Pathogenesis of congenital cytomegalovirus infection : finding prognostic markers and correlates of protection

Rovito, R.

Citation

Rovito, R. (2018, October 16). *Pathogenesis of congenital cytomegalovirus infection : finding prognostic markers and correlates of protection*. Retrieved from https://hdl.handle.net/1887/66319

Version:	Not Applicable (or Unknown)
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/66319

Note: To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/66319 holds various files of this Leiden University dissertation.

Author: Rovito, R.
Title: esis of congenital cytomegalovirus infection : finding prognostic markers and correlates of protection
Issue Date: 2018-10-16

CHAPTER 2

T AND B CELL MARKERS IN DRIED BLOOD SPOTS OF NEONATES WITH CONGENITAL CYTOMEGALOVIRUS INFECTION: B CELL NUMBERS AT BIRTH ARE ASSOCIATED WITH LONG-TERM OUTCOME

Roberta Rovito[1], Marjolein J. Korndewal[1,2], Menno C. van Zelm[3], Dimitrios Ziagkos[4], Els Wessels[1], Mirjam van der Burg[5], Aloys C.M. Kroes[1], Anton W. Langerak[5], Ann C.T.M Vossen[1]

[1] Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands;
[2] Centre for Infectious Diseases, Epidemiology and Surveillance, National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands;
[3] Department of Immunology and Pathology, Central Clinical School, Monash University, Melbourne, Victoria, Australia;
[4] Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, The Netherlands;
[5] Department of Immunology, Erasmus MC University Medical Centre Rotterdam, Rotterdam, The Netherlands.

J Immunol 2017 198(1):102-109

ABSTRACT

Congenital cytomegalovirus infection (cCMV) is the most common congenital infection which can cause long-term impairment (LTI). The pathogenesis of LTI is not completely understood. Fetal immunity may play a role in controlling the infection and preventing LTI, though immune activation may also contribute to fetal immunopathology. In this study we analyzed various molecular markers of T and B cell numbers in neonatal Dried Blood Spots (DBS) of 99 children with cCMV and 54 children without cCMV: δRec-ψJα signal joints on T cell receptor excision circles, intronRSS-Kde signal joints on Igκ-deleting recombination excision circles, genomic intronRSS-Kde coding joint, genomic Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements. Of this cohort clinical symptoms at birth and LTI at 6 y of age were recorded. Neonates with cCMV had less TRECs in their blood than non-infected controls. Furthermore, cCMV infection was associated with increased numbers of γδ T cells and B cells, and these numbers were positively correlated with CMV viral load in the DBS. Infected children with a better long-term outcome had higher numbers of B cells at birth than those who developed LTI; no differences in B cell replication were observed. The potential protective role of B cells in controlling cCMV-related disease and the clinical value of this marker as a predictor of long-term outcome merit further evaluation.

2.1. INTRODUCTION

Human cytomegalovirus (CMV) is the most common cause of congenital infections worldwide with an overall birth prevalence in industrialized countries between 0.6-0.7% (1, 2). Among congenitally infected children, 12.7% are estimated to be symptomatic at birth with the most common symptoms being petechiae, jaundice, hepatosplenomegaly, thrombocytopenia, chorioretinitis, and microcephaly (1, 2). An estimated 40-58% of these symptomatic children will develop permanent sequelae, such as hearing loss, mental retardation, and developmental delay (1). Importantly, out of the 87.3% of neonates that are asymptomatic at birth, approximately 13.5% will develop permanent sequelae as well (1). Defining markers that may help to predict whether a neonate will develop long-term impairment (LTI) will have profound impact on postnatal policy.

The pathogenesis of fetal damage during congenital infection is still poorly understood due to the complex interplay between viral, maternal, placental and fetal factors. It has been shown that infections occurring predominantly in the first half of pregnancy are associated with sequelae (3, 4). The developing fetal immune system may play a role in controlling the infection later in pregnancy, thereby preventing the development of long-term sequelae (5). There is evidence of fetal and neonatal immune system activation in the context of congenital CMV infection (cCMV). Previous studies have shown expansions of fetal γδ T cells (6), $CD8^+$ T cells (7, 8) and $CD4^+$ T cells (7, 9) in cCMV. Upon congenital infection similar types of effector $CD4^+$ and $CD8^+$ T cells as in adults are generated (10-12) but they appear to be functionally impaired (7), the $CD4^+$ T cell response being more impaired than the $CD8^+$ T cells (8, 13). Despite the fetal capacity to generate IgM against CMV (14), the fetal B cell response has not been extensively studied in cCMV. Previous studies on cellular and humoral immunity in cCMV have not related their findings to either symptoms at birth or LTI.

In recent years, molecular markers for T and B cells have been used to address several clinical questions. These markers specifically involve the circular excision products produced upon the most common TCR and Ig gene rearrangements during T and B progenitor cell generation in thymus and bone marrow, respectively. δRec-ΨJα signal joints on TCR excision circles (TRECs) are used in newborn screening on dried blood spots (DBS) for primary immunodeficiency such as SCID (15, 16) because they can be readily detected by quantitative PCR (17-19). Furthermore, studies have been carried out to implement screening for agammaglobulinemia (20), using quantitative PCR on DBS for Igκ-deleting recombination excision circles (KRECs). In addition, measurement of TRECs has been used in HIV patients to monitor the cellular recovery after initiation of highly active antiretroviral therapy (21), and in stem cell transplantation patients with primary immunodeficiency, TRECs and KRECs were used to monitor the thymic T cell and bone marrow B cell neogenesis (22, 23). Finally, the replication history of isolated B cell subsets has been used to improve the characterization of immunological disease with aberrant B lymphoid maturation (24-26).

For this study, neonatal DBS from a large cohort of children with cCMV and from non-infected controls were analyzed for molecular markers of T and B cells and the results were related to clinical data from birth until 6 y of age. Using quantitative real time PCR on DNA isolated from DBS, TCR and Ig gene rearrangements were detected. δRec-ΨJα signal joints on TRECs were detected as a measure of αβ T cell thymogenesis and coding joints of Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements

in the genome as a measure of circulating γδ T cells. Furthermore, intronRSS-Kde coding joints (cj intronRSS-Kde) in the genome and intronRSS-Kde signal joints on KRECs were quantified as a measure of circulating B cells and newly-derived bone marrow B cells, respectively. KRECs were additionally used to determine the B cell replication history (27).

In the present study, the quantification of TRECs and KRECs, as well as molecular markers for γδ T cells, was applied to provide new insights into the immune regulation of cCMV and to identify early markers to predict LTI at 6 y of age, such as neurodevelopmental impairment.

2.2. MATERIALS AND METHODS

2.2.1. Study population and clinical data

A previously described, nationwide, retrospective cohort was used in this study. This cohort was derived from a total group of 31,484 children, born in 2008 in the Netherlands, that was retrospectively tested for cCMV infection by PCR for CMV DNA in neonatal DBS at 5 y of age (28). After approval by the Medical Ethics Committee of the Leiden University Medical Center, the parents of 125 congenitally CMV infected children and of 263 non-infected children were asked to participate. Parents of 99 congenitally infected children gave informed consent for the use of DBS and the use of clinical data for this study. In addition, 54 controls without cCMV were randomly sampled from a control group matched for gender-, month-of-birth and region. Children were defined as symptomatic at birth if they had one or more of the following signs or symptoms in the neonatal period: prematurity, being small for gestational age, microcephaly, hepato- or splenomegaly, generalized petechiae or purpura, hypotonia, abnormal laboratory findings (elevated liver transaminases, hyperbilirubinemia, neutropenia or thrombocytopenia), cerebral ultrasound abnormalities, ophthalmologic abnormalities or neonatal hearing impairment. LTI was defined as the presence of impairment in one or more domain (hearing, visual, neurological, motor, cognitive and speech-language). Additionally, the severity of the LTI was assessed by accumulating the number of domains affected and indicated as the presence of LTI in two or more domains. The same definitions were used for children with and without cCMV. Finally, in this cohort maternal seroimmunity to CMV before birth was unknown, therefore it was assumed that cCMV infection could have resulted from either maternal primary or secondary infection.

