL-2]) was measured by intracytoplasmic staining and flow cytometry. The proportion of Ags recognized by CD4+ and CD8+ T cells from each subject in the 3 study groups is shown in the left panel. Bars denote median values. Individual responses of CD4+ and CD8+ T cells producing any cytokine to each CMV Ags are shown in the right panel. Significant differences between CB^i and MB (*) and between MB and chronic subjects (°) are shown. $^{*,\circ}P < .05$, $^{**,\circ\circ}P < .01$, and $^{***,\circ\circ\circ}P < .001$. Bars denote median values. *B*, Representative example of flow cytometry dot plots showing cytokine staining within unstimulated (upper panel) and CMV antigen (US3)–stimulated (lower panel) newborn CD4+ T cells. Numbers in quadrants indicate percentages of CD4+ T cells.

T cells expressing a late-differentiation effector phenotype were detected in infected newborns. Differentiated newborn T cells expressed high levels of the Th1/Tc1 transcription factor T-bet and the chemokine receptor CCR5. This phenotype is identical to the one expressed by adult CMV-specific T cells during primary or chronic infection [29–31]. Differentiated

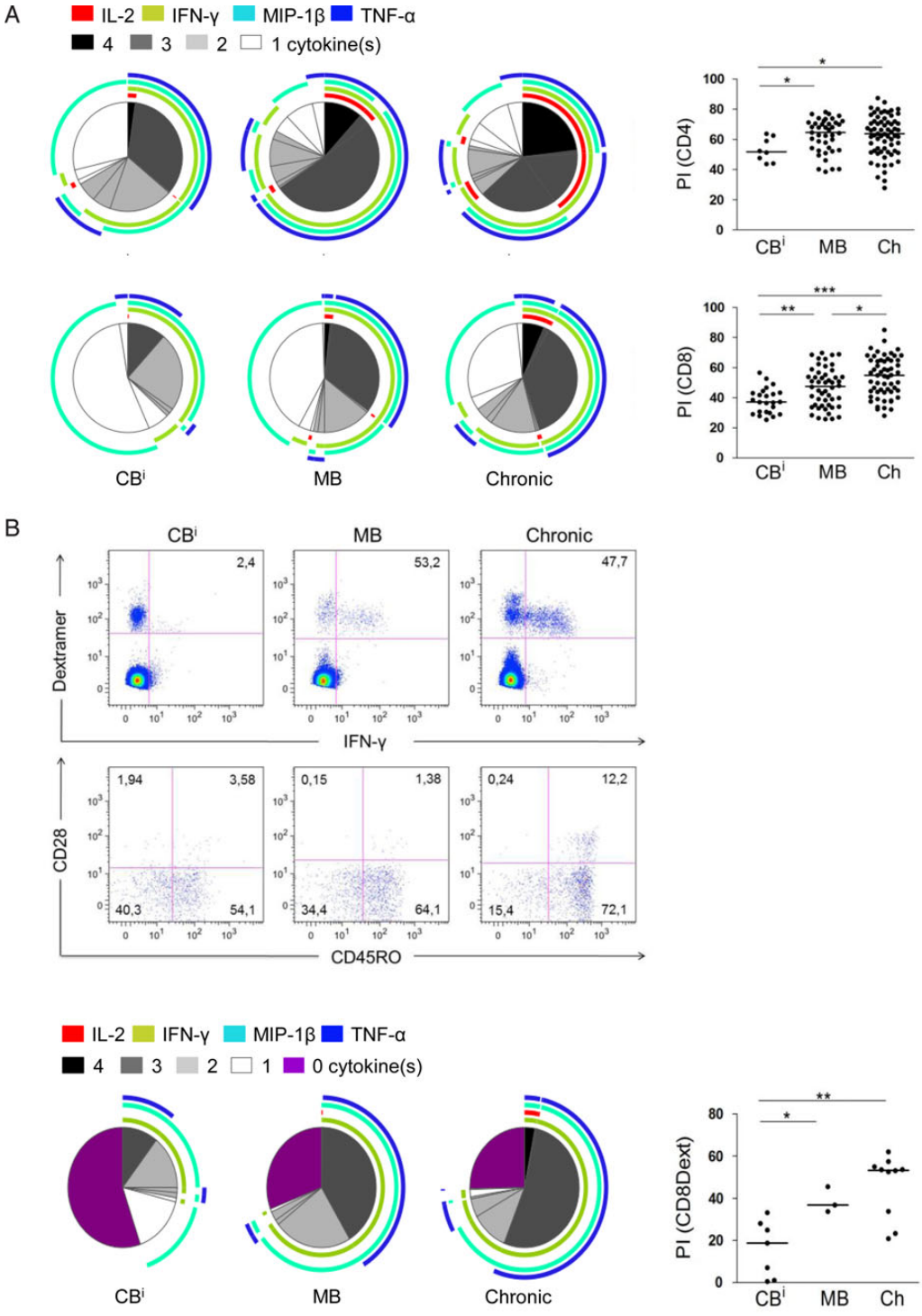

Figure 4. Newborn CD4$^+$ and CD8$^+$ T lymphocytes have paucifunctional cytokine responses to cytomegalovirus (CMV) antigens. *A*, The polyfunctionality of CMV-specific CD4$^+$ and CD8$^+$ T cells producing at least one of the 4 cytokines (interferon γ [IFN-γ], macrophage inflammatory protein 1β [MIP-1β], tumor necrosis factor α [TNF-α], and interleukin 2 [IL-2]) in response to any pool of peptides in CMV-infected newborns (CB^i), their mothers (MB), and healthy adults with chronic CMV infection (chronic) was analyzed using SPICE software (left panel). The median proportion of CD4$^+$ and CD8$^+$ T cells producing 1, 2, 3, or 4 cytokines is shown for each study group. Nonresponders were excluded from the analysis, as defined in "Materials and Methods". The polyfunctionality index (PI) of the cells was calculated using FUNKY CELLS software (right panel). *B*, The same analysis was performed on CMV dextramer$^+$ CD8$^+$ T cells, except that the total population of dextramer$^+$ cells, including cells producing no detectable cytokine (0 cytokine), was included in the analysis. Bars denote median values. **P* < .05, ***P* < .01, and ****P* < .001. The upper part of Figure 4B presents a representative example of flow cytometry dot plots showing the production of IFN-γ by and the phenotype (expression of the differentiation markers CD28 and CD45RO) of tetramer$^+$ CD8$^+$ T cells. Numbers in quadrants indicate percentages of tetramer$^+$ CD8$^+$ T cells.

