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The effect of early life cytomegalovirus infection on the immune profile of children

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ABSTRACT

Cytomegalovirus (CMV) infection has a life-long impact on the immune system, particularly on memory T cells. However, the effect of early life CMV infection on the phenotype and functionality of T cells in infants and especially longitudinal changes occurring during childhood have not been explored in detail.

The phenotype and functionality of peripheral blood CD8+ and CD4+ T cells from children infected with CMV in early life (< 6 months of age) was analyzed using high-dimensional flow cytometry. Samples from CMV IgG-seropositive (CMV+) children were collected at 6 months and 6 years of age and compared to samples from CMV-seronegative (CMV-) children.

Early life CMV infection caused multiple alterations within T cells. These include downregulation of CD28 expression and upregulation of CD57 expression within both CD27+ early and CD27- late effector memory CD8+ and CD4+ T-cells at 6 months of age. Of these changes, only alterations within the highly differentiated late effector memory compartment persisted at the age of 6 years.

Early life CMV-infection has a distinct impact on developing CD8+ and CD4+ memory T cell compartments. It appears to induce both temporary as well as longer-lasting alterations, which may affect the functionality of the immune system throughout life.

1. Introduction

The early stages of life play a crucial role in the development of a functional immune system. The genetic background of an individual, but even more dominantly environmental exposures, such as gut microbiota composition, infections and vaccinations influence how the immune system, in particular adaptive immunity, develops during the first months and years of life [31,32]. In addition to protective immunity, this period is believed to strongly influence the susceptibility to develop immune-mediated diseases, such as allergies [40] and autoimmunity [50].

Human cytomegalovirus (CMV) is a ubiquitous herpes virus with adult seroprevalence ranging from 60 to 90% globally [51]. It establishes a lifelong latent infection, with reactivations and reinfections sporadically [6]. CMV infection is commonly acquired during the first months of life [1]. Congenital CMV infection is rare but can cause

Abbreviations: CM, central memory; CMV, cytomegalovirus; PI, Polyfunctionality index; TEM, T effector memory; TEMRA, T effector memory re-expressing CD45RA; tSNE, T-Distributed Stochastic Neighbor Embedding.

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symptomatic, even severe infection [36]. The vast majority of early life CMV infections are asymptomatic and are transmitted postnatally through saliva or breast milk following reactivation of the latent virus in the mother [36].

CMV infection is one of the strongest environmental factors affecting the immune system. Previous studies have shown that between 13 and 50% of immune parameters studied are affected by CMV infection [4,33]. The most notable changes occur among T cells, in particular CD8+ T cells. CMV infection leads to a drastic inflation of T-cell memory, with up to 30 to 40% of all memory T cells targeting CMV epitopes in infected individuals [38,45].

Upon initial activation, naïve T cells (CD45RA+CCR7+CD27+) undergo phenotypic changes and progressive differentiation from the central memory (CM) T-cell subset (CD45RA-CCR7+CD27+) to CD27+ early effector memory (TEM) subset, then to CD27- late TEM T-cell subset (both CD45RA-CCR7-), and finally to the effector memory re-expressing CD45RA (TEMRA) subset (CD45RA+CCR7-CD27-) [23]. CMV-specific T cells are predominantly found within the highly differentiated CD27- late TEM and TEMRA memory T-cell subsets and exhibit a specific "advanced differentiation phenotype" characterized by increased expression of CD57, KLRG1, CX3CR1, CD56 and NKG2C [2,16,17,43,46,49]. Both CMV-specific CD8+ and CD4+ T cells also display strong polyfunctionality, producing high levels of proinflammatory cytokines such as IFN-γ and TNF-α [7,34,35].

While most studies on the effects of CMV infection on blood immune cell frequencies and phenotype have focused on adults [2,16,17,43,46,49], it appears that early life CMV infection induces similar changes also in children [29,30,47]. However, longitudinal studies tracking changes in blood immune cells shortly after infection and throughout the latent phase in children are still limited.

In this study, we analyzed samples from children with confirmed early life CMV infection, i.e., infection before the age of 6 months, at the time points of 6 months and 6 years of age. Our aim was to study the impact of CMV infection on the development of T-cell memory during the maturation of the immune system.