2.2.2. DNA extraction from DBS and quantitative PCR for CMV

After a first initial CMV PCR screening performed at the National Institute for Public Health and the Environment, a second confirmatory PCR was performed at the Leiden University Medical Center (28). For this purpose, DNA was extracted from DBS by using the QIAamp DNA minikit according to the previously described protocol (29). For each test one full DBS was punched by using an automated DBS puncher (1296-071, Perkin Elmer-Wallac, Zaventem, Belgium). CMV DNA amplification of a 126-bp fragment from the immediate-early antigen region was performed using an internally controlled quantitative real-time PCR as described previously (30, 31) on a CFX96 Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands). The PCR was performed in triplicate and the CMV viral load expressed in IU/ml.

2.2.3. Quantification of TCR and Ig gene rearrangements

To study the numbers of T and B cells in neonatal DBS, the most frequently formed TCR and Ig gene rearrangements were quantified by TaqMan-based quantitative PCR. The δRec-ΨJα rearrangement occurs in 70-80% of TCRD alleles in mature αβ T lymphocytes, resulting in TRECs in nearly all newly formed αβ T cells (32). Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements are collectively present in nearly all mature γδ T cells in neonates, but absent in αβ T cells (6, 33). The intronRSS-Kde rearrangement occurs in ~30% of $Ig\kappa^+$ and in almost all $Ig\lambda^+$ mature B lymphocytes (27).

These rearrangements as well as the β-globin housekeeping gene were quantified in triplicate from each sample using two multiplex real-time PCR assays (Table 1). Each multiplex assay consisted of 5 µl of DNA extract and 20 µl reaction mixture containing 3.5 mM of $MgCl_2$, 0.04 mg/ml of BSA and 12.5 µl HotStar Master mix (QIAGEN, Hilden, Germany). Both mixtures were optimized by primer limitation, and for probe and $MgCl_2$ concentration with primers and probes specific for TREC, KREC, cj intronRSS-Kde, Vδ1-Jδ1, Vδ2-Jδ1 and β-globin to ensure equal amplification efficiencies. The Phocine Herpes Virus (PhHV) amplification was used to check for inhibition of the PCR and β-globin amplification was used to control for the number of nucleated cells. The following PCR mixes were used: the first mix contained 300 nM primers with 200 nM probe for cj intronRSS-Kde, Vδ1-Jδ1 and 500 nM primers with 200nM probe for β-globin. The second mix contained 300 nM KREC, Vδ2-δ1 and PhHV primers, 900 nM TREC primers, 200 nM KREC, TREC and Vδ2-δ1 probes, 50 nM PhHV probe.

Quantification was performed using dilution series of DNA from the following cell lines: DB01+T, a modification of the previously published U698 DB01 cell line (27) that contains one TREC, one KREC and one cj intronRSS-Kde rearrangement copy per genome; Peer (34), a T lymphoid cell line, containing one Vδ1-Jδ1 rearrangement copy per genome and T-ALL T032 (34), a T-acute lymphoblastic leukemia cell line, containing one Vδ1-Jδ1 rearrangement construct per genome. For TRECs two positive controls (cord blood with two different levels of TRECs spotted on filter paper) and a negative control (leukocyte-reduced adult blood produced by filtration and spotted on filter paper) were included in each run. These materials were kindly provided by the Centers for Disease Control and Prevention, Atlanta. The real-time PCR was performed on a CFX96 Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands) in a 96-well plate using a thermocycling profile as follows: 15 min 95°C followed by 45 cycles of 95°C (30 s), 55°C (30 s) and 72°C (30 s). Data were analyzed with CFX Manager 3.1 software.

The percentage of cells that contained the rearrangement in relation to the total amount of nucleated cells in blood was calculated using the ΔΔCt method with the formula as previously described (35): $2^\wedge((CT_{\beta\text{-globin}} - CT_{target})_{sample} - (CT_{\beta\text{-globin}} - CT_{target})_{cell\ line})*100\%$. The housekeeping gene β-globin was quantified in both the sample and cell line used as standard.

The average number of B cell divisions, B cell replication history, was calculated with the formula as previously described (35): $(CT_{sj} - CT_{cj})_{sample} - (CT_{sj} - CT_{cj})_{cell\ line}$.

The copies/µl of whole blood were calculated based on the theoretical recovery of 1 µg DNA from approximately 150,000 cells (19, 36) and the assumption that one full spot contains 50 µl of whole blood.

Table 1. Primers and probes used for the two internally-controlled multiplex rea- time PCRs.

	Targets	Forward primer (5'- 3')	Reverse primer (5'- 3')	Probe (5'- 3')
Mix I	cj[a] int-Kde (144 bp) Ref (35)	CCCGATTAATGCTGCCGTAG	CCTAGGGAGCAGGGAGGCTT	FAM[b]-AGCTGCATTTTTGCCATATCCACTATTTGGAGT-BHQ[c]-1
	Vδ1-Jδ1 (200-300 bp) Ref (33)	ATGCAAAAAGTGGTCGCTATT	TTAGATGGAGGATGCCTTAACCTTA	TXR[d]-CCCGTGTGACTGTGGAACCAAGTAAGTAACTC-BHQ-2
	β-globin (97 bp)	AAGTGCTCGGTGCCTTTAGTG	ACGTGCAGCTTGTCACAGTG	YAK[e]-TGGCCTGGCTCACCTGGACAACCT-BHQ-1
Mix II	sj[f] KREC int-Kde (148 bp) Ref (35)	TCAGCGCCCATTACGTTTCT	GTGAGGGACACGCAGCC	YAK-CCAGCTCTTACCCTAGAGTTTCTGCACGG-BHQ-1
	Vδ2-Jδ1 (around 200 bp) Ref (33)	ATACCGAGAAAAGGACATCTATG	TTAGATGGAGGATGCCTTAACCTTA	TXR-CCCGTGTGACTGTGGAACCAAGTAAGTAACTC-BHQ-2
	sj TREC ΨJα -δRec (131 bp) Ref (35)	CCATGCTGACACCTCTGGTT	TCGTGAGAACGGTGAATGAAG	FAM-CACGGTGATGCATAGGCACCTGC-BHQ-1
	PhHV[g] (89 bp)	GGGCGAATCACAGATTGAATC	GCGGTTCCAAACGTACCAA	CY5[h]-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ-1

[a] coding joint; [b] 6-carboxyfluorescein; [c] Black Hole Quencher; [d] Texas Red; [e] Yakima Yellow; [f] signal joint; [g] phocine herpesvirus type 1; [h] Cyanine.

2.2.4. Sensitivity and efficiency of the quantitative PCR assays

Ten-fold serial dilutions of DNA from the various control cell lines carrying the target rearrangements were used to determine the assay sensitivity. The analytical sensitivity, expressed as the lower limit of detection, was assessed by testing the dilutions in triplicate. Quantification of β-globin levels was possible with 0.05 ng total DNA with fewer than 36 PCR cycles in DB01+T, Peer and T-ALL 032 cell lines. Quantification of TREC and KREC levels was possible with 0.05 ng total DNA with fewer than 37 PCR cycles. Quantification of cj intronRSS-Kde, Vδ1-Jδ1 and Vδ2-Jδ1 levels was possible with 0.05 ng total DNA with < 36 PCR cycles.

The similar efficiency between each target and the β-globin is required to use the ΔCt method for correct quantification of normalized targets levels (37-39). The amplification efficiency, determined from the slope of the log-linear portion of the calibration curve, was assessed by testing the dilutions in triplicate. The efficiencies of the assays were very similar: 0.99 ± 0.02 for cj intronRSS-Kde, 0.98 ± 0.01 for KREC, 0.96 ± 0.02 for TREC and 0.99 ± 0.01 for β-globin in DB01+T cell line; 1.04 ± 0.01 for Vδ1-Jδ1 and 1.03 ± 0.03 for β-globin in Peer cell line; 1.06 ± 0.03 for Vδ2-Jδ1 and 0.98 ± 0.01 for β-globin in T-ALL 032 cell line; all $R^2 \geq 0.98$.

