

Human cytomegalovirus elicits fetal $\gamma\delta$ T cell responses in utero

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The fetus and infant are highly susceptible to viral infections. Several viruses, including human cytomegalovirus (CMV), cause more severe disease in early life compared with later life. It is generally accepted that this is a result of the immaturity of the immune system. $\gamma\delta$ T cells are unconventional T cells that can react rapidly upon activation and show major histocompatibility complex-unrestricted activity. We show that upon CMV infection in utero, fetal $\gamma\delta$ T cells expand and become differentiated. The expansion was restricted to V γ 9-negative $\gamma\delta$ T cells, irrespective of their V δ chain expression. Differentiated $\gamma\delta$ T cells expressed high levels of IFN- γ , transcription factors T-bet and eomes, natural killer receptors, and cytotoxic mediators. CMV infection induced a striking enrichment of a public V γ 8V δ 1-TCR, containing the germline-encoded complementary-determining-region-3 (CDR3) δ 1-CALGELGDDKLIF/CDR3 γ 8-CATWDTTGWFKIF. Public V γ 8V δ 1-TCR-expressing cell clones produced IFN- γ upon coinubation with CMV-infected target cells in a TCR/CD3-dependent manner and showed antiviral activity. Differentiated $\gamma\delta$ T cells and public V γ 8V δ 1-TCR were detected as early as after 21 wk of gestation. Our results indicate that functional fetal $\gamma\delta$ T cell responses can be generated during development in utero and suggest that this T cell subset could participate in antiviral defense in early life.

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Abbreviations used: CDR3, complementary-determining-region-3; KIR, killer immunoglobulin receptor; MFI, mean fluorescence intensity; NKR, NK receptor.

The fetus and young infant have a high susceptibility to infections with intracellular pathogens, suggesting that T cell-mediated immune responses are different in early life. A number of viruses, including human CMV, herpes simplex type 2, respiratory syncytial virus, and HIV, cause more severe or rapidly progressive disease in early life as compared with later life (Stagno, 2001; Marchant and Goldman, 2005). It is generally accepted that this increased susceptibility to viral infections is related to the immaturity of the neonatal immune system. This includes intrinsic defects of conventional T cells, especially CD4 $\alpha\beta$ T cells, and impaired DC responses (Lewis and Wilson, 2001; White et al., 2002; Maródi, 2006; Levy, 2007; Lee et al., 2008). CMV is the most common cause of congenital infection, affecting 0.2% of all live births in industrialized countries and up to 3% in developing countries (Stagno, 2001). Although CMV infection causes no detectable symptoms in immunocompetent adults, ~20%

of newborns with congenital infection develop serious symptoms, including cerebral malformations, multiple organ failure, deafness, and mental retardation (Stagno, 2001; Dollard et al., 2007).

$\gamma\delta$ T cells are T cells expressing γ and δ chains as a TCR on their cell surface instead of α and β chains as in conventional CD4 and CD8 $\alpha\beta$ T cells. Together with $\alpha\beta$ T cells, they have been conserved for >450 million years of evolution (Hayday, 2000). $\gamma\delta$ T cells are the prototype of unconventional T cells; they can react rapidly upon activation and show MHC-unrestricted activity (Hayday, 2000; Holtmeier and Kabelitz, 2005). Thus, they are not influenced by MHC down-regulation strategies used by viruses such as CMV to escape conventional T cells (Wilkinson et al., 2008).

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Studies in several species have shown an important role for $\gamma\delta$ T cells in protection against infection, in tumor surveillance, in immunoregulation, and in tissue repair (Hayday, 2000; Wang et al., 2001; Holtmeier and Kabelitz, 2005; Pennington et al., 2005; Toulon et al., 2009). In general, they show a rapid and robust response before the development of the adaptive immunity mediated by conventional T cells. In comparison with $\alpha\beta$ T cells, $\gamma\delta$ T cells are not abundant in the peripheral blood but are highly enriched in tissues like the gut epithelium (Hayday, 2000; Holtmeier and Kabelitz, 2005). The majority of $\gamma\delta$ T cells in human adult peripheral blood use the TCR V region pair V γ 9V δ 2 (note that according to an alternative nomenclature the V γ 9 chain is also termed V γ 2 [Holtmeier and Kabelitz, 2005]). This subset has been shown to react specifically toward nonpeptide low molecular weight phosphorylated metabolites (so-called phosphoantigens) and has been the subject of several clinical trials (Wilhelm et al., 2003; Dieli et al., 2007; Kabelitz et al., 2007).

Probably in all species, $\gamma\delta$ T cells are the first T cells to develop (Hayday, 2000). In contrast to adult peripheral blood $\gamma\delta$ T cells, human neonatal cord blood $\gamma\delta$ T cells express diverse V γ and V δ chains paired in a variety of combinations (Morita et al., 1994). Thus the adult-like V γ 9V δ 2 subpopulation only represents a small fraction of the neonatal $\gamma\delta$ T cells (Parker et al., 1990; Morita et al., 1994; Cairo et al., 2008). Further illustrating the differences between adult and neonatal $\gamma\delta$ T cells, is the demonstration that in vitro exposure toward the same pathogen (*Escherichia coli* or *Pseudomonas aeruginosa*) results in expansion of V δ 2⁺ $\gamma\delta$ T cells in adult peripheral blood but of V δ 1⁺ $\gamma\delta$ T cells in cord blood (Kersten et al., 1996). In mice, $\gamma\delta$ T cells are important for the protection against an intestinal parasite infection in early life but not in adult life (Ramsburg et al., 2003), and during human T cell ontogeny $\gamma\delta$ T cells mature before $\alpha\beta$ T cells (De Rosa et al., 2004). However, so far it is not known whether pathogens in early life can activate human $\gamma\delta$ T cells. To gain insight into the ability of $\gamma\delta$ T cells to mount responses to viruses during fetal life, we studied the changes occurring in the $\gamma\delta$ T cell compartment during congenital CMV infection.

RESULTS

CMV infection in utero induces expansion of fetal $\gamma\delta$ T cells in newborns

To address whether human fetal $\gamma\delta$ T cells are responsive to CMV infection in utero, we first compared the percentage of $\gamma\delta$ T cells among all T cells in cord blood samples derived from 19 CMV-infected newborns versus 22 control CMV-uninfected newborns. In CMV-infected newborns, the percentage of $\gamma\delta$ T cells was significantly higher than in CMV-uninfected newborns (Fig. 1 A). To exclude the possibility that this higher percentage of $\gamma\delta$ T cells was the result of a decreased number of $\alpha\beta$ T cells, we determined the absolute number of $\gamma\delta$ T cells per microliter of blood. Indeed, significantly more $\gamma\delta$ T cells were present per microliter of cord blood in CMV-infected newborns in comparison with controls (Fig. 1 B). The higher number of $\gamma\delta$ T cells

correlated with a higher percentage of $\gamma\delta$ T cells expressing the proliferation marker Ki-67 in CMV-infected newborns (Fig. 1 C).

The expansion of $\gamma\delta$ T cells in CMV-infected newborns is restricted to V γ 9⁻ cells, irrespective of the usage of the V δ chain

To further define specific subsets of $\gamma\delta$ T cells in cord blood of CMV-infected newborns, flow cytometry analysis was performed with antibodies specific against V γ 9, V δ 1, V δ 2, and V δ 3. In combination with the pan- $\gamma\delta$ TCR antibody, the V γ 9 antibody can make distinction between V γ 9⁺ and V γ 9⁻

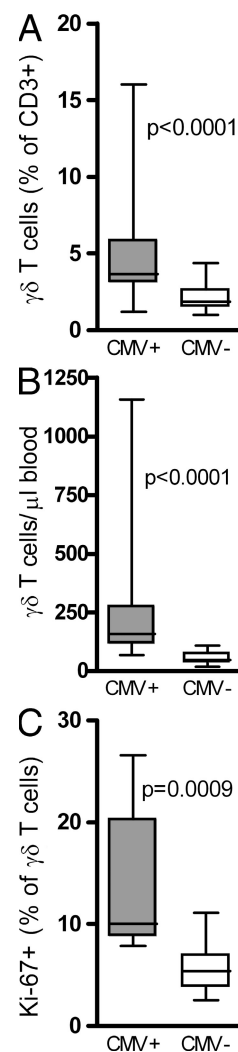


Figure 1. CMV infection in utero induces an expansion of $\gamma\delta$ T cells in newborns. (A) Percentage of $\gamma\delta$ T cells of total T cells (CMV⁺, $n = 19$; CMV⁻, $n = 22$). (B) Absolute number of $\gamma\delta$ T cells per microliter of cord blood (CMV⁺, $n = 13$; CMV⁻, $n = 15$). (C) Percentage of $\gamma\delta$ T cells which are Ki-67⁺ (CMV⁺, $n = 9$; CMV⁻, $n = 15$) in CMV-infected (gray boxes) and CMV-uninfected (white boxes) newborns. In box-and-whisker graphs, the line at the middle is the median, the box extends from the 25th to 75th percentile, and the error bars, or whiskers, extend down to the lowest value and up to the highest.