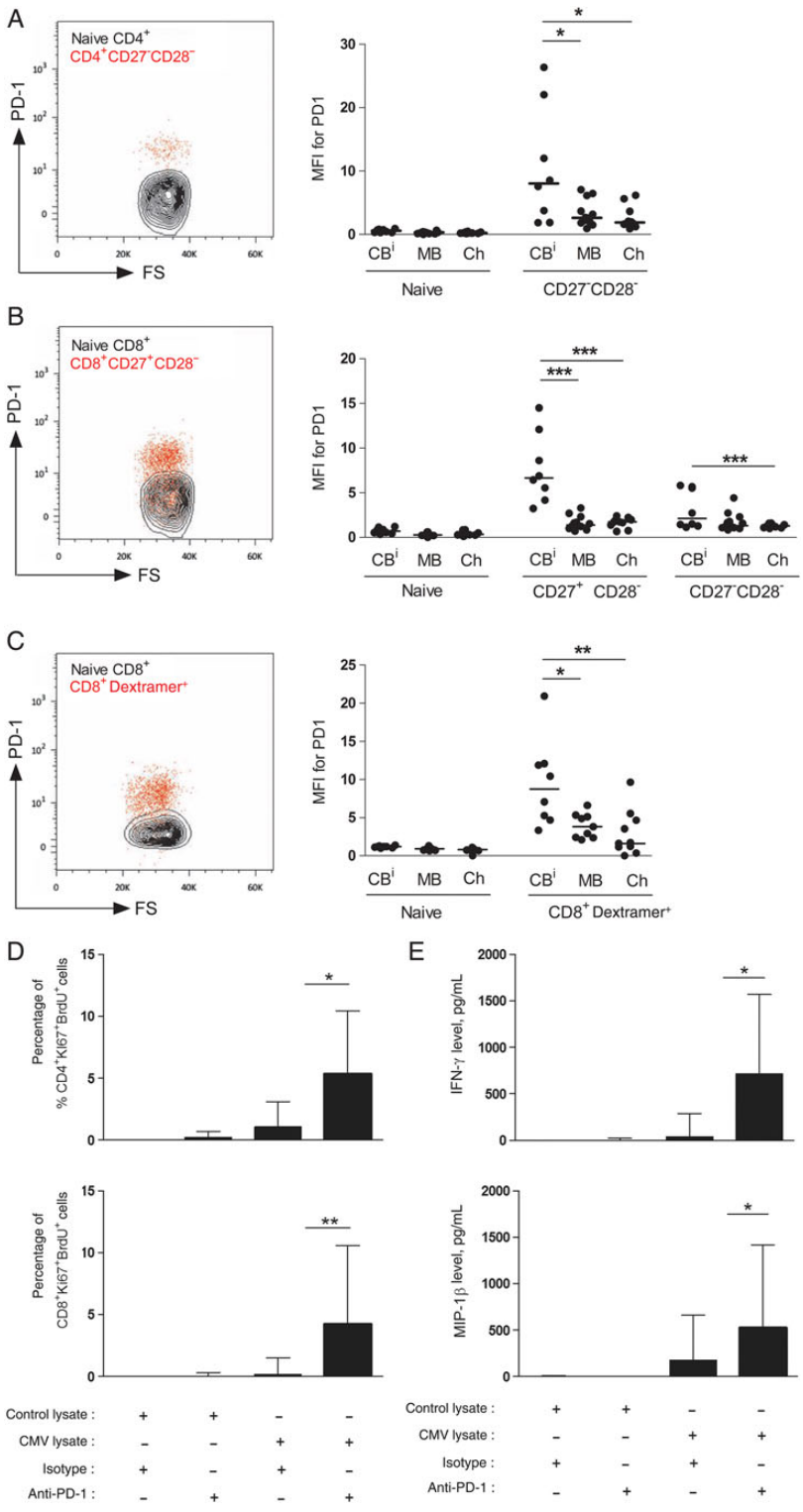

Figure 5. CD4$^+$ and CD8$^+$ T lymphocytes from cytomegalovirus (CMV)–infected newborns express high levels of programmed death 1 (PD-1). *A–C*, The expression of PD-1 by naive and differentiated CD4$^+$ T cells (*A*), CD8$^+$ T cells (*B*), and CMV dextramer$^+$ CD8$^+$ T cells (*C*) from infected newborns (CB^i; n = 8) and of adults with primary infection (MB; n = 12) or chronic infection (Ch; n = 10–11) was measured by flow cytometry. Representative dot plots of cells from CMV-infected newborns are shown in the left panels. Shown are individual data and median values. *D* and *E*, The proliferative responses of CD4$^+$ and CD8$^+$ T cells (*D*) and the secretion of cytokines (*E*) were measured in CMV-infected newborns after 7 days of stimulation with CMV Ags. Proliferation was measured using the BrdU incorporation assay, and cytokine secretion was measured by enzyme-linked immunosorbent assay. Data are median values ± interquartile ranges for 10 subjects. *$P < .05$, **$P < .01$, and ***$P < .001$. Abbreviations: FS, forward scatter; IFN-γ, interferon γ; MFI, mean fluorescence intensity; MIP-1β, macrophage inflammatory protein 1β.

newborn $CD4^+$ and $CD8^+$ T-cell populations were enriched in expansions involving specific TCR Vβ families, supporting the notion that the responses are antigen specific and not the result of a bystander activation. This notion was further supported by the observation that most newborn $CD4^+$ T cells producing cytokines in response to CMV antigens and that CMV-tetramer$^+$ newborn $CD8^+$ T cells expressed a late-differentiation phenotype.