2.2.5. Statistics

The differences in the levels of immunological markers between the different categories - CMV status, viral load, symptoms at birth and LTI - were assessed by using a linear mixed model with random effects in order to account for the repeated measurements on the same patient. A Pearson's correlation analysis between viral loads, log(IU/ml), and the different immunological markers was carried out. P-values <0.05 were considered statistically significant. Due to the exploratory nature of this study the correction for multiple comparison was not applied for multiple statistical testing. Data were analyzed by using the Statistical Package for Social Sciences (SPSS, version 23, Chicago, IL).

2.3. RESULTS

2.3.1. Study population and clinical data

DBS of 99 children with cCMV were tested for using two multiplex real-time PCR assays (Table 1), as were 54 controls. The study population and the presence of symptoms at birth and LTI are shown in Table 2. In the control group, seven (12.9%) children showed symptoms at birth and five (9%) had LTI. In the children with cCMV, 16 (16%) children were symptomatic at birth and 22 (22%) had LTI.

2.3.2. TCR and Ig gene rearrangements in DBS of children with cCMV versus controls

First, the effect of cCMV was assessed by quantifying TCR and Ig gene rearrangements on DNA from DBS using real time PCR and comparing children with cCMV (cCMV+) and children without cCMV (cCMV-). Supplemental Table 1 shows the estimated means for the markers in our cohort. The cCMV+ group had a trend towards significant decrease percentage of cells that contained

Table 2. Long-term impairments in the group with cCMV and controls.

	Congenital CMV infection			No congenital CMV infection		
Long term impairment	**n = 99**	**Asympt.[a] n = 83**	**Sympt.[b] n = 16**	**n = 54**	**Asympt.[a] n = 47**	**Sympt.[b] n = 7**
Hearing impairment[c]	1	1	0	0	0	0
Visual impairment[d]	2	2	0	0	0	0
Neurological impairment[e]	4	2	2	4	4	0
Motor impairment[f]	10	7	3	1	1	0
Cognitive impairment[g]	4	2	2	2	2	0
Speech/language problem[h]	15	9	6	3	3	0
One or more impairment[i]	22	14	8	5	5	0
More than one impairmentl[j]	7	5	2	3	3	0

[a] Asymptomatic (Asympt.) at birth; [b] Symptomatic (Sympt.) at birth; [c] Sensorineural hearing loss; [d] Optic nerve atrophy, cortical visual impairment, congenital cataract; [e] Cerebral palsy, epilepsy, microcephaly, ADHD, autism; [f] Motor impairment (fine, gross or balance) based on test or diagnosis or sensory processing disorder or developmental coordination disorder; [g] Cognitive impairment based on test or diagnosis; [h] Language impairment based on test or diagnosis, speech-impairment, oral motor skill difficulties or auditory processing disorder; [i] Any long-term impairment, in one or more domains; [j] Impairment in two or more domains.

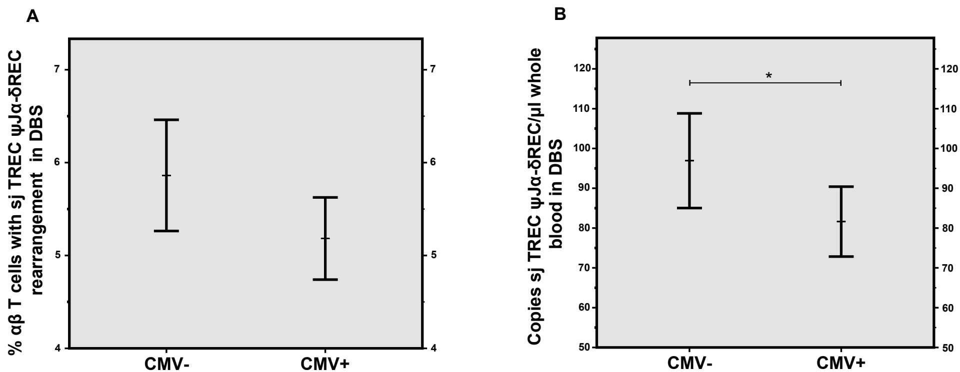

Figure 1. TRECs in DBS of CMV infected children and uninfected controls. (**A**) Frequency of TREC rearrangements in the group with cCMV (CMV+) and controls (CMV-). p = 0.073. (**B**) Absolute TREC numbers per µl of whole blood. The estimated means from each group of patients ± 2 SE are shown. *p = 0.043.

the TREC rearrangement normalized for the presence of β-globin (p = 0.073) (Fig. 1A). In accordance with this finding, the number of TRECs per µl of whole blood was lower than the control group (p = 0.043) (Fig. 1B).

It has been shown that prematurity, defined as birth before 37 wk gestational age, is related to a lower amount of TRECs (15, 40) and that intra-uterine growth retardation, or dysmaturity, is associated with a small thymus (41). To exclude their influence on the differences in TRECs' levels, an additional sensitivity analysis was performed by first excluding the group of premature newborns, 10 (10%) with cCMV and 4 (7.4 %) in the control group, and then by excluding the group of dysmature

newborns, 2 (2%) with cCMV and 2 (3.7%) in the control group. No differences were found in the estimates and significance compared to the whole cohort (data not shown). Therefore, our data suggest that the reduction in the amount of TRECs is not confounded by prematurity or by being small for gestational age.

The percentage of γδ T cells that contained the Vδ1-Jδ1 rearrangement was significantly higher in the cCMV+ group ($p = 0.019$). To exclude the possibility that this higher percentage of Vδ1-Jδ1 rearrangements was the results of fewer αβ T cells, we determined the absolute number of Vδ1-Jδ1 per microliter of whole blood. Indeed, significantly more Vδ1-Jδ1 copies per µl of whole blood were present in the infected group ($p = 0.038$). No statistically significant difference between the groups was observed for the percentage of cells with the Vδ2-Jδ1 rearrangement nor for the Vδ2-Jδ1 copies per µl of whole blood.

In the cCMV+ group, the percentage of cells that contained the cj intronRSS-Kde rearrangement was higher than in the cCMV- group ($p = 0.055$). As the cj intronRSS-Kde copies per µl of whole blood were slightly higher in the cCMV+ group, but not statistically significant, the small increase in percentage might be due to a decrease in αβ T cells. The KREC copies and the percentage of cells that carried a KREC were not different between the cCMV+ and cCMV- groups ($p = 0.297$, $p = 0.139$ respectively) even though the same trend of higher numbers in the cCMV+ group was observed. Consequently, the B cell replication history was not significantly different between cCMV+ and controls.

2.3.3. TCR and Ig gene rearrangements in DBS of children with cCMV in relation to CMV viral load

To study the relation between T and B cell numbers with CMV viral load, the cCMV+ group was divided into three by taking two cut-points at the first and third quartile of the viral load in DBS, resulting in a low (n=24), medium (n=50) and high (n=25) viral loads groups. The mean viral loads for each group were 254, 2907 and 27121 IU/ml, and the estimated means for the molecular T and B cell markers according to these groups are shown in Table 3.

The high viral load group did not show statistically significant differences compared with the low and medium viral load groups for the TRECs copies per µl whole blood or the percentage of cells that contained the TREC rearrangement.

DBS from neonates with high CMV viral loads showed a significantly higher percentage of cells containing Vδ1-Jδ1 rearrangements than the low viral load group ($p = 0.022$), and slightly higher than the medium viral load group ($p = 0.124$) (Fig. 2A). In addition, the Vδ1- Jδ1 copies per µl of whole blood were significantly higher in this group than in the low and medium viral load groups ($p = 0.033$, $p = 0.041$, respectively) (Fig. 2B). Furthermore, a Pearson's *r* data analysis suggested a positive correlation between Vδ1- Jδ1 percentage as well as Vδ1- Jδ1 copies per microliter, and viral loads ($p < 0.001$, $r = 0.25$, and $r = 0.23$, respectively). No statistically significant differences between the groups were observed for the percentage of cells that carried Vδ2-Jδ1 rearrangements nor in the copies Vδ2- Jδ1 copies per microliter of whole blood.