$\gamma\delta$ T cells (Fig. 2 A). $V\gamma 9$ is the only member of the $V\gamma$ II family; thus the $V\gamma 9^-$ cells express $V\gamma$ chains of the $V\gamma$ I family (Hayday, 2000). The combination of $V\delta 1$, $V\delta 2$, and $V\delta 3$ antibodies stained the vast majority ($\sim 90\%$; unpublished data) of the cord blood $\gamma\delta$ T cells. This approach allows us to identify six $\gamma\delta$ T cell subpopulations in cord blood: $V\gamma 9^+V\delta 1^+$, $V\gamma 9^-V\delta 1^+$, $V\gamma 9^+V\delta 2^+$, $V\gamma 9^-V\delta 2^+$, $V\gamma 9^+V\delta 3^+$, and $V\gamma 9^-V\delta 3^+$. We detected higher percentages of $\gamma\delta$ T

cells negative for $V\gamma 9$, including $V\gamma 9^-V\delta 1^+$, $V\gamma 9^-V\delta 2^+$, and $V\gamma 9^-V\delta 3^+$ $\gamma\delta$ T cells in CMV-infected newborns compared with uninfected newborns (Fig. 2, A and B). On a selected number of CMV-uninfected and CMV-infected newborns, we performed a more detailed analysis of the γ chain usage. In CMV-uninfected newborns, there was a slight preference for $V\gamma 4$ and $V\gamma 9$, whereas upon CMV infection the $V\gamma$ I family members $V\gamma 4$ and $V\gamma 8$ were highly expanded (Fig. S1).

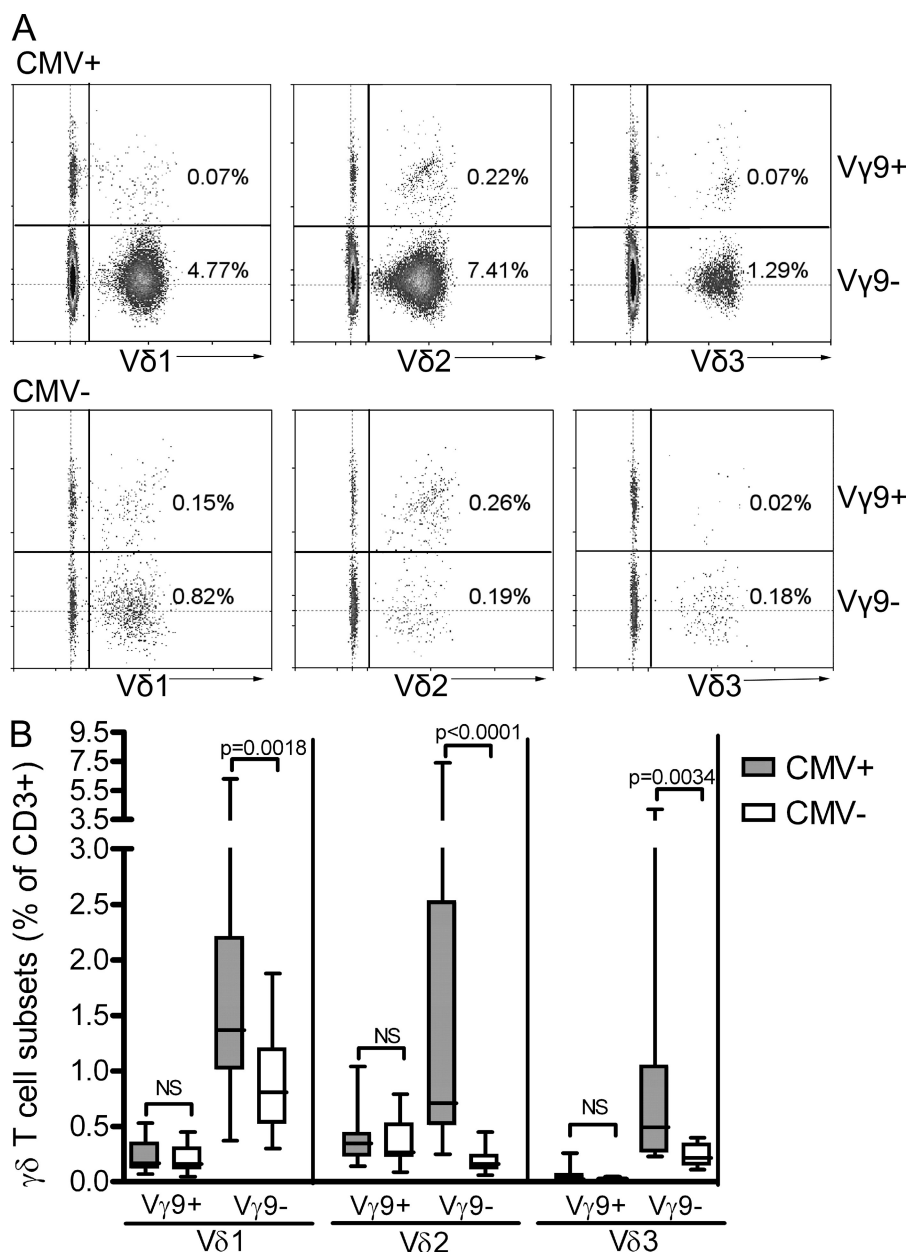


Figure 2. The expansion of $\gamma\delta$ T cells in CMV-infected newborns is restricted to $V\gamma 9^-$ $\gamma\delta$ T cells, irrespective of the usage of the $V\delta$ chain. (A) Expression of $V\gamma 9$ versus $V\delta 1$, $V\delta 2$, or $V\delta 3$ by $\gamma\delta$ T cells from CMV-infected newborn Pos13 (top) and CMV-uninfected newborn Neg4 (bottom). Numbers in dot plots are the percentages of total T cells. (B) Box-and-whisker graph (defined as in Fig. 1) of the percentages of $V\gamma 9^+V\delta 1^+$, $V\gamma 9^+V\delta 2^+$, $V\gamma 9^+V\delta 3^+$, $V\gamma 9^-V\delta 1^+$, $V\gamma 9^-V\delta 2^+$, and $V\gamma 9^-V\delta 3^+$ $\gamma\delta$ T cell subpopulations of $\gamma\delta$ T cells expressed as a percentage of total T cells. CMV+, $n = 10-18$; CMV-, $n = 14-22$.

Table I. Percentage of activation (HLA-DR) and differentiation (CD27, CD28) markers, NKR (CD94, NKG2A, NKG2C, CD158, NKG2D, and KLRG1), cytotoxic mediators (perforin and granzyme A), and chemokine receptor CX3CR1 on $\gamma\delta$ T cells derived from CMV-infected and CMV-uninfected newborns

Marker	CMV ⁺	CMV ⁻	p (CMV ⁺ versus CMV ⁻)
HLA-DR ⁺	6.34 (2.87–10.68)	0.58 (0.27–0.80)	<0.0001
CD27 ⁻ CD28 ⁻	43.82 (31.97–48.98)	0.22 (0.13–0.45)	<0.0001
CD94 ⁺	33.21 (23.68–57.20)	4.44 (3.61–5.56)	<0.0001
NKG2A ⁺	10.99 (4.27–20.64)	3.84 (2.33–5.11)	0.0464
NKG2C ⁺	28.00 (10.77–35.54)	1.17 (0.91–3.68)	<0.0001
CD158a/h ⁺	14.70 (6.61–30.53)	1.56 (0.94–3.34)	<0.0001
CD158b/j ⁺	46.60 (29.07–51.38)	2.75 (2.56–3.32)	<0.0001
NKG2D ⁺	69.18 (64.42–76.76)	52.38 (46.26–63.08)	0.0001
KLRG1 ⁺	38.85 (27.80–45.41)	24.70 (16.73–28.72)	0.0009
perforin ⁺	61.50 (51.97–73.32)	0.67 (0.48–1.77)	<0.0001
granzyme A ⁺	69.82 (57.97–79.63)	4.38 (2.82–6.28)	<0.0001
CX3CR1 ⁺	43.21 (25.08–57.96)	1.11 (0.65–2.10)	0.0003

Data for CMV⁺ and CMV⁻ represent median (25–75% percentile). The gate is put on CD3⁺ $\gamma\delta$ ⁺. CMV⁺, *n* = 8–18; CMV⁻, *n* = 9–22.

$\gamma\delta$ T cells from CMV-infected newborns are activated and differentiated

Next, we evaluated whether the expansion of fetal $\gamma\delta$ T cells after congenital CMV infection was accompanied by activation and/or differentiation of these cells. A significant proportion of $\gamma\delta$ T cells from CMV-infected newborns expressed the activation marker HLA-DR, whereas expression was virtually absent in uninfected controls (Table I). Down-regulation of CD27 and CD28 expression has been shown to be associated with advanced or late differentiation in CD8 $\alpha\beta$ T cells upon CMV infection (Appay et al., 2002; Marchant et al., 2003; van Leeuwen et al., 2006). These markers have also been used to identify differentiated human $\gamma\delta$ T cells (Morita et al., 2007). Although CD27⁻CD28⁻ $\gamma\delta$ T cells were absent from CMV-uninfected newborns, a large proportion of $\gamma\delta$ T cells showed this phenotype in CMV-infected newborns (Table I). This differentiation was most pronounced in the V γ 9⁻ $\gamma\delta$ T cell subpopulation (unpublished data). Collectively, these data clearly show that upon congenital CMV infection, $\gamma\delta$ T cells are activated, undergo cell division, and become differentiated.

Expression of NK receptors (NKR), cytotoxic mediators, and IFN- γ is highly increased in $\gamma\delta$ T cells of CMV-infected newborns

To gain insight into the function of fetal $\gamma\delta$ T cells in newborns with congenital CMV infection, we compared the gene expression profiles of $\gamma\delta$ T cells derived from three CMV-infected newborns versus three CMV-uninfected newborns. 1,622 genes were increased and 654 decreased upon infection (using the selection criteria described in Materials and methods; *M* > 0.05, *P* < 0.05). More than 100 genes associated with cell cycle showed increased expression upon CMV infection (as analyzed with DAVID; not depicted), coinciding with the expansion data (Fig. 1).