Previous studies reported low or undetectable responses of $CD4^+$ T cells to congenital CMV infection, and it has been proposed that the fetal immune system may be particularly unable to develop antiviral Th1 responses [22]. Supporting this possibility, several factors have been described that could limit the differentiation of Th1 cells in early life, including a reduced production of IL-12 by dendritic cells and a hypermethylation of the promoter in the gene encoding IFN-γ [32–35]. Our observations indicate that these factors do not prevent the differentiation of Th1 cells following CMV infection. However, cytokine responses to CMV antigens were markedly lower in newborns than in adults with primary CMV infection. Importantly, this difference affected both $CD4^+$ and $CD8^+$ T cells. Using the largest panel of viral antigens tested so far in primary CMV infection, we observed that the breadth of CMV antigens stimulating T-cell responses and the frequencies of T cells recognizing individual antigens were lower in newborns than in adults. These results are in keeping with those reported by Gibson et al and indicating that the number of CMV pp65 and IE1 epitopes stimulating $CD8^+$ T-cell responses increased with time following congenital CMV infection [18]. In our study, the differences in the breadth and magnitude of the responses were observed despite the fact that similar frequencies of differentiated $CD8^+$ T cells were detected in infected newborns and adults. In contrast, newborns had lower frequencies of differentiated ($CD27^-CD28^-$) $CD4^+$ T cells than adults. This difference may be related to a more limited cell expansion and/or a reduced cell survival of CMV-specific $CD4^+$ T cells in vivo and could contribute to the lower breadth and magnitude of the newborn $CD4^+$ T-cell responses.