Table 3. Estimated means and SEs of all immunological markers in viral load.

Estimated means (SE)	Viral load cCMV infection		
	Low[a] (n = 24)	Medium[b] (n = 50)	High[c] (n = 25)
T cell markers			
TREC (%)	5.2 (0.5)	5.3 (0.3)	5.0 (0.4)
TREC copies/μl	79 (9)	81 (6)	83 (8)
Vδ1-Jδ1 (%)	0.42 (0.08)	0.53 (0.05)	0.68 (0.08)
Vδ1-Jδ1 copies/μl	10 (2)	12 (1)	17 (2)
Vδ2- Jδ1 (%)	0.6 (0.08)	0.71 (0.06)	0.71 (0.08)
Vδ2-Jδ1 copies/μl	19 (2)	20 (2)	22 (2)
β-glob copies/μl	2307 (141)	2304 (98)	2439 (138)
B cell markers			
KREC (%)	2.6 (0.3)	2.7 (0.2)	4.0 (0.3)
KREC copies/μl	34 (5)	38 (3)	65 (5)
cj int-Kde (%)	2.06 (0.31)	2.37 (0.21)	3.06 (0.3)
cj int-Kde copies/μl	27 (4)	32 (3)	48 (4)
B rep. history	-0.39 (0.09)	-0.23 (0.06)	-0.4 (0.09)

[a] Viral loads below first quartile; [b] Viral loads between first and third quartile; [c] Viral loads above third quartile.

The percentage of cells that contained KRECs (Fig. 2C) and the KREC copies per microliter (Fig. 2D) were significantly higher in the high viral load group than in the low and medium viral load groups ($p = 0.002$, $p = 0.001$, and $p<0.001$, respectively). A Pearson's *r* data analysis suggested a positive correlation between percentage of KREC, as well as KREC copies per microliter, and viral load ($p < 0.001$, $r = 0.33$, and $r=0.41$, respectively). Additionally, in the high viral load group, the percentage of cells that contained the cj intronRSS-Kde rearrangement was significantly higher than the low viral load group ($p = 0.024$), and trend significantly higher than the medium viral load group ($p = 0.068$) (Fig. 2E). These differences were even more significant for the cj intronRSS-Kde copies per μl of whole blood ($p = 0.001$, $p = 0.004$, respectively) (Fig. 2F). A Pearson's *r* data analysis suggested a positive correlation between percentage cj intronRSS-Kde, as well as cj intronRSS-Kde copies per microliter, and viral loads ($p < 0.01$, $r = 0.26$, and $r=0.37$, respectively). No differences in the B cell replication history were observed between the viral load groups.

2.3.4. TCR and Ig gene rearrangements in DBS of children in relation to symptoms at birth

Next, TCR and Ig gene rearrangements were studied in relation to symptoms at birth to evaluate whether they reflected differences in symptoms (Supplemental Table 1). The comparison of T and B cell markers between asymptomatic cCMV+ and symptomatic cCMV+ individuals did not show statistical differences for any of the markers. Additionally, when comparing the asymptomatic cCMV+ with asymptomatic cCMV- groups a similar trend was observed in the same markers as

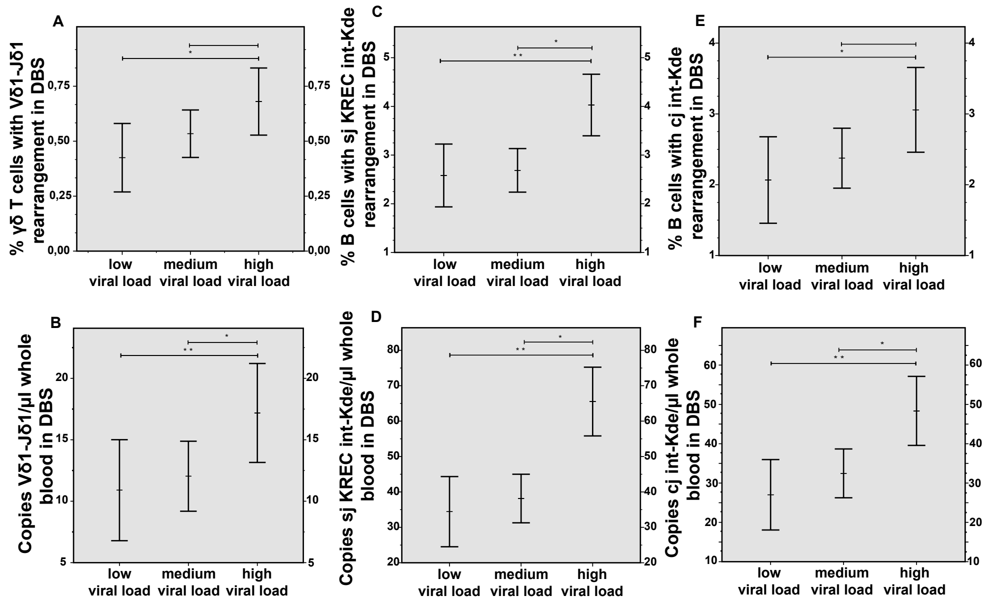

Figure 2. Vδ1-Jδ1, sj KREC int-Kde, and cj intronRSS-Kde in DBS of CMV infected children with different viral loads. (**A**) Frequency of Vδ1-Jδ1 rearrangements in the infected group (CMV+) with low, medium and high viral load on DBS. *p = 0.022. (**B**) Absolute Vδ1-Jδ1 numbers per microliter of whole blood in the infected group (CMV+) with low, medium and high viral load on DBS. *p = 0.041, **p = 0.033. (**C**) Frequency of sj KREC int-Kde rearrangements in the infected group (CMV+) with low, median and high viral load on DBS. *p = 0.001, **p = 0.002. (**D**) Absolute sj KREC int-Kde numbers per µl of whole blood in the infected group (CMV+) with low, median and high viral load on DBS, both p < 0.001. (**E**) Frequency of cj intronRSS-Kde rearrangements in the infected group (CMV+) with low, median and high viral load on DBS. *p = 0.024. (**F**) Absolute cj intronRSS-Kde numbers per µl of whole blood. *p = 0.004, **p = 0.001. The estimated means from each group of patients ± 2 SE are shown.

shown in the overall comparison between cCMV+ and cCMV- children (data not shown). Finally, no significant differences in viral loads were found between symptomatic and asymptomatic subjects.

2.3.5. TCR and Ig gene rearrangements in DBS of children in relation to long-term impairment

Next, the TCR and Ig gene rearrangements were correlated with LTI (Supplemental Table 1). First, we evaluated the immunological markers in relation to the development of any disorder in one or more of the following domains of impairment: hearing, visual, neurological, motor, cognitive and speech-language. When comparing the group of children with cCMV infection that develop any LTI to those who do not, a significantly lower percentage of cells that contain the KREC rearrangement was observed (p = 0.008) (Fig. 3A). Also the KRECs copies per µl of whole blood were significantly lower in the patients with LTI (p = 0.005) (Fig. 3B). A similar trend, although not statistically significant, was observed for the percentage of cells that contained the cj intronRSS-Kde

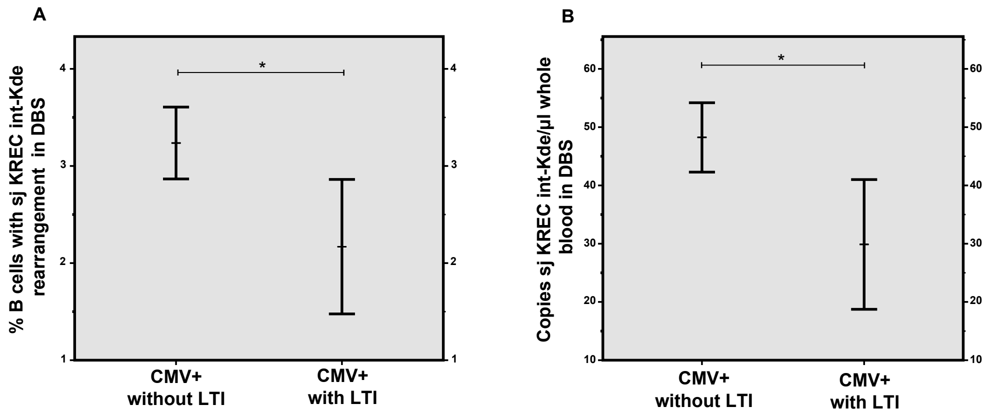

Figure 3. B cells and long-term impairments in cCMV infected children. (**A**) Frequency of sj int-Kde KREC rearrangement on DBS in the cCMV+ group without and with any long term impairment (LTI). *p = 0.008. (**B**) Absolute sj KREC int-Kde numbers per microliter of whole blood on DBS in the cCMV+ group without and with any LTI. *p = 0.005.