NKRs. Expression of a range of NKR genes was increased in $\gamma\delta$ T cells from CMV-infected newborns in comparison with $\gamma\delta$ T cells from CMV-uninfected newborns (Fig. 3, KIR2DL1; Table S1). This included both activating and inhibitory receptors (Table S1) and involved all the NKR families: the killer immunoglobulin receptor (KIR) family, the C-type lectin family (CD94/NKG2A/NKG2C), and the natural cytotoxicity receptor (NCR) family (NKp46; Lanier, 2008). In CMV-uninfected newborns, there were either no or very few $\gamma\delta$ T cells (CD94/NKG2A/NKG2C, CD158a/h [KIR2DL1/KIR2DS1], and CD158b/j [KIR2DL2/KIR2DS2]) or a significant fraction of $\gamma\delta$ T cells (NKG2D and KLRG1) expressing NKR on their membrane, as determined by flow cytometry (Table I). In CMV-infected newborns, significantly more $\gamma\delta$ T cells expressed all these NKRs (Table I).

Cytotoxic mediators. In general, cytotoxic lymphocytes can kill target cells by two main mechanisms: exocytosis of granule-associated molecules, such as granzymes, perforin, and granulysin, or binding to receptors with ligands of the TNF superfamily (e.g., FasL and TRAIL). Among the >47,000 transcripts analyzed, the two genes displaying the most increased expression upon CMV infection were members of the granzyme family: granzyme B and granzyme H (Fig. 3). In addition, other granzyme family members (granzyme A and granzyme M), perforin, granulysin, FasL, and TRAIL were increased (Table S1). In CMV-uninfected newborns, there was either no or only a low percentage of $\gamma\delta$ T cells expressing perforin and granzyme A, as demonstrated by flow cytometry (Table I). In CMV-infected newborns, the percentages of $\gamma\delta$ T cells expressing perforin or granzyme A were highly increased (Table I). This expression was clearly associated with the late differentiation status of the $\gamma\delta$ T cells (Fig. S2 A).

Chemokines and chemokine receptors. The genes for the chemokines CCL3 (MIP-1 α), CCL4 (MIP-1 β), and CCL5

(RANTES), all ligands for CCR5, and two chemokine receptor genes (CCR5 and CX3CR1) showed increased expression in $\gamma\delta$ T cells from CMV-infected newborns in comparison with $\gamma\delta$ T cells from CMV-uninfected newborns (Table S1). CCR7 gene expression was decreased upon CMV infection ($M = -2.27$, $A = 8.07$, $P = 0.03$). In CMV-uninfected newborns, there were no or only a low percentage of $\gamma\delta$ T cells expressing CX3CR1 (fractalkine receptor) on their membrane, as determined by flow cytometry (Table I). In CMV-infected newborns, the percentage of $\gamma\delta$ T cells expressing CX3CR1 was highly increased (Table I), which was clearly associated with the late differentiation phenotype of the $\gamma\delta$ T cells (Fig. S2 B).

Cytokines. Only a limited number of cytokine genes showed increased expression in $\gamma\delta$ T cells derived from CMV-infected newborns in comparison with $\gamma\delta$ T cells derived from CMV-uninfected newborns (Table S1). IFN- γ was one of the most increased expressed genes (Fig. 3 and Table S1). Strikingly, gene expression of transcription factors known to be implicated in IFN- γ production, namely T-bet ($M = 2.37$, $A = 6.64$, $P = 0.000112$) and eomes ($M = 3.02$, $A = 6.31$, $P = 0.000246$), was highly increased. The high expression of T-bet was confirmed at protein level by flow cytometry in $\gamma\delta$ T cells from CMV-infected newborns and was associated with the differentiation status of $\gamma\delta$ T cells (Fig. 4 A). Almost all CD27⁻CD28⁻ $\gamma\delta$ T cells expressed T-bet, whereas CD27⁺CD28⁺ $\gamma\delta$ T cells expressed significantly lower levels of this transcription factor (median within CD27⁻CD28⁻ $\gamma\delta$ T cells, 97%; median within CD27⁺CD28⁺ $\gamma\delta$ T cells, 28%; $P = 0.0006$). In addition, T-bet expression per cell, as

measured by the mean fluorescence intensity (MFI), was consistently much higher in CD27⁻CD28⁻ $\gamma\delta$ T cells than in CD27⁺CD28⁺ $\gamma\delta$ T cells (median within CD27⁻CD28⁻ $\gamma\delta$ T cells, 318 MFI; median within CD27⁺CD28⁺ $\gamma\delta$ T cells, 102 MFI; $P = 0.0023$). Upon a brief polyclonal stimulation in vitro, the majority of CD27⁻CD28⁻ differentiated $\gamma\delta$ T cells of CMV-infected newborns produced IFN- γ (Fig. 4 B), whereas the CD27⁺CD28⁺ $\gamma\delta$ T cells produced significantly less IFN- γ (median within CD27⁻CD28⁻ $\gamma\delta$ T cells, 68%; median within CD27⁺CD28⁺ $\gamma\delta$ T cells, 22%; $P = 0.0006$). T-bet and IFN- γ expression within $\gamma\delta$ T cells from CMV-uninfected newborns were similar to the expression found within CD27⁺CD28⁺ $\gamma\delta$ T cells from CMV-infected newborns (unpublished data).

The CDR3 δ 1 and CDR3 δ 2 are highly restricted upon congenital CMV infection

To study the impact of CMV infection during fetal life on the TCR repertoire of $\gamma\delta$ T cells, we assessed the degree of junctional diversity of the complementary-determining-region-3 (CDR3) of the V δ 1 (CDR3 δ 1) and V δ 2 (CDR3 δ 2) chains by spectratyping on 11 CMV-uninfected and 13 CMV-infected cord blood samples. CMV-uninfected cord blood samples showed polyclonal profiles for both CDR3 δ 1 (as described previously; Beldjord et al., 1993) and CDR3 δ 2. In contrast, the CDR3 δ 1 and CDR3 δ 2 repertoires became highly restricted in the vast majority of CMV-infected newborns (Fig. 5 A and Fig. S3). To quantify this restriction, we calculated an index of oligoclonality for CDR3 δ 1 and CDR3 δ 2 as described previously (Déchanet et al., 1999; Pitard et al., 2008). For both CDR3s, the index of oligoclonality

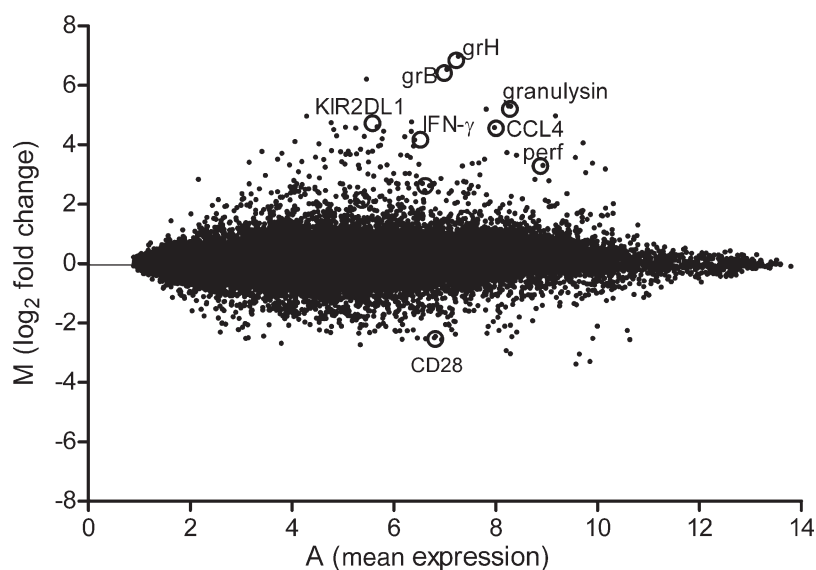


Figure 3. Gene expression analysis of $\gamma\delta$ T cells derived from three CMV-infected newborns versus $\gamma\delta$ T cells derived from three CMV-uninfected newborns. MA plot of differentially expressed genes in $\gamma\delta$ T cells upon CMV infection. M (\log_2 of fold change) reflects the differential expression of a gene. Positive and negative values indicate genes which are up- and down-regulated, respectively, upon CMV infection. A (mean expression) reflects the overall expression level of a gene. Each dot represents one gene. Examples of highly expressed NKR (KIR2DL1), cytotoxic mediators (granzyme B, granzyme H, perforin, and granulysin), cytokines (IFN- γ), and chemokines (CCL4) are indicated.

was significantly higher in CMV-infected newborns than in CMV-uninfected newborns (Fig. 5 B). Moreover, the restriction of CDR3 δ 1 of CMV-infected newborns was enriched for the same length (11 aa) in all CMV-positive newborns (Fig. 5 A, arrows). This length was either absent or minimally present in CMV-uninfected newborns (Fig. 5 A). In contrast to CDR3 δ 1, the length of the enriched CDR3 δ 2 sequences in CMV-infected newborns varied from newborn to newborn (Fig. S3). It is of note that CMV-infected newborn Pos10 showed a polyclonal CDR3 δ 1 repertoire corresponding with the absence of CD27⁻CD28⁻ differentiated V δ 1⁺ $\gamma\delta$ T cells (Fig. 5 A). In contrast, V δ 2⁺ $\gamma\delta$ T cells of this newborn were well differentiated, corresponding to a restricted CDR3 δ 2 repertoire (Fig. S3).