2. Materials and methods

2.1. Study cohort

The samples used in the study were collected during the years 2009–2016 from healthy children participating in the prospective Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study [18]. All children carried HLA class II genotypes associated with increased risk for type 1 diabetes but remained negative for disease-associated autoantibodies during the follow-up. 139 children with available frozen peripheral blood mononuclear cell (PBMC) samples collected at the age of 6 months were chosen to be screened for CMV-specific IgG antibodies utilizing plasma samples collected at the same time point. Since maternal CMV IgG antibodies may still be detectable from sera at 6 months after birth, serum samples collected at the age of 12 months from children with CMV IgG antibodies were also tested to confirm CMV-seropositivity (Supplementary Table 1). The children positive for CMV antibodies at both time points ($n = 20$) were considered to have had an early life CMV infection, i.e., infection at 0–6 months of age. All the CMV+ cases were matched with two CMV antibody negative (CMV-) subjects (age and year of sample collection). Sex and HLA class II genotypes of the study subjects are shown in Supplementary Table 2. Follow-up samples were selected based on availability of a PBMC sample at 6 years of age and persistent CMV IgG antibody positivity ($n = 10$) or negativity ($n = 11$). The study protocol for the DIPP study was approved by the ethics committee of the Hospital District of Northern Ostrobothnia. All children and/or their legal guardians provided written informed consent, as mandated by the Declaration of Helsinki.

2.2. CMV IgG-antibody measurement

CMV-specific IgG antibodies were measured from plasma samples stored at −80 °C at the University of Turku with the VIDAS CMV IgG assay (bioMérieux S.A.), according to the manufacturer's instructions.

2.3. HLA sequencing

For the selection of samples for HLA class I tetramer staining, HLA-A*02 genotyping was performed in samples from the study subjects, as previously described [5].

2.4. Sample preparation

PBMCs were isolated from blood samples by Ficoll gradient centrifugation and stored at −150 °C until analyses. Samples were thawed at a +37 °C water bath and resuspended in 10 ml of prewarmed RPMI 1640 (Sigma-Aldrich) medium supplemented with 5% inactivated human AB serum (Sigma-Aldrich), 2 mM L-glutamine, 20 μM 2-mercaptoethanol, 1 mM sodium pyruvate, nonessential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (all from Lonza). After washing, the samples were treated with 10% DNase I (Stemcell) in RPMI+5% AB for 15 min at RT and washed again with RPMI+5% AB.

Cells were counted and rested overnight in RPMI+5% AB at a density of 2 million cells/ml at +37 °C and 5% CO_2 before flow cytometry analyses. The viability of the PBMCs before flow cytometry analyses was routinely >94%, as assessed by viability staining.

2.5. Surface and HLA class I tetramer staining

Samples were first stained with the Zombie Aqua viability dye (BioLegend) at a 1:800 dilution for 15 min at RT and then washed with 1 ml of PBS + 0.5% BSA. Next, the samples were resuspended in 30 μl of PBS + 0.5% BSA together with antibody cocktails (Supplementary Table 3) in the presence of Brilliant Stain Buffer Plus (BD Biosciences). Samples were incubated for 20 min at RT and washed twice, first with 1 ml of PBS + 0.5% BSA and then with 1 ml of FACS buffer (PBS with 2% FCS and 1% of NaN_3), and subsequently analyzed.

Samples from HLA-A*02-positive children were additionally stained with HLA class I tetramers prior to the surface staining step described above. These samples were first incubated with 50 nM dasatinib (Sigma-Aldrich) in 50 μl at +37 °C 5% CO_2 for 10 min. Then 2.5 μl of HLA-A*02:01 CMV pp65(NLVPMVATV) iTAg MHC Class I tetramer-PE (Beckman Coulter) was added for the detection CMV-specific CD8+ T cells. Samples were incubated for 30 mins in RT and washed once with 1 ml 0.5% BSA + PBS.

2.6. Stimulation and intracellular cytokine staining

Samples were stimulated for 4 h at +37 °C 5% CO_2 in 1 ml RPMI+5% AB together with PMA (50 ng/ml) and ionomycin (1 μg/ml) (both from Sigma). Anti-human CD107a BB700 (BD) was added at the beginning of the stimulation and 1 h later brefeldin A (3 μg/ml) (eBioscience) and monensin (20 μM) (BioLegend) were added.

After stimulation, samples were first stained for surface markers and in some cases also with HLA class I tetramers, as described above, followed by fixation in 100 μl of fixative (Intracellular fixation & permeabilization buffer set, eBioscience) for 30 min in RT. After this, the samples were washed once with 1 ml PBS + 0.5% BSA and stored at +4 °C overnight. The next day, the samples were washed twice with 1 ml Perm buffer (eBioscience) and resuspended in 30 μl of Perm buffer for intracellular staining. Antibody cocktail (Supplementary Table 3) was added, and samples were incubated for 30 min at RT. After incubation, samples were first washed with Perm buffer and then with FACS buffer before analysis.