rearrangement as well as cj intronRSS-Kde copies per µl of whole blood (p = 0.137, p=0.073). Second, TCR and Ig gene rearrangements were assessed in relation to the development of more extensive impairments, defined as LTI in two or more domains. Similar trends were observed when children with more extensive impairments were compared to children without any LTI. Lower percentages and numbers of KRECs (p = 0.04, p = 0.02, respectively) and slightly lower percentages and numbers of cells containing cj intronRSS-Kde rearrangements were detected in cCMV+ children with more extensive LTI than in cCMV+ children without any LTI. No differences in B cell replication history and TCR rearrangements were observed in relation to LTI.

Next, we evaluated if the same markers that were significant in the overall cCMV+ and cCMV- comparison were still present in absence of any LTI. Indeed, a similar trend was observed in the same markers, TREC and Vδ1-Jδ1 (data not shown). In addition, the cCMV+ group had a significant increase in the percentage of cells that contains the KREC rearrangement (p = 0.021) and KREC copies (p = 0.044) as well as percentage of cells that contains the cj intronRSS-Kde rearrangement (p = 0.017) and slightly higher cj intronRSS-Kde copies per microliter of whole blood (p = 0.075).

Finally, no significant differences in viral loads were found between congenitally infected children with and without any LTI.

2.4. DISCUSSION

The analysis of molecular markers for T and B cells from DBS in this large cohort of children shows that cCMV resulted in reduced thymic production of αβ T cells, increased numbers of γδ T cells and a trend towards increased numbers of B cells. Children with cCMV and LTI did show lower number of B cells. The observed trend of B cells number increase in the infected group was further emphasized when excluding the patients with LTI, who had a lower number of B cells and might have diluted the effect.

The reduced number of TRECs in the DBS from children with cCMV suggests that intrauterine infection leads to reduced thymic production of T cells. Indeed, the sensitivity analysis indicated that the reduction in the amount of TRECs in our cohort is not confounded by prematurity or by being small for gestational age. CMV infection is known to induce a shift from naïve towards more differentiated αβ T cells with a reduction in the pool of naïve T cells, as has been shown in immunosuppressed individuals and in elderly (42, 43) as well as in pregnant CMV IgG-seropositive women (44). However, these all concern adults in whom the reduction of the pool of naïve T cells might result from a process that takes place over a longer period of time than the gestational period and where the longevity of naïve T cells might also play a role in masking differences in thymic output, with the reduced output being visible only after several years (45). Therefore, this process is unlikely to be the cause of the reduced amount of TREC in our cohort. Interestingly, in vitro CMV has been shown to be capable of infecting thymic epithelial cells that play a central role in T cell development and maturation, both during gestation and early stage of life (46, 47). Moreover, in newborn infants with cCMV, hypoplastic thymuses have been described and both in guinea pig and mouse models pathologic changes of the thymus have been shown (48-50). Although the TREC numbers were only moderately lowered, the effect of cCMV on thymopoiesis certainly deserves further study.

In our study we did not find an increase in Vδ2 T cells in the cCMV infected group, however, we looked at Vδ2-Jδ1 so a role of γδ T cells with other Vδ-Jδ rearrangements, as reported previously, cannot be excluded (6). Our observation of increased percentages of Vδ1 T cells in children with cCMV is in accordance with a previous study that showed a γδ T cell response upon cCMV after primary maternal CMV infection. These fetal γδ T cells, detected as early as 21 wk of gestation, were shown to be activated, to undergo cell division and to become differentiated with highly restricted repertoires (6). Moreover, γδ clones derived from cCMV-infected newborns showed antiviral activity when incubated with CMV-infected cells (6) suggesting a role in controlling viral replication (51). These unconventional cells, react rapidly upon activation (52) and develop earlier than αβ T cells during immune ontogeny. Therefore, they might have an important role in early life (51) and, possibly, in a context where the αβ T-cell response is impaired, they might be more efficient in controlling the early phases of cCMV. In the mouse model, when adaptive mechanisms are impaired or absent, γδ T cells can provide effective control over CMV infection (53, 54). To further support this, γδ T cell expansion in solid organ transplanted patients in response to CMV was associated with the resolution of infection and less symptomatic CMV disease, whereas late γδ T cell expansion correlated with a more intense and durable CMV infection (55). In our cohort, this was not associated with fewer symptoms at birth or LTI. However, the symptoms that define CMV disease in solid organ-transplanted patients are different to the clinical signs in the cCMV setting and the ability of γδ T cells to control long-term CMV disease, has not been elucidated (52).

CMV viral load in DBS was not correlated to symptoms at birth or to LTI in our cohort. Some previous studies have demonstrated a relation between CMV viral load with clinical outcome (56, 57), whereas others have not (58-60). The predictive role of CMV viral load in blood for congenital CMV disease may differ depending on the timing of infection and whether there was primary maternal infection or recurrent infection and therefore it still needs to be clarified. In our cohort

it is impossible to establish the trimester of infection or if a maternal primary infection occurred. Interestingly, none of our tested molecular B and T cell markers were associated with symptoms at birth in cCMV infected children. cCMV infected children with LTI did show significantly lower absolute and relative numbers of KRECs as well as slightly decreased, though not statistically significant, absolute and relative numbers of cj intronRSS-Kde, compared with cCMV infected children without LTI. However, there were no differences in B cell replication. The same trend was observed when considering more severe LTI. These findings suggest that cCMV infection does not induce a notable intrauterine B cell proliferation and possibly no considerable Ab production, but rather hint to an increase in B cell production. Unfortunately, little information is available on fetal B cell immunity in relation to cCMV. IgM positive B cells have been shown to emerge in the peripheral circulation as early as 12 wk of gestation (61) and CMV infected fetuses can produce IgM (62-64), but the antiviral activity and the role in CMV disease control have not yet been evaluated (5). Whether the different numbers of B cells are associated with postnatal differences in the capacity to generate long-lived plasma cells, memory B cells or support effector functions of immune cells remains to be elucidated. The potential protective role of Ab can be illustrated by the fact that primary CMV infection in pregnancy is associated with a vertical transmission rate of 30-40% and that this risk is at least 10-fold-lower in seropositive pregnant women (2). Although it is uncertain whether Abs are capable of influencing an ongoing CMV infection, a possible better initial B cell response might be beneficial in controlling the progressive tissue damage responsible for LTI development, possibly due to a sustained viral replication and spread. A positive correlation between B cells number and viral load was observed. In a group of pregnant women with primary infection, an expansion of a large pool of activated memory B cells enriched for CMV specificity and higher in viremic women was shown, further supporting a causal relationship between high viral loads and cells activation (65). On the other hand, the difference in B cell numbers that we observed at birth between infected children with and without LTI may also be related to a different timing of infection, with earlier infections leading to a more extensive cCMV and inflammation influencing the early B cell lymphopoiesis in the fetal liver.

To our knowledge, this is the first study on molecular markers for T and B cells in neonatal DBS of cCMV infected children in relation to long-term outcome. A reliable marker for long-term outcome could provide the means to introduce the long debated (66) newborn screening program for CMV in DBS by defining subgroups that would benefit from clinical, audiological follow-up and possibly antiviral treatment. Whether KREC, that was related to LTI, has enough discriminative power needs to be assessed in other CMV cohorts. Finally, this study on molecular markers generates new hypotheses on the effects of CMV infection on fetal, and possibly child, immunity and on the potential protective role of B cells in cCMV infection.