Public germline-encoded CDR3 δ 1 and CDR3 γ 8 sequences are highly enriched in CMV-infected newborns

Because the CDR3 δ 1 of CMV-infected newborns was highly enriched at 11 aa, we wondered whether this region included the same or similar sequences. Strikingly, at amino acid level in all 12 sequenced CMV-infected newborns the CDR3 δ 1 of 11 aa had exactly the same sequence: CALGELGDDKLIF, or ELGDD for short (Table S2; Fig. 5 A). At the nucleotide level, two variants were observed: the first D of ELGDD was either formed by the ga of the diversity gene δ 3 (D δ 3) and

the c of the joining gene δ 1 (J δ 1) or completely formed by the gat of the D δ 3 (Table S2, dark gray). Besides ELGDD itself, few longer variants were present in some CMV-infected newborns, which were enriched as well, containing one (Pos3) or two (Pos3 and Pos11) extra Ts after the first D of ELGDD (Table S2). In contrast to the other CDR3 δ 1 sequences, the highly enriched ELGDD sequence did not contain P/N additions and was thus completely germline encoded (Table S2).

In comparison with CDR3 δ 1, the degree of shared CDR3 δ 2 sequences among the CMV-infected newborns was much less clear (Table S3). Among eight CMV-infected newborns, three exhibited enrichment of the same CDR3 δ 2 sequence (Pos8, Pos9, and Pos10; Table S3). No other obvious similarities were found between different enriched CDR3 δ 2 sequences of different CMV-infected newborns. V δ 1 almost always paired with J δ 1, whereas V δ 2 had a preference for J δ 3 (Table S2 and Table S3). Sequencing of CDR3 δ 1 and CDR3 δ 2 of CMV-uninfected newborns confirmed the polyclonal repertoire as found by spectratyping (Table S2 and Table S3).

We wondered whether the V δ 1 chain, containing the public CDR3 δ 1, from CMV-infected newborns cells had a preference for pairing with specific V γ chains. By costaining of V δ 1 and V γ 2/3/4, V γ 5/3, V γ 8, or V γ 9, we determined that within CMV-uninfected newborns V δ 1 had a preference

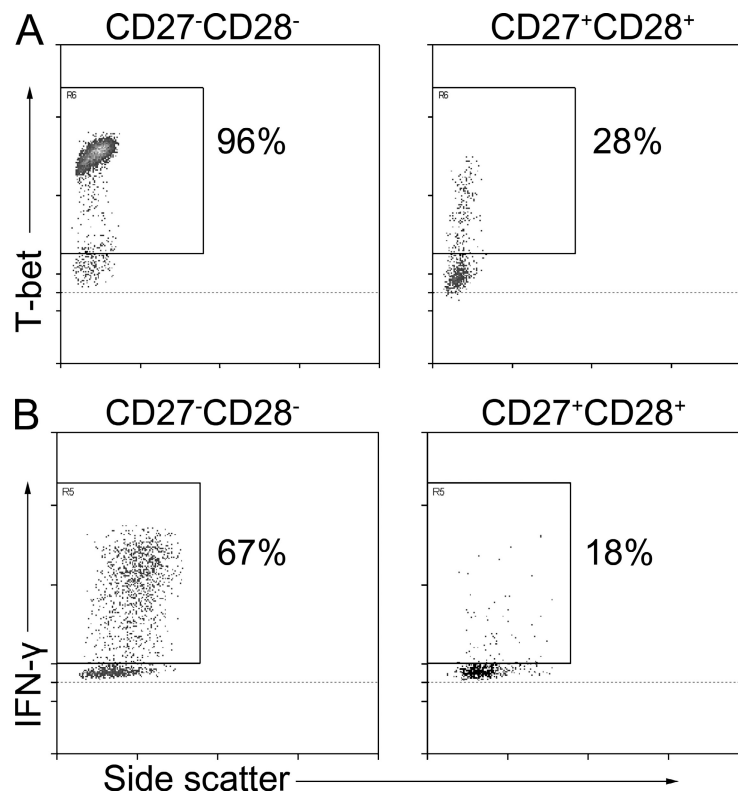


Figure 4. Differentiated (CD27⁻CD28⁻) $\gamma\delta$ T cells from CMV-infected newborns express highly the transcription factor T-bet and produce high levels of IFN- γ . Flow cytometry plots for T-bet (A) and IFN- γ (B), gated on CD27⁻CD28⁻ versus gated on CD27⁺CD28⁺ $\gamma\delta$ T cells of a CMV-positive newborn (Pos12), representative of seven CMV-infected newborns. Cells were stimulated for 4 h with PMA/ionomycin before intracellular staining for IFN- γ . Unstimulated cells showed no IFN- γ staining.

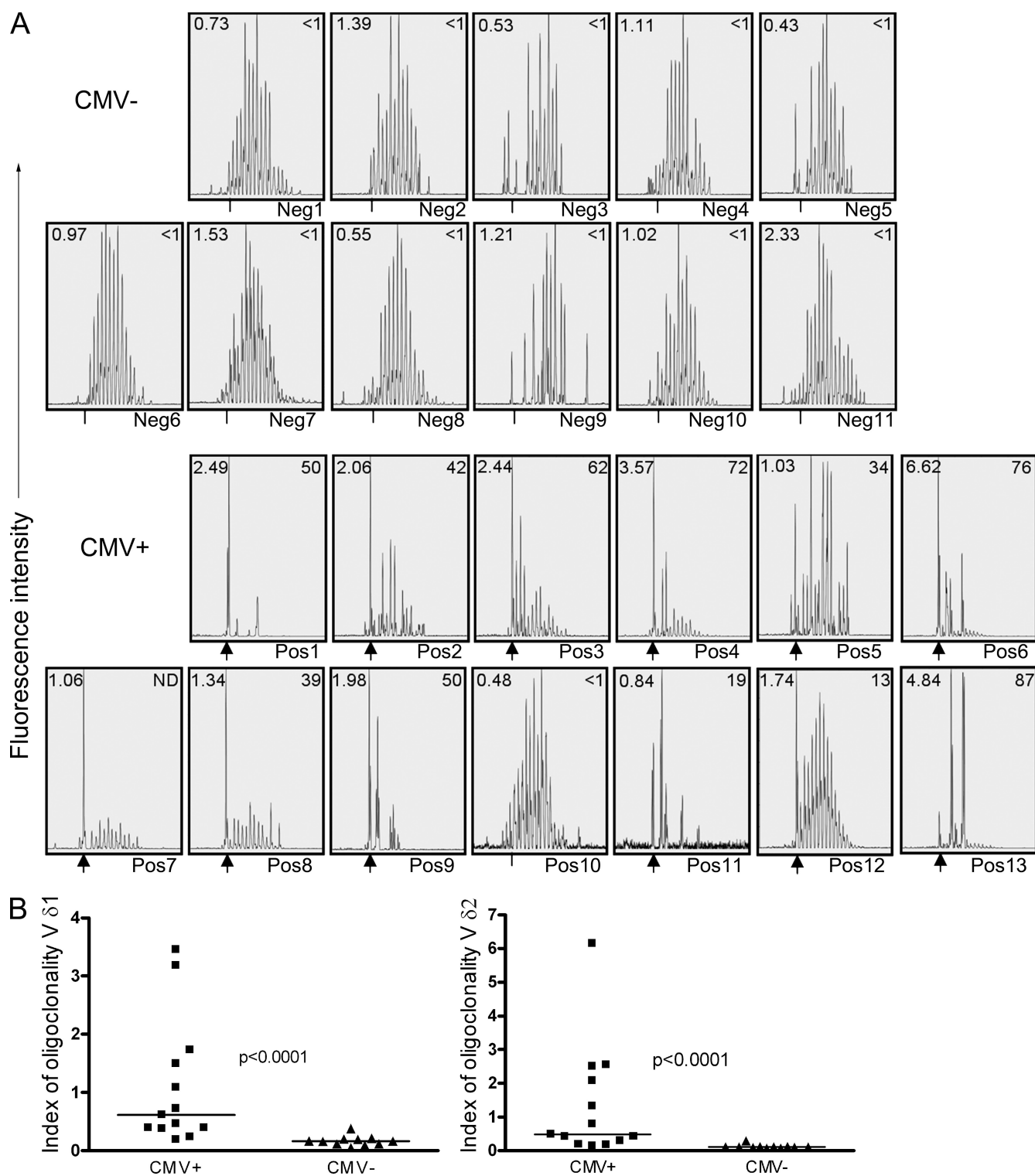


Figure 5. The CDR3 δ 1 and CDR3 δ 2 repertoire of $\gamma\delta$ T cells from CMV-infected newborns are oligoclonal, and CDR3 δ 1 is highly enriched for a single sequence. (A) Spectratyping plots of the CDR3 δ 1 of CMV-uninfected and CMV-infected newborns. Each box represents one donor. The numbers at the left top of each box represents the percentage of $V\delta 1^+$ $\gamma\delta$ T cells, expressed as percentage of total T cells. The numbers at the right top of each box represent the percentage of $CD27^-CD28^-$ cells of $V\delta 1^+$ $\gamma\delta$ T cells. The arrows indicate the sequences at CDR3 δ 1 of 11-aa size of the CMV-infected newborns that have been sequenced: CALGELGDDKLIF (Table S2). (B) Index of oligoclonality for CDR3 δ 1 and CDR3 δ 2, determined as described in Materials and methods. Lines indicate medians.

for pairing with V γ 2/3/4 (Fig. 6 A), whereas V δ 2 and V δ 3 had rather a preference for V γ 9 and V γ 5/3, respectively (not depicted). In contrast, in CMV-infected newborns with highly expanded $\gamma\delta$ T cells, V δ 1 had a clear preference for pairing with V γ 8 (Fig. 6 A), whereas V δ 2 and V δ 3 had rather a preference for V γ 2/3/4 (not depicted). Because of this preferential pairing of V δ 1 with V γ 8, we performed spectratyping for CDR3 γ 8 on six CMV-uninfected and six CMV-infected newborns. The CDR3 γ 8 of CMV-uninfected newborns showed a polyclonal repertoire. In contrast, the CDR3 γ 8 repertoire became highly restricted in CMV-infected newborns, showing a high enrichment at a length of 11 aa in five out of six CMV-infected newborns. The sixth CMV-infected newborn (Pos13) had a high enrichment at 12 aa (Fig. 6 B). Sequencing revealed that the CDR3 γ 8 sequences at 11 aa contained all the same sequence: CATWDTTGWFKIF (DTTGW for short). Pos13 had an

extra Y after D (Fig. 6 B; Table S4). This sequence was not detected in CMV-uninfected newborns (Table S4). As for the public CDR3 δ 1 sequence, the public CDR3 γ 8 was completely germline encoded (Table S4).