The lower magnitude and breadth of newborn $CD4^+$ and $CD8^+$ T-cell responses to CMV antigens were associated with a markedly lower polyfunctionality as compared to adult cells. This paucifunctionality may have led to an underestimation of the frequencies of CMV-specific T cells in the experiments where the cells were identified through their production of cytokines. The detection of CMV dextramer$^+$ $CD8^+$ T cells with a polyfunctionality index close or equal to 0 in several infected newborns supports this possibility. On the other hand, we cannot exclude the possibility that fetal T cells responded to CMV proteins that were not included in our analysis.

The paucifunctionality of newborn $CD4^+$ and $CD8^+$ T cells was associated with the expression of high levels of the inhibitory receptor PD-1, and blockade of this receptor increased T-cell responses to CMV antigens. Polymorphism in the gene encoding PD-1 was recently associated with CMV infection in kidney transplanted patients [36]. To our knowledge, this is the first observation of increased PD-1 expression during a fetal immune response. Reduced polyfunctionality and high expression of PD-1 are central characteristics of functionally exhausted T cells [37, 38]. T-cell exhaustion is induced by chronic viral infections involving a prolonged exposure to high antigen loads. It is characterized by the hierarchical loss of T-cell functions, with lower antigen loads inducing the loss of IL-2 production and higher antigen loads progressively inducing the loss of TNF-α, IFN-γ, and finally MIP-1β production [28]. This hierarchy of cytokine production is the same as the one observed in CMV-infected newborns, further supporting the notion that fetal $CD4^+$ and $CD8^+$ effector T cells are functionally exhausted. PD-1 blockade increased the proliferative and cytokine responses of newborn cells after 7 days of antigen stimulation but did not significantly influence cytokine responses after short-term stimulation (data not shown). Similar differences in the impact of PD-1 blockade on short-term and long-term in vitro stimulation were observed in adults with chronic viral infections and indicate that factors other than PD-1 contribute to the functional exhaustion of newborn cells [37, 39]. Adult T cells also had reduced polyfunctionality during primary as compared to chronic CMV infection. Together, these data indicate that primary CMV infection induces the functional exhaustion of both fetal and adult T cells and that the level of exhaustion is more pronounced in the fetus. As the level of T-cell exhaustion has been linked to the strength of T-cell stimulation [28], this difference may be related to the exposure of fetal T cells to higher CMV antigen loads than adult cells. Supporting this possibility, higher viral loads have been reported in CMV-infected fetuses as compared to their mother with primary infection [40–42]. Such difference may be related to a lower control of CMV replication by the fetal immune system but may also involve the ingestion of high CMV loads excreted in the amniotic fluid [41, 42]. Alternatively, the quality of the signals provided by fetal antigen-presenting cells may favor the emergence of functionally regulated T cells in utero, as observed in animal models of adaptive tolerance [43]. Finally, fetal T cells are programmed differently than adult cells and may therefore be intrinsically more susceptible to functional exhaustion [44].

The functional exhaustion of newborn T cells is likely to reduce their capacity to control viral replication and may therefore play an important role in the prolonged viral excretion associated with CMV infection in early life. Indeed, CMV-specific $CD4^+$ T-cell responses increase during the first 2 years of life, and the cessation of viruria correlates with the acquisition of proliferative responses to CMV antigens [3, 45]. A similar association between functional exhaustion and intense viral excretion was recently observed following primary CMV infection of juvenile rhesus macaques [46]. Studies

suggest that functional exhaustion may also limit T-lymphocyte responses to other viral pathogens that are poorly controlled in early life, including HIV [21, 22, 47–49]. The identification of functional exhaustion as a mechanism limiting effector T-cell responses in early life has important implications for our understanding of the pathogenesis of CMV infection, as well as other chronic viral infections affecting the fetus and the young infant.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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