2.5. ACKNOWLEDGEMENTS

We thank professor Arnaud Marchant MD, PhD for critically reading the manuscript.

2.6. DISCLOSURES

The authors have no financial conflicts of interest.

2.7. FUNDING

This work was supported by European Union Seventh Framework Programme FP7/2012–2016 under grant agreement number 316655 (VACTRAIN).

REFERENCES

1. Dollard, S. C., S. D. Grosse, and D. S. Ross. 2007. New estimates of the prevalence of neurological and sensory sequelae and mortality associated with congenital cytomegalovirus infection. *Rev.Med.Virol*. 17: 355-363.
2. Kenneson, A., and M. J. Cannon. 2007. Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection. *Rev.Med.Virol*. 17: 253-276.
3. Pass, R. F., K. B. Fowler, S. B. Boppana, W. J. Britt, and S. Stagno. 2006. Congenital cytomegalovirus infection following first trimester maternal infection: symptoms at birth and outcome. *J.Clin.Virol*. 35: 216-220.
4. Enders, G., A. Daiminger, U. Bader, S. Exler, and M. Enders. 2011. Intrauterine transmission and clinical outcome of 248 pregnancies with primary cytomegalovirus infection in relation to gestational age. *Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology* 52: 244-246.
5. Schleiss, M. R. 2013. Cytomegalovirus in the neonate: immune correlates of infection and protection. *Clin.Dev.Immunol*. 2013: 501801.
6. Vermijlen, D., M. Brouwer, C. Donner, C. Liesnard, M. Tackoen, R. M. Van, N. Twite, M. Goldman, A. Marchant, and F. Willems. 2010. Human cytomegalovirus elicits fetal gammadelta T cell responses in utero. *J.Exp. Med*. 207: 807-821.
7. Huygens, A., S. Lecomte, M. Tackoen, V. Olislagers, Y. Demarcelle, W. Burny, R. M. Van, C. Liesnard, M. Larsen, V. Appay, C. Donner, and A. Marchant. 2015. Functional exhaustion limits CD4+ and CD8+ T cell responses to congenital cytomegalovirus infection. *J.Infect.Dis*.
8. Lidehall, A. K., M. L. Engman, F. Sund, G. Malm, I. Lewensohn-Fuchs, U. Ewald, T. H. Totterman, E. Karltorp, O. Korsgren, and B. M. Eriksson. 2013. Cytomegalovirus-specific CD4 and CD8 T cell responses in infants and children. *Scand.J.Immunol*. 77: 135-143.
9. Fujikawa, T., K. Numazaki, H. Asanuma, and H. Tsutsumi. 2003. Human cytomegalovirus infection during pregnancy and detection of specific T cells by intracellular cytokine staining. *Int.J.Infect.Dis*. 7: 215-221.
10. 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, V. Cerundolo, M. Callan, K. P. McAdam, S. L. Rowland-Jones, C. Donner, A. J. McMichael, and H. Whittle. 2003. Mature CD8(+) T lymphocyte response to viral infection during fetal life. *J.Clin.Invest* 111: 1747-1755.
11. Pedron, B., V. Guerin, F. Jacquemard, A. Munier, F. Daffos, P. Thulliez, Y. Aujard, D. Luton, and G. Sterkers. 2007. Comparison of CD8+ T Cell responses to cytomegalovirus between human fetuses and their transmitter mothers. *J.Infect. Dis*. 196: 1033-1043.
12. Elbou Ould, M. A., D. Luton, M. Yadini, B. Pedron, Y. Aujard, E. Jacqz-Aigrain, F. Jacquemard, and G. Sterkers. 2004. Cellular immune response of fetuses to cytomegalovirus. *Pediatr. Res*. 55: 280-286.
13. Prendergast, A. J., P. Klenerman, and P. J. Goulder. 2012. The impact of differential antiviral immunity in children and adults. *Nature reviews. Immunology* 12: 636-648.
14. Neto, E. C., R. Rubin, J. Schulte, and R. Giugliani. 2004. Newborn screening for congenital infectious diseases. *Emerg.Infect.Dis*. 10: 1068-1073.
15. Routes, J. M., W. J. Grossman, J. Verbsky, R. H. Laessig, G. L. Hoffman, C. D. Brokopp, and M. W. Baker. 2009. Statewide newborn screening for severe T-cell lymphopenia. *JAMA* 302: 2465-2470.
16. Verbsky, J. W., M. W. Baker, W. J. Grossman, M. Hintermeyer, T. Dasu, B. Bonacci, S. Reddy, D. Margolis, J. Casper, M. Gries, K. Desantes, G. L. Hoffman, C. D. Brokopp, C. M. Seroogy, and J. M. Routes. 2012. Newborn screening for severe combined immunodeficiency; the Wisconsin experience (2008-2011). *J.Clin.Immunol*. 32: 82-88.
17. Douek, D. C., R. A. Vescio, M. R. Betts, J. M. Brenchley, B. J. Hill, L. Zhang, J. R. Berenson, R. H. Collins, and R. A. Koup. 2000. Assessment of thymic output in adults after haematopoietic

stem-cell transplantation and prediction of T-cell reconstitution. *Lancet* 355: 1875-1881.

18. Hazenberg, M. D., S. A. Otto, J. W. Cohen Stuart, M. C. Verschuren, J. C. Borleffs, C. A. Boucher, R. A. Coutinho, J. M. Lange, T. F. Rinke de Wit, A. Tsegaye, J. J. van Dongen, D. Hamann, R. J. de Boer, and F. Miedema. 2000. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. *Nature medicine* 6: 1036-1042.

19. Hazenberg, M. D., S. A. Otto, E. S. de Pauw, H. Roelofs, W. E. Fibbe, D. Hamann, and F. Miedema. 2002. T-cell receptor excision circle and T-cell dynamics after allogeneic stem cell transplantation are related to clinical events. *Blood* 99: 3449-3453.

20. Nakagawa, N., K. Imai, H. Kanegane, H. Sato, M. Yamada, K. Kondoh, S. Okada, M. Kobayashi, K. Agematsu, H. Takada, N. Mitsuiki, K. Oshima, O. Ohara, D. Suri, A. Rawat, S. Singh, Q. Pan-Hammarstrom, L. Hammarstrom, J. Reichenbach, R. Seger, T. Ariga, T. Hara, T. Miyawaki, and S. Nonoyama. 2011. Quantification of kappa-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects. *J.Allergy Clin.Immunol*. 128: 223-225.

21. Di Mascio, M., I. Sereti, L. T. Matthews, V. Natarajan, J. Adelsberger, R. Lempicki, C. Yoder, E. Jones, C. Chow, J. A. Metcalf, I. A. Sidorov, D. S. Dimitrov, M. A. Polis, and J. A. Kovacs. 2006. Naive T-cell dynamics in human immunodeficiency virus type 1 infection: effects of highly active antiretroviral therapy provide insights into the mechanisms of naive T-cell depletion. *Journal of virology* 80: 2665-2674.

22. Patel, D. D., M. E. Gooding, R. E. Parrott, K. M. Curtis, B. F. Haynes, and R. H. Buckley. 2000. Thymic function after hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. *The New England journal of medicine* 342: 1325-1332.

23. Sottini, A., C. Ghidini, C. Zanotti, M. Chiarini, L. Caimi, A. Lanfranchi, D. Moratto, F. Porta, and L. Imberti. 2010. Simultaneous quantification of recent thymic T-cell and bone marrow B-cell emigrants in patients with primary immunodeficiency undergone to stem cell transplantation. *Clin.Immunol*. 136: 217-227.

24. van der Burg, M., M. Pac, M. A. Berkowska, B. Goryluk-Kozakiewicz, A. Wakulinska, B. Dembowska-Baginska, H. Gregorek, B. H. Barendregt, M. Krajewska-Walasek, E. Bernatowska, J. J. van Dongen, K. H. Chrzanowska, and A. W. Langerak. 2010. Loss of juxtaposition of RAG-induced immunoglobulin DNA ends is implicated in the precursor B-cell differentiation defect in NBS patients. *Blood* 115: 4770-4777.