The public V γ 8V δ 1 TCR reacts against CMV-infected target cells

To verify whether the public V γ 8V δ 1 TCR reacts against CMV-infected target cells, we generated $\gamma\delta$ T cell clones expressing the public TCR containing the CDR3 δ 1-ELGDD and CDR3 γ 8-DTTGW from CMV-infected newborns Pos4 (11 public clones) and Pos6 (21 public clones). All clones expressing CDR3 δ 1-ELGDD coexpressed CDR3 γ 8-DTTGW, whereas clones with a different CDR3 δ 1 expressed other CDR3 γ 's (unpublished data), showing in a direct way the preferential pairing between CDR3 δ 1-ELGDD and CDR3 γ 8-DTTGW. A brief cocubation (6 h) of public

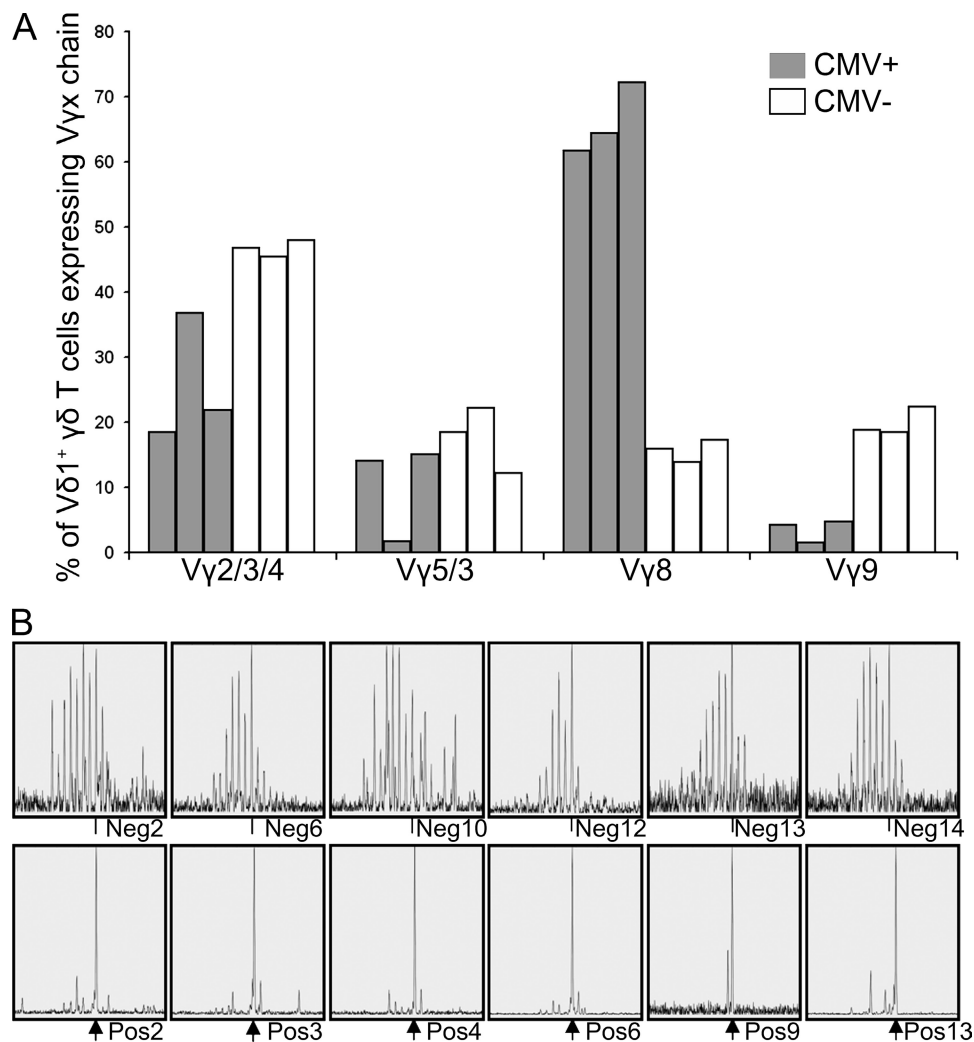


Figure 6. The V δ 1 chain on $\gamma\delta$ T cells of CMV-infected newborns preferentially pairs with a public V γ 8 chain. (A) The percentage of V δ 1⁺ $\gamma\delta$ T cells positive for V γ 2/3/4, V γ 5/3, V γ 8, or V γ 9 determined in three CMV-infected (Pos4, Pos6, and Pos13) and three CMV-uninfected newborns. (B) Spectratyping for CDR3 γ 8 of six CMV-uninfected newborns (top row) and six CMV-infected newborns (bottom row). The arrow indicates the public CDR3 γ 8 sequence CATWDTTGWFKIF of 11 aa (Table S3). The CDR3 γ 8 of Pos13 contains 1 aa more (Y).

clones with CMV-infected human embryonic lung fibroblasts induced IFN- γ production, which was blocked by the presence of a soluble anti-CD3 antibody (OKT3) showing the involvement of the public V γ 8V δ 1 TCR/CD3 complex in the recognition of CMV-infected target cells (Fig. 7 and Fig. S4). Control $\gamma\delta$ T cell clones of CMV-uninfected newborns did not show CMV-induced IFN- γ production. To gain insight into the antiviral activity of the $\gamma\delta$ T cell clones, we conducted additional experiments. Public clones killed infected target cells (Fig. 8 A) and inhibited CMV replication (between one and two log₁₀ inhibition; Fig. 8 B), whereas control V γ 9V δ 2 T clones had no or only a moderate effect (Fig. 8).

Differentiation and oligoclonal expansion of fetal $\gamma\delta$ T cells can occur early during gestation

To explore the possibility that $\gamma\delta$ T cells could develop a response toward CMV infection early during fetal life, we analyzed the $\gamma\delta$ T cells from fetal cord blood samples collected between 20 and 29 wk of gestation (from 12 CMV-

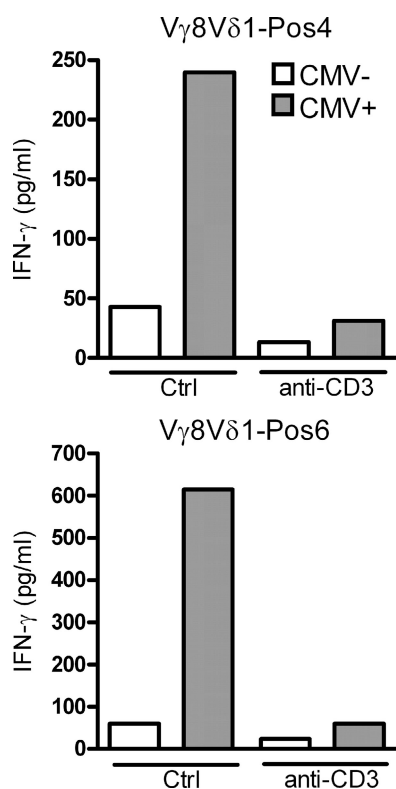


Figure 7. $\gamma\delta$ T cell clones expressing the public V γ 8V δ 1 TCR display reactivity against CMV-infected cells via TCR/CD3. Clones were cocultured for 6 h with human embryonic fibroblasts not infected (white bars) or infected (gray bars) with CMV (TB40/E). During cocultivation either a control IgG2a antibody (ctrl) or the anti-CD3 antibody OKT3 (anti-CD3) was present in soluble form. Results are shown for one public clone from CMV-infected newborn Pos4 (V γ 8V δ 1-Pos4) and for one public clone of CMV-infected newborn Pos6 (V γ 8V δ 1-Pos6) and are representative of five independent experiments involving 11 different public V γ 8V δ 1 clones.

negative and 13 CMV-positive fetuses). At these earlier gestation times, the $\gamma\delta$ T cells were already clearly differentiated (down-regulation of CD27 and CD28) and showed high expression of perforin (Fig. 9 A), granzyme A, and NKR (not depicted). From four CMV-infected fetuses, we performed spectratyping and sequencing for CDR3 δ 1 at time of delivery and at earlier gestation time (Fig. 9 B). We found that the CDR3 δ 1-ELGDD sequence was already enriched at as early as 21 wk of gestation (Fig. 9 B and Table S2). Few other CDR3 δ 1 sequences that were present at early gestation time were also present at time of delivery (Table S2, fetus Pos4 [14 aa] and fetus Pos13 [17 aa]). As observed at time of