25. Rakhmanov, M., B. Keller, S. Gutenberger, C. Foerster, M. Hoenig, G. Driessen, M. van der Burg, J. J. van Dongen, E. Wiech, M. Visentini, I. Quinti, A. Prasse, N. Voelxen, U. Salzer, S. Goldacker, P. Fisch, H. Eibel, K. Schwarz, H. H. Peter, and K. Warnatz. 2009. Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells. *Proceedings of the National Academy of Sciences of the United States of America* 106: 13451-13456.

26. Moir, S., J. Ho, A. Malaspina, W. Wang, A. C. DiPoto, M. A. O'Shea, G. Roby, S. Kottilil, J. Arthos, M. A. Proschan, T. W. Chun, and A. S. Fauci. 2008. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *The Journal of experimental medicine* 205: 1797-1805.

27. van Zelm, M. C., T. Szczepanski, M. van der Burg, and J. J. van Dongen. 2007. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. *J.Exp.Med*. 204: 645-655.

28. Korndewal, M. J., A. C. Vossen, J. Cremer, V. A. N. B. RS, A. C. Kroes, V. D. S. MA, A. M. Oudesluys-Murphy, and D. E. M. HE. 2015. Disease burden of congenital cytomegalovirus infection at school entry age: study design, participation rate and birth prevalence. *Epidemiology and infection*: 1-8.

29. de Vries, J. J., M. Barbi, S. Binda, and E. C. Claas. 2012. Extraction of DNA from dried blood in

the diagnosis of congenital CMV infection. *Methods Mol.Biol*. 903: 169-175.

30. de Vries, J. J., E. C. Claas, A. C. Kroes, and A. C. Vossen. 2009. Evaluation of DNA extraction methods for dried blood spots in the diagnosis of congenital cytomegalovirus infection. *J.Clin. Virol*. 46 Suppl 4: S37-S42.

31. Kalpoe, J. S., A. C. Kroes, M. D. de Jong, J. Schinkel, C. S. de Brouwer, M. F. Beersma, and E. C. Claas. 2004. Validation of clinical application of cytomegalovirus plasma DNA load measurement and definition of treatment criteria by analysis of correlation to antigen detection. *J.Clin.Microbiol*. 42: 1498-1504.

32. Verschuren, M. C., I. L. Wolvers-Tettero, T. M. Breit, J. Noordzij, E. R. van Wering, and J. J. van Dongen. 1997. Preferential rearrangements of the T cell receptor-delta-deleting elements in human T cells. *J.Immunol*. 158: 1208-1216.

33. Dik, W. A., K. Pike-Overzet, F. Weerkamp, R. D. de, E. F. de Haas, M. R. Baert, P. van der Spek, E. E. Koster, M. J. Reinders, J. J. van Dongen, A. W. Langerak, and F. J. Staal. 2005. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. *J.Exp.Med*. 201: 1715-1723.

34. Sandberg, Y., B. Verhaaf, E. J. van Gastel-Mol, I. L. Wolvers-Tettero, V. J. de, R. A. Macleod, J. G. Noordzij, W. A. Dik, J. J. van Dongen, and A. W. Langerak. 2007. Human T-cell lines with well-defined T-cell receptor gene rearrangements as controls for the BIOMED-2 multiplex polymerase chain reaction tubes. *Leukemia* 21: 230-237.

35. van Zelm, M. C., M. van der Burg, A. W. Langerak, and J. J. van Dongen. 2011. PID comes full circle: applications of V(D)J recombination excision circles in research, diagnostics and newborn screening of primary immunodeficiency disorders. *Front Immunol*. 2: 12.

36. Bains, I., R. Thiebaut, A. J. Yates, and R. Callard. 2009. Quantifying thymic export: combining models of naive T cell proliferation and TCR excision circle dynamics gives an explicit measure of thymic output. *J.Immunol*. 183: 4329-4336.

37. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 29: e45.

38. Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem*. 55: 611-622.

39. Zubakov, D., F. Liu, M. C. van Zelm, J. Vermeulen, B. A. Oostra, C. M. van Duijn, G. J. Driessen, J. J. van Dongen, M. Kayser, and A. W. Langerak. 2010. Estimating human age from T-cell DNA rearrangements. *Curr.Biol*. 20: R970-R971.

40. Chase, N. M., J. W. Verbsky, and J. M. Routes. 2011. Newborn screening for SCID: three years of experience. *Ann.N.Y.Acad.Sci*. 1238: 99-105.

41. Cromi, A., F. Ghezzi, R. Raffaelli, V. Bergamini, G. Siesto, and P. Bolis. 2009. Ultrasonographic measurement of thymus size in IUGR fetuses: a marker of the fetal immunoendocrine response to malnutrition. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology* 33: 421-426.

42. Weinberger, B., L. Lazuardi, I. Weiskirchner, M. Keller, C. Neuner, K. H. Fischer, B. Neuman, R. Wurzner, and B. Grubeck-Loebenstein. 2007. Healthy aging and latent infection with CMV lead to distinct changes in CD8+ and CD4+ T-cell subsets in the elderly. *Human immunology* 68: 86-90.

43. Almanzar, G., S. Schwaiger, B. Jenewein, M. Keller, D. Herndler-Brandstetter, R. Wurzner, D. Schonitzer, and B. Grubeck-Loebenstein. 2005. Long-term cytomegalovirus infection leads to significant changes in the composition of the CD8+ T-cell repertoire, which may be the basis for an imbalance in the cytokine production profile in elderly persons. *Journal of virology* 79: 3675-3683.

44. Lissauer, D., M. Choudhary, A. Pachnio, O. Goodyear, P. A. Moss, and M. D. Kilby. 2011. Cytomegalovirus sero positivity dramatically alters the maternal CD8+ T cell repertoire

and leads to the accumulation of highly differentiated memory cells during human pregnancy. *Hum.Reprod*. 26: 3355-3365.

45. Hazenberg, M. D., M. C. Verschuren, D. Hamann, F. Miedema, and J. J. van Dongen. 2001. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. *Journal of molecular medicine (Berlin, Germany)* 79: 631-640.

46. Wainberg, M. A., K. Numazaki, L. Destephano, I. Wong, and H. Goldman. 1988. Infection of human thymic epithelial cells by human cytomegalovirus and other viruses: effect on secretion of interleukin 1-like activity. *Clinical and experimental immunology* 72: 415-421.

47. Numazaki, K., L. DeStephano, I. Wong, H. Goldman, B. Spira, and M. A. Wainberg. 1989. Replication of cytomegalovirus in human thymic epithelial cells. *Medical microbiology and immunology* 178: 89-98.

48. Griffith, B. P., H. L. Lucia, and G. D. Hsiung. 1982. Brain and visceral involvement during congenital cytomegalovirus infection of guinea pigs. *Pediatric research* 16: 455-459.

49. Naeye, R. L. 1967. Cytomegalic inclusion disease. The fetal disorder. *American journal of clinical pathology* 47: 738-744.

50. Schwartz, J. N., C. A. Daniels, and G. K. Klintworth. 1975. Lymphoid cell necrosis, thymic atrophy, and growth retardation in newborn mice inoculated with murine cytomegalovirus. *The American journal of pathology* 79: 509-522.

51. Huygens, A., N. Dauby, D. Vermijlen, and A. Marchant. 2014. Immunity to cytomegalovirus in early life. *Front Immunol*. 5: 552.

52. Born, W. K., N. Jin, M. K. Aydintug, J. M. Wands, J. D. French, C. L. Roark, and R. L. O'Brien. 2007. gammadelta T lymphocytes-selectable cells within the innate system? *Journal of clinical immunology* 27: 133-144.

53. Sell, S., M. Dietz, A. Schneider, R. Holtappels, M. Mach, and T. H. Winkler. 2015. Control of murine cytomegalovirus infection by gammadelta T cells. *PLoS pathogens* 11: e1004481.