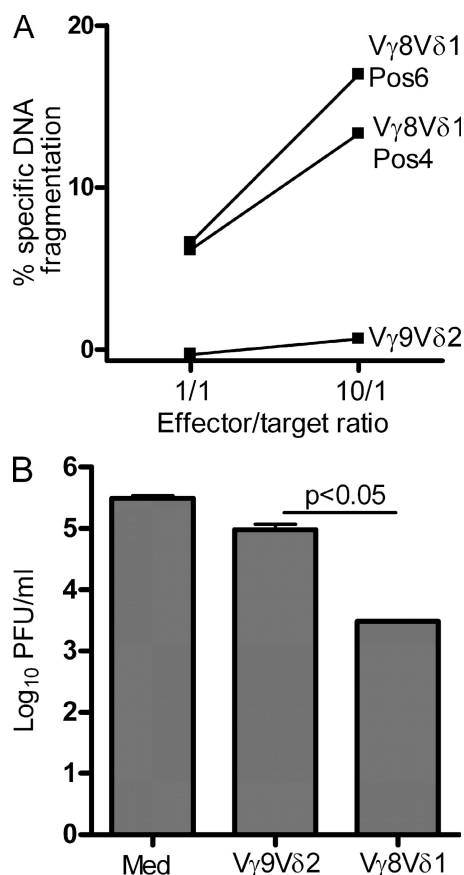


Figure 8. Public V γ 8V δ 1 clones kill CMV-infected target cells and inhibit CMV replication in vitro. (A) CMV-infected (TB40/E) human embryonic fibroblasts were cocultured with either $\gamma\delta$ T cell clones expressing the public V γ 8V δ 1 TCR (derived from CMV-infected newborns Pos4 and Pos6) or a control V γ 9V δ 2 clone (derived from a CMV-uninfected newborn) at the indicated effector to target ratios. After 4 h of cocultivation, the level of DNA fragmentation in the target cells was quantified. Results are representative of three independent experiments. (B) Human embryonic fibroblasts were incubated with CMV for 2 h, washed, and incubated with medium alone, with a public V γ 8V δ 1 clone from CMV-infected newborn Pos6 or with a control V γ 9V δ 2 clone from a CMV-uninfected newborn. After 7 d, the quantity of infectious CMV from the supernatant was determined by a plaque assay (PFU, plaque forming units). Shown are the mean \pm SEM of quadruplicate determinations. Results are representative of two independent experiments.

delivery, CDR3 δ 2 spectratyping showed more variability between fetuses (unpublished data). Furthermore, CDR3 δ 2 appeared to vary with time within the same fetus (Table S3, compare CDR3 δ 2 sequencing data of Pos4 at 20 wk, 5 d and at 40 wk, 0 d of gestation). Thus, the enriched CDR3 δ 1-ELGDD sequence appeared early during gestation in CMV-

infected fetuses and remained present with time. In contrast, the enriched sequences of the CDR3 δ 2 were variable from one fetus to the other and changed during gestation time. Furthermore, the CDR3 γ sequence associated with the CDR3 δ 1-ELGDD sequence, namely CDR3 γ 8-DTTGW, was also already enriched at as early as 21 wk of gestation

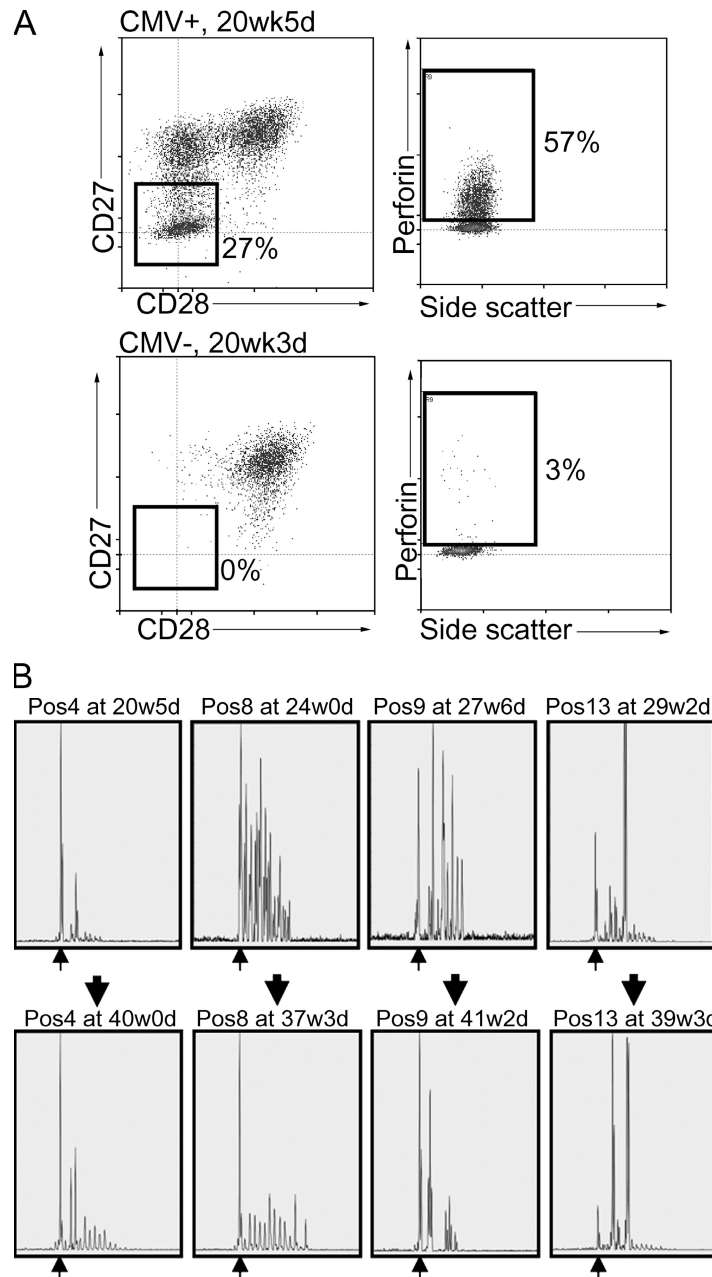


Figure 9. Differentiation and oligoclonal (CALGELGDDKLIF) expansion of fetal $\gamma\delta$ T cells can occur early during gestation. (A) Expression of CD27/CD28 and perforin by $\gamma\delta$ T cells from a representative CMV-uninfected (bottom) and a representative CMV-infected fetus (Pos4; top), both at the gestational age of 20–21 wk. Dot plots are presented and numbers indicate the percentages of $\gamma\delta$ T cells negative for CD27 and CD28 and positive for perforin. CMV⁻, results are representative of 12 (CD27CD28) and 7 (perforin) fetuses (gestation range: 20 wk, 3 d–29 wk, 2 d); CMV⁺, results are representative of 13 (CD27CD28) and 5 (perforin) fetuses (gestation range: 20 wk, 5 d–29 wk, 2 d). (B) Spectratyping for CDR3 δ 1 of four CMV-infected fetuses for which we had blood samples both at the time of delivery and at earlier gestation times. The enrichment for the CDR3 δ 1 size of 11 aa consists of the sequence CALGELGDDKLIF (Table S2).

(Table S4; CMV-infected newborn Pos4). Thus, despite the recent description of selective impairments of $\gamma\delta$ T cells in preterm infants (Gibbons et al., 2009), $\gamma\delta$ T cells are able to develop robust responses toward CMV infection in utero as early as 21 wk of gestation.

DISCUSSION

In this study, we demonstrate that CMV infection in utero leads to the oligoclonal expansion and differentiation of fetal $\gamma\delta$ T cells, which express high levels of NKR and cytotoxic mediators and produce IFN- γ . Both activating (e.g., activating KIR, NKG2C, and NKG2D) and inhibitory (e.g., inhibitory KIR, NKG2A, and KLRG1) NKRs were highly expressed in $\gamma\delta$ T cells derived from congenitally infected newborns. This would allow them to sense CMV-induced changes in infected target cells; HLA-E (ligand for NKG2A/NKG2C) expression is increased upon CMV infection, whereas classical MHC class I (ligands for KIR) expression is decreased (Wilkinson et al., 2008). In comparison with conventional T cells, it has been described that adult $\gamma\delta$ T cells express high levels of NKR, like members of the C-type lectin and the KIR family (Battistini et al., 1997; De Libero, 1999; Pennington et al., 2005). We confirmed the expression of KLRG1 on $\gamma\delta$ T cells in CMV-uninfected newborns (Eberl et al., 2005) and also showed that NKG2D is constitutively expressed. In contrast, unlike NK cells (Dalle et al., 2005), other NKRs (CD94/NKG2x and KIR family members) were not expressed or were expressed at very low levels on $\gamma\delta$ T cells from CMV-uninfected newborns. Thus the majority of NKR expression on adult $\gamma\delta$ T cells is likely to be the consequence of infections after birth. CMV infection in utero induced the up-regulation of various cytotoxic mediators in fetal $\gamma\delta$ T cells, including almost all members of the granzyme family, perforin, granulysin, FasL, and TRAIL. Perforin and granulysin are membrane-disrupting molecules and most granzymes have been shown to be involved in killing of target cells, with most evidence for granzyme B (Lieberman, 2003; Chowdhury and Lieberman, 2008). In addition, other granzyme-mediated antiviral mechanisms have been recently described: granzyme A plays a proinflammatory role (Metkar et al., 2008), granzyme M targets α -tubulin (Bovenschen et al., 2008), and granzyme H cleaves La, a phosphoprotein involved in cellular and viral RNA metabolism (Romero et al., 2009). It is of note that granzyme H cleaves an adenovirus-encoded granzyme B inhibitor (Andrade et al., 2007). Analysis of the profile of cytokine genes expressed in fetal $\gamma\delta$ T cells derived from CMV-infected newborns revealed the restricted high expression of IFN- γ . In parallel, we detected elevated levels of the T-box transcription factors T-bet and eomes, which are involved in the rapid and vigorous IFN- γ production by $\gamma\delta$ T cells (Yin et al., 2002; Chen et al., 2007). In contrast, expression of other transcription factor genes like GATA3 (Th2) or ROR- γ t (Th17) was not affected, coinciding with the absence of modulation of cytokine genes associated with these Th subsets. Only two chemokine receptors were significantly increased

in the microarray analysis: CCR5 and CX3CR1 (fractalkine receptor). Fractalkine can be produced by endothelial cells in the context of CMV infection (Bolovan-Fritts et al., 2004), thus possibly attracting differentiated CX3CR1⁺ fetal $\gamma\delta$ T cells to the site of infection. Together, our data indicate that fetal $\gamma\delta$ T cells generated in utero during CMV infection are equipped with a range of antiviral effector mechanisms, including IFN- γ production and granule-mediated cytotoxicity. Indeed, $\gamma\delta$ T cell clones generated from CMV-infected newborns killed CMV-infected cells and limited CMV replication in vitro. It is therefore likely that they participate in the limitation of the viral spread in the fetus. In kidney-transplanted patients with acute CMV infection, expansion of $\gamma\delta$ T cells is associated with the clinical resolution, suggesting a protective role of the expanded $\gamma\delta$ T cells (Lafarge et al., 2001; Halary et al., 2005).

We demonstrated that CMV infection during fetal life leads to the oligoclonal expansion of $\gamma\delta$ T cells, which is characterized by highly restricted CDR3 δ 1 and CDR3 δ 2 repertoires and by the high enrichment of a public CDR3 δ 1-CDR3 γ 8 sequence. Expanded $\gamma\delta$ T cells were negative for V γ 9 and included V δ 1⁺, V δ 2⁺, and V δ 3⁺ cells. In contrast, in adult CMV-infected kidney transplanted patients, expanded $\gamma\delta$ T cells do not include V δ 2⁺ cells and there is no restriction of CDR3 δ 2 (Déchanet et al., 1999). V γ 9-V δ 2⁺ $\gamma\delta$ T cells are very rare in the adult (Morita et al., 1994), providing a possible explanation of why Déchanet et al. (1999) did not detect any expansion of this subset in adults (Pitard et al., 2008).

TCR- δ chains have the highest potential diversity in the CDR3 loop ($\sim 10^{16}$ combinations) among all antigen receptor chains (TCR- α , TCR- β , TCR- γ , TCR- δ , IgH, and IgL) because multiple D gene segments can join together, all D gene segments can be read in all three open reading frames, and N nucleotides can be inserted into the junctions of each of the joining segments (Chien and Konigshofer, 2007). Therefore, it was surprising to identify a high enrichment of exactly the same CDR3 δ 1 sequence (i.e., public CDR3) in all fetuses with differentiated V δ 1⁺ $\gamma\delta$ T cells upon congenital CMV infection (ELGDD). It has been suggested that much of the diversity of the CDR3 junctions of the δ chain may confer different affinities of the $\gamma\delta$ TCR rather than the ability to recognize different ligands (Chien and Konigshofer, 2007). In addition, in adult CD8 $\alpha\beta$ T cells, public CMV-reactive TCR sequences bind the MHC-peptide complexes with higher affinity than private MHC peptide-specific TCR sequences (Trautmann et al., 2005; Day et al., 2007). This suggests that the public CDR3 δ 1-ELGDD is enriched by recognition of a CMV-induced ligand with high affinity. Our results show for the first time, to our knowledge, the expansion of a public $\gamma\delta$ TCR CDR3 in the context of an infection. Furthermore, we demonstrated that the public CDR3 δ 1 pairs with a public CDR3 γ 8 sequence (DTTGW), indicating that both the γ and δ chain are important for the recognition of the putative ligand. In addition, this public V γ 8V δ 1 TCR showed reactivity against CMV-infected

target cells *in vitro*. It is of note that both the CDR3 δ 1-ELGDD and CDR3 γ 8-DTTGW were germline encoded, as the CDR3 δ 1 was only formed by the V δ 1 gene, one D δ gene (D δ 3) and the J δ 1 gene, and the CDR3 γ 8 by the V γ 8 gene and the J γ P1 gene, without any addition of P/N nucleotides. Similarly, the mouse T22/T10-binding CDR3 δ does not contain N nucleotides (Adams et al., 2005; Chien and Konigshofer, 2007). This contrasts highly with $\alpha\beta$ T cells, where the most critical amino acids in the CDR3 α and CDR3 β involved in the recognition of the MHC-peptide complex are encoded either completely or partially by N nucleotides (Davis et al., 1998; Chien and Konigshofer, 2007).

Despite the immaturity of the neonatal immune system and possible mechanisms of immunosuppression by regulatory T cells (Mold et al., 2008), CMV infection is efficient in stimulating vigorous responses of both $\gamma\delta$ T cells and CD8 $\alpha\beta$ T cells (Marchant et al., 2003) during fetal life. Studies in mice show that the protective role of $\gamma\delta$ T cells in early life is not dependent on $\alpha\beta$ T cells (Ramsburg et al., 2003). Conversely, in a model of West Nile virus infection, it has been shown that $\gamma\delta$ T cells facilitate the CD8 $\alpha\beta$ T cell response (Wang et al., 2006). In comparison with adult DC, fetal DC shows impaired functions (Goriely et al., 2004; Levy, 2007). Because $\gamma\delta$ T cell differentiation is not dependent or is less dependent on DC in comparison with $\alpha\beta$ T cells, it is reasonable to believe that fetal DC defects would not prevent $\gamma\delta$ T cell differentiation upon fetal exposure to CMV. Instead, $\gamma\delta$ T cells may recognize their ligands directly on infected cells in tissues (Hayday, 2009). Such activated fetal $\gamma\delta$ T cells could, in turn, induce fetal DC maturation (Ismaili et al., 2002; Conti et al., 2005; Caccamo et al., 2006; Devilder et al., 2006; Eberl et al., 2009) and/or directly activate naive CD8 $\alpha\beta$ T cells via antigen cross-presentation (Brandes et al., 2009), as shown in adult cells. Such mechanisms could contribute to the development of functional CD8 $\alpha\beta$ T cell responses to CMV infection during fetal life (Marchant et al., 2003).

We conclude that human $\gamma\delta$ T cells can mount a vigorous response to CMV infection during development *in utero*, providing an important mechanism by which the fetus can fight pathogens. Identification of the $\gamma\delta$ TCR ligands induced upon CMV infection, like the putative ligand of the public V γ 8V δ 1 TCR, will likely be useful to design novel vaccination strategies against viral infection in early life.

MATERIALS AND METHODS

Study population. This study was approved by the Hôpital Erasme and Hôpital Saint-Pierre ethical committees. Women with suspected primary CMV infection were referred to the Fetal Medicine Units of the Hôpital Erasme or Hôpital Saint-Pierre. Diagnosis of primary maternal infection was based on anti-CMV IgG seroconversion or on the detection of high titers of anti-CMV-specific IgM, as described previously (Liesnard et al., 2000). After maternal informed consent, 20–50 ml of cord blood was collected at birth (full term, >37 wk gestation). In some cases, fetal cord blood was collected at earlier gestation ages (~1 ml by cordocentesis). Diagnosis of congenital infection was based on the detection of CMV genome by PCR and/or by viral culture on amniotic fluid and/or on newborn urine collected during the first week of life. The study included 19 CMV-infected

newborns and 22 uninfected control newborns as well as 13 infected and 12 uninfected fetuses. Symptomatic congenital infection was diagnosed in fetus Pos12 who had brain lesions at antenatal magnetic resonance imaging and an abnormal postnatal neurological development and in fetus Pos5 who had an abnormal postnatal neurological development.

Flow cytometry. The following antibodies were used: CD3-pacific blue (clone SP34-2), $\gamma\delta$ -PE (11F2), $\gamma\delta$ -FITC (11F2), CD27-APC (L128), CD27-FITC (L128), CD94-APC (HP-3D9), CD158a-FITC (HP-3E4), CD158b-FITC (CH-L), HLA-DR-APC-Cy7 (L243), NKG2D-APC (1D11), perforin-FITC (δ G9), granzyme A-FITC (CB9), Ki-67-FITC (B56), and IFN- γ -FITC (25723.11; BD); V δ 2-FITC (IMMU389), NKG2A-PE (Z199), CD3-ECD (UCHT1), and CD28-ECD (CD28.2; Beckman Coulter); V δ 1-FITC (TS1; Thermo Fisher Scientific); NKG2C-APC (134591; R&D Systems); CX3CR1-PE (2A9-1; MBL International); and T-bet-PE (4B10; eBioscience). V γ 5-PC5 (IMMU360) and V δ 3-FITC (P11.5B) were derived from Beckman Coulter via custom design service. V γ 2/3/4-biotin and V γ 2/3/4-FITC (23D12), V γ 4-FITC, V γ 5/3-biotin (56.3), and V γ 8-biotin (R4.5.1) were provided by D. Wesch (Institute of Immunology, University of Kiel, Kiel, Germany; Kabelitz et al., 1994; Hinz et al., 1997; Wesch et al., 1998). KLRG1-Alexa Fluor 488 (13F12F2) was provided by H. Pircher (University of Freiburg, Freiburg, Germany; Marcolino et al., 2004) and unlabeled V δ 3 antibody by E. Scotet (Institut National de la Santé et de la Recherche Médicale U601, Nantes, France; Peyrat et al., 1995). Staining was done on whole blood. Red blood cells were lysed using FACS Lysing solution (BD). The absolute number of $\gamma\delta$ T cells in whole blood was determined using Trucount beads (BD). Intracellular staining for perforin-FITC, granzyme A-FITC, and Ki-67-FITC was performed with the Perm 2 kit (BD) and for T-bet-PE with the Foxp3 staining buffer set (eBioscience). For the detection of IFN- γ , PBMCs were stimulated for 4 h with 10 ng/ml PMA and 2 μ M ionomycin in the presence of 2 μ M monensin. Staining was done using the Cytotfix/Cytoperm kit (BD). Cells were run on the CyAn flow cytometer equipped with three lasers (405, 488, and 633 nm) and data were analyzed using Summit 4.3 (Dako).