54. Khairallah, C., S. Netzer, A. Villacreces, M. Juzan, B. Rousseau, S. Dulanto, A. Giese, P. Costet, V. Praloran, J. F. Moreau, P. Dubus, D. Vermijlen, J. Dechanet-Merville, and M. Capone. 2015. gammadelta T cells confer protection against murine cytomegalovirus (MCMV). *PLoS pathogens* 11: e1004702.

55. Lafarge, X., P. Merville, M. C. Cazin, F. Berge, L. Potaux, J. F. Moreau, and J. Dechanet-Merville. 2001. Cytomegalovirus infection in transplant recipients resolves when circulating gammadelta T lymphocytes expand, suggesting a protective antiviral role. *J.Infect. Dis*. 184: 533-541.

56. Lanari, M., T. Lazzarotto, V. Venturi, I. Papa, L. Gabrielli, B. Guerra, M. P. Landini, and G. Faldella. 2006. Neonatal cytomegalovirus blood load and risk of sequelae in symptomatic and asymptomatic congenitally infected newborns. *Pediatrics* 117: e76-83.

57. Forner, G., D. Abate, C. Mengoli, G. Palu, and N. Gussetti. 2015. High Cytomegalovirus (CMV) DNAemia Predicts CMV Sequelae in Asymptomatic Congenitally Infected Newborns Born to Women With Primary Infection During Pregnancy. *The Journal of infectious diseases* 212: 67-71.

58. Halwachs-Baumann, G., B. Genser, S. Pailer, H. Engele, H. Rosegger, A. Schalk, H. H. Kessler, and M. Truschnig-Wilders. 2002. Human cytomegalovirus load in various body fluids of congenitally infected newborns. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 25 Suppl 3: S81-87.

59. Binda, S., A. Mammoliti, V. Primache, P. Dido, C. Corbetta, F. Mosca, L. Pugni, A. Bossi, C. Ricci, and M. Barbi. 2010. Pp65 antigenemia, plasma real-time PCR and DBS test in symptomatic and asymptomatic cytomegalovirus congenitally infected newborns. *BMC infectious diseases* 10: 24.

60. Ross, S. A., Z. Novak, K. B. Fowler, N. Arora, W. J. Britt, and S. B. Boppana. 2009. Cytomegalovirus blood viral load and hearing loss in young children with congenital infection. *The Pediatric infectious disease journal* 28: 588-592.

61. Holt, P. G., and C. A. Jones. 2000. The development of the immune system during pregnancy and early life. *Allergy* 55: 688-697.

62. Hassan, J., S. Dooley, and W. Hall. 2007. Immunological response to cytomegalovirus in congenitally infected neonates. *Clinical and experimental immunology* 147: 465-471.

63. Griffiths, P. D., S. Stagno, R. F. Pass, R. J. Smith, and C. A. Alford, Jr. 1982. Congenital cytomegalovirus infection: diagnostic and prognostic significance of the detection of specific immunoglobulin M antibodies in cord serum. *Pediatrics* 69: 544-549.

64. Ahlfors, K., M. Forsgren, P. Griffiths, and C. M. Nielsen. 1987. Comparison of four serological tests for the detection of specific immunoglobulin M in cord sera of infants congenitally infected with cytomegalovirus. *Scandinavian journal of infectious diseases* 19: 303-308.

65. Dauby, N., C. Kummert, S. Lecomte, C. Liesnard, M. L. Delforge, C. Donner, and A. Marchant. 2014. Primary human cytomegalovirus infection induces the expansion of virus-specific activated and atypical memory B cells. *J.Infect. Dis*. 210: 1275-1285.

66. Cannon, M. J., P. D. Griffiths, V. Aston, and W. D. Rawlinson. 2014. Universal newborn screening for congenital CMV infection: what is the evidence of potential benefit? *Reviews in medical virology* 24: 291-307.

SUPPLEMETARY DATA

Table S1. Estimated means and standard errors of all immunological markers in the different categories

	Congenital CMV infection						No congenital CMV infection					
Estimated means (SE)	**Overall n = 99**	**Asympt.[1] n = 83**	**Sympt.[2] n = 16**	**No LTI[3] n = 77**	**LTI (≥ 1)[4] n = 22**	**LTI (≥ 2)[5] n = 7**	**Overall n = 54**	**Asympt. n = 47**	**Sympt. n = 7**	**No LTI n = 49**	**LTI (≥ 1) n = 5**	**LTI (≥ 2) n = 3**
T cell markers												
TREC (%)	5.2 (0.2)	5.2 (0.2)	5.0 (0.6)	5.2 (0.3)	5.1 (0.5)	4.9 (0.8)	5.9 (0.3)	6.0 (0.3)	4.7 (0.8)	5.8 (0.3)	6.9 (0.9)	5.6 (1.2)
TREC copies/µl	81 (4)	82 (5)	78 (11)	82 (5)	82 (9)	69 (16)	96 (6)	99 (6)	84 (17)	96 (6)	103 (20)	80 (26)
Vδ1-Jδ1 (%)	0.54 (0.03)	0.56 (0.04)	0.45 (0.1)	0.56 (0.05)	0.51 (0.08)	0.56 (0.15)	0.40 (0.05)	0.44 (0.03)	0.15 (0.09)	0.39 (0.04)	0.56 (0.12)	0.35 (0.15)
Vδ1-Jδ1 copies/µl	13 (1)	14 (1)	10 (3)	14 (1)	11 (2)	10 (4)	9 (1)	11 (1)	4 (2)	9 (1)	13 (3)	8 (4)
Vδ2- Jδ1 (%)	0.68 (0.04)	0.68 (0.05)	0.66 (0.10)	0.65 (0.05)	0.77 (0.9)	0.81 (0.16)	0.77 (0.06)	0.79 (0.06)	0.66 (0.16)	0.77 (0.06)	0.79 (0.19)	0.61 (0.24)
Vδ2-Jδ1 copies/µl	20 (1)	21 (2)	19 (4)	20 (2)	24 (3)	23 (5)	23 (2)	24 (2)	18 (6)	23 (2)	21 (7)	17 (9)
[6]β-glob copies/µl	2339 (70)	2344 (76)	2314 (173)	2339 (79)	2339 (147)	2014 (259)	2450 (94)	2421 (103)	2639 (267)	2461 (102)	2340 (318)	2275 (410)
B cell markers												
KREC (%)	3.0 (0.2)	2.9 (0.2)	3.1 (0.4)	3.24 (0.19)	2.17 (0.35)	1.8 (0.6)	2.6 (0.2)	2.6 (0.2)	2.8 (0.5)	2.55 (0.19)	3.1 (0.6)	2.8 (0.8)
KREC copies/µl	44 (2)	44 (3)	46 (7)	48 (3)	30 (6)	22 (10)	39 (3)	39 (3)	44 (9)	38 (3)	50 (10)	36 (13)
cj int-Kde (%)	2.5 (0.1)	2.5 (0.17)	2.5 (0.40)	2.6 (0.17)	2.0 (0.33)	1.9 (0.6)	2.0 (0.2)	2.01 (0.16)	2.04 (0.42)	1.96 (0.16)	2.56 (0.49)	2.28 (0.63)
cj int-Kde copies/µl	35 (2)	35 (3)	34 (6)	37 (3)	27 (5)	24 (9)	30 (3)	31 (3)	30 (8)	30 (3)	39 (9)	31 (12)
B rep. history	-0.31 (0.04)	-0.31(0.05)	-0.34 (0.12)	-0.36 (0.05)	-0.16 (0.09)	-0.03 (0.17)	-0.38 (0.06)	-0.37(0.05)	-0.43 (0.14)	-0.39 (0.05)	-0.33 (0.17)	-0.30 (0.22)

[1] Asymptomatic at birth; [2] Symptomatic at birth; [3] No long-term impairment in any of the domain: hearing, visual, neurological, motor, cognitive and speech-language; [4] Any long-term impairment, in one or more domain; [5] Impairment in two or more domains; [6] biallelic β-globin copies.