Microarray analysis. PBMCs were isolated from cord blood by Lymphoprep gradient centrifugation (Axis-Shield). After depletion of remaining red blood cells and CD4⁺ cells by magnetic cell sorting (Miltenyi Biotec), CD3⁺ $\gamma\delta$ ⁺ lymphocytes were sorted till high purity (>99%) with a MoFlo sorter (Dako). The $\gamma\delta$ T cell yield varied from 80,000–300,000 cells per cord blood sample. Total RNA was isolated using the RNeasy Micro kit (QIAGEN) from sorted $\gamma\delta$ T cells derived from three CMV-infected newborns and three CMV-uninfected newborns. RNA concentration was measured using the NanoDrop (Thermo Fisher Scientific) and RNA quality was assessed using the Bioanalyzer 2100 (Agilent Technologies). RNA was amplified into biotin-labeled complementary RNA (cRNA) by one-round *in vitro* transcription using the Premier kit (Applied Biosystems). The cRNA was fragmented and hybridized on the Human Genome U133 Plus 2.0 GeneChip (Affymetrix). Staining and scanning was done on the Affymetrix platform. The procedures, from RNA quality control to generation of raw data (CEL files), were performed at DNAVision (Gosselies, Belgium). The raw data were analyzed using the Affy package of Limma (linear models for microarray data; www.bioconductor.org), including fitting a linear model (lmfit) as described previously (Vermijlen et al., 2007). M- and A-values for each gene were generated. M (\log_2 of the fold change) is related to the degree of differential expression between the $\gamma\delta$ T cells from CMV-infected newborns versus $\gamma\delta$ T cells from CMV-uninfected newborns, whereas A is a measurement of the mean signal intensity. Genes were regarded as differentially expressed if the absolute M-value was >0.5 with a p-value <0.05. Genes with M-values >0.5 are enriched in the $\gamma\delta$ T cells derived from CMV-infected newborns, whereas genes with M-values <–0.5 are enriched in the $\gamma\delta$ T cells derived from CMV-uninfected newborns. The Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) was used to assist in the discovery of functionally related groups of differentially expressed genes. Microarray data and procedures

were deposited at Array Express (www.ebi.ac.uk/arrayexpress) under accession no. E-MEXP-2055.

Spectratyping. Total RNA was isolated from PBMC of cord blood of CMV-infected newborns and CMV-uninfected newborns, after which cDNA was generated using the First Strand cDNA synthesis kit (Fermentas). PCR (40 cycles) was performed with C δ (5'-GTAGAATTCCTTCAC-CAGACAAG-3') and V δ 1 (5'-CTGTCAACTTCAAGAAAGCAGC-GAAATC-3') or V δ 2 (5'-ATACCGAGAAAAGGACATCTATG-3') primers, resulting in amplification of the sequences containing the CDR3 δ 1 or CDR3 δ 2, respectively. For amplification of sequences containing the CDR3 γ 8, PCR was performed with C γ (5'-CAAGAAGACAAAGGTAT-GTTCCAG-3') and V γ 8 (5'-GCAAGCACAGGGAAGAGCCTTAA-3'). Then a run-off reaction (one cycle) was performed using the fluorescently labeled C δ -FAM primer (5'-ACGGATGGTTTGGTATGAGGCTGA-3') for CDR3 δ 1 and CDR3 δ 2 and with the C γ -FAM primer (5'-CTTCTG-GAGYTTTGTTCAGC-3') for CDR3 γ 8 (Déchanet et al., 1999; www.imgt.org). The labeled reaction products were run on a capillary sequencer (ABI3730xl or ABI3130xl analyzer) at DNAMVision. The fluorescence intensity was analyzed using Peak Scanner 1.0 (Applied Biosystems). The index of oligoclonality was calculated as described previously (Déchanet et al., 1999; Pitard et al., 2008).

Sequencing. As described in Spectratyping, PCR (40 cycles) was performed on cDNA to amplify the sequences that contain the CDR3 δ 1, CDR3 δ 2, or CDR3 γ 8. PCR products were TA cloned according to the instructions of the manufacturer (Invitrogen). Sequencing was performed on recombinant plasmids purified from bacterial clones by cycle sequencing (BigDye kit; Applied Biosystems). Electrophoresis of the sequencing reaction products was performed on the 96-capillary 3730xl DNA analyzer (Applied Biosystems) at DNAMVision. The CDR3 length, V-gene, P/N nucleotides, D gene segments, and J gene segments were determined using the IMGT/V-QUEST tool (www.imgt.org; Brochet et al., 2008). The CDR3 is delimited by (but does not include) the anchor positions 2nd-Cys(C) 104 and J-PHE(F) 118. Only sequences in frame were included in Tables S2, S3, and S4.

Generation of $\gamma\delta$ T cell clones. $\gamma\delta$ T cells (V δ 1⁺ or V γ 9⁺V δ 2⁺) from CMV-infected and CMV-uninfected newborns were sorted into wells at 1, 3, or 10 cells/well in X-VIVO 15 medium (Lonza) containing 10% fetal calf serum (PAA Laboratories), penicillin (100 U/ml), streptomycin (100 U/ml), (Lonza), and 2.5 μ g/ml fungizone (Invitrogen) and stimulated with 4 μ g/ml PHA-P (Sigma-Aldrich) and irradiated feeder cells (mix of two allogeneic PBMCs [100,000 cells/well] and irradiated B cell line JY [10,000 cells/well; Vanhecke et al., 1995; Halary et al., 2005; Gibbons et al., 2009]). At day 3, T cell growth factor (ZeptoMetrix) was added until it reached a final concentration of 5% of the medium, and at day 10, again, feeder cells and PHA were added. Restimulation with PHA/feeder cells was performed every 2–3 wk. 25 ng/ml IL-15 and 10 ng/ml IL-7 (R&D Systems) were added to maintain expanded $\gamma\delta$ T cell clones.

Coincubation of $\gamma\delta$ T cell clones with CMV-infected target cells. Confluent monolayers of the human embryonic lung fibroblasts (HEL299; American Type Culture Collection) in flat-bottom 96-well plates were incubated with CMV TB40/E strain (gift from Z. Tabi, Cardiff School of Medicine, Cardiff, UK; Tabi et al., 2001) at a multiplicity of infection (MOI) of 0.1 or 0.01 for 2 h, washed, and cultured for 5 d. Before coincubation with $\gamma\delta$ T cell clones, the infected and uninfected fibroblasts were washed two times with PBS. Verification of infection was performed by evaluating cytopathic effect by microscopy and analyzing the expression of immediate early antigen by flow cytometry (antibody clone E13; Argon). $\gamma\delta$ T cells were preincubated with either control IgG2a or anti-CD3 antibody (clone OKT3; eBioscience) for 10 min at 10 μ g/ml. The antibodies remained present during the coincubation at 5 μ g/ml. Treatment with soluble OKT3 did not influence the viability of the clones. $\gamma\delta$ T cell clones were added at

30,000 cells/well and, after 6 h, supernatant was collected. Release of IFN- γ into the supernatant was quantified by ELISA (Invitrogen). The killing assay was performed as described previously (Vermijlen et al., 2002) with some modifications. Fibroblasts with [methyl-³H]thymidine-labeled DNA were infected with CMV for 5 d and coincubated with $\gamma\delta$ T cells at the indicated effector to target ratios. After 4 h of coincubation, the level of DNA fragmentation induced by the $\gamma\delta$ T cells in the fibroblasts was determined as previously described (Vermijlen et al., 2002).

CMV replication assay. Confluent monolayers of human embryonic lung fibroblasts (HEL299) in flat-bottom 96-well plates were incubated with CMV (TB40/E) for 2 h (MOI 0.1), washed, and incubated with medium alone, with a public V γ 8V δ 1 clone or with a control V γ 9V δ 2 clone (150,000 cells per well). After 7 d, the quantity of infectious CMV from the supernatant was determined in quadruplicate by standard plaque assay titration (in plaque forming units).

Statistical analysis. Differences between CMV-infected newborns and CMV-uninfected newborns were determined using the nonparametric Mann-Whitney test using InStat software (GraphPad Software, Inc.). Differences were regarded as significant at $P < 0.05$.

Online supplemental material. Fig. S1 shows the V γ chain expression of CMV-infected and CMV-uninfected newborns. Fig. S2 shows the association of the late differentiation phenotype of $\gamma\delta$ T cells with the expression of cytotoxic mediators and chemokine receptor CX3CR1. Fig. S3 shows the CDR3 δ 2 repertoire. Fig. S4 shows TCR/CD3-dependent IFN- γ production by more public V γ 8V δ 1 clones upon coincubation with CMV-infected target cells. Table S1 provides an overview of differentially expressed genes in $\gamma\delta$ T cells from CMV-infected newborns versus CMV-uninfected newborns. Tables S2–S4 contain the sequencing data for CDR3 δ 1, CDR3 δ 2, and CDR3 γ 8 of CMV-infected and CMV-uninfected newborns. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20090348/DC1>.

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