2.7. Flow cytometry analyses

The samples were acquired with the Novocyte Quanteon flow cytometer (Agilent) and the FCS files were analyzed with FlowJo (version 10.8.1, BD Biosciences). The manual gating strategy for the staining panels is shown in Supplementary Figs. 1–3. Dimensionality reduction was performed with FlowJo using the native platform of T-Distributed Stochastic Neighbor Embedding (tSNE) and Barnes-Hut gradient algorithm in FlowJo. The clustering algorithm FlowSOM [48] was implemented separately for pregated total CD8+ and CD4+ T cells in staining panel 1 and for CD8+ and CD4+ memory T cells in staining panel 2. All flow cytometry assays and analyses were performed in accordance to the 'Guidelines for the use of flow cytometry and cell sorting in immunological studies' [8].

2.8. Statistics

Statistical analyses were performed with Prism software (GraphPad). The Mann-Whitney *U* test was used. *P* values of <0.05 were considered as statistically significant. Polyfunctionality index (PI) was calculated as previously described [24].

3. Results

3.1. Early life CMV infection leads to the persistent expansion of CD27- effector memory CD8+ and CD4+ T cells

139 children were screened for CMV-specific IgG antibodies and 20 of them (14.4%) had antibodies against CMV both at the age of 6 and 12 months and were thus considered to have had an early life CMV infection.

We analyzed the immune cell composition of these 20 CMV+ children, as well as 41 CMV- control children at the age of 6 months by flow cytometry. A sequential sample collected at the age of 6 years was also analyzed from 10 of the 20 CMV+ and 11 of the 41 CMV- children.

First, we used unsupervised clustering to separate distinct differentiation states of CD8+ and CD4+ T cells based on the expression of CD45RA, CCR7, CD27, CD28, CD38, and CD57 (Fig. 1A and B). Six separate clusters were detected in CD8+ T cells, representing CD45RA+CCR7+ naïve T cells (cluster 1), CD45RA-CCR7+ central memory (CM) T cells (cluster 2), and four different clusters of CD45RA+/-CCR7- effector memory T (TEM) cells (clusters 3–6; Fig. 1B). After early life CMV infection the frequency of naïve CD8+ T cells at 6 months decreased compared to CMV- control children due to increase in total memory CD8+ T cells. However, at 6 years of age the difference between the groups had disappeared (Fig. 1C).

The inflated memory compartment in CMV+ children at 6 months was caused by the accumulation of highly differentiated late TEM cells lacking CD27 and CD28 expression but expressing the activation marker CD38 (cluster 5), and particularly a subset of these also upregulating CD45RA and CD57 (cluster 6). The clusters representing less differentiated CD27+ early TEM cells (clusters 3 and 4) were correspondingly decreased in CMV+ children. At the age of 6 years, only the increase in the most highly differentiated CD57+ TEM cluster 6 persisted in CMV+ children (Fig. 1C).

CD4+ T cells could similarly be separated into five clusters representing CD45RA+CCR7+ naïve T cells (cluster 1), CD45RA-CCR7+ CM T cells (cluster 2), and three different clusters of CD45RA+/-CCR7- TEM cells (clusters 3–5). Of these, cluster 5, representing the most highly differentiated CD57+ late TEM cells, was expanded at both the age of 6 months and 6 years in CMV+ children but was absent in CMV- children (Suppl. Fig. 4).

These findings were corroborated through manual gating of the flow cytometry data. (Suppl. Fig. 5). Specifically, when we separated memory T cells into CCR7+CD45RA- CM, CD27+/-CCR7-CD45RA- late/early TEM and CD27-CCR7-CD45RA+ effector memory re-expressing CD45RA (TEMRA) T-cell subsets, an increase in CD27- late TEM was detected in CMV+ children only at 6 months but an increase in TEMRA also at 6 years within both CD8+ and CD4+ T cells. Consistent with this, the analysis of CD8+CCR7- and CD4+CCR7- memory T cells (TEM and TEMRA subsets) demonstrated a higher frequency of highly differentiated CD27- T cells in CMV+ compared to CMV- children at both 6 months and 6 years (Suppl. Fig. 5).

To further analyze the CMV-associated memory T cells we performed unsupervised clustering of memory CD4+ and CD8+ T cells based on the expression of CD45RA, CCR7, CD27, as well as additional activation/differentiation markers KLRG1, TIGIT, CX3CR1 and PD-1 using a separate staining panel (Fig. 1D and E). Within CD8+ memory T cells, CD45RA+ TEMRA cells expressing CX3CR1 (cluster 7) and a subset of these expressing also KLRG1 (cluster 8) were increased in CMV+ children at the ages of 6 month and 6 years (Fig. 1F). The CD45RA-CD27- late TEM cell cluster lacking KLRG1 and CX3CR1 expression (cluster 6) was only increased at 6 months but not at 6 years in CMV+ children (Fig. 1F). Within CD4+ T cells, CD27- TEM cells expressing KLRG1 and CX3CR1 (cluster 8) were also expanded in CMV+ children at 6 months. (Suppl. Fig. 6).

Taken together, both CD8+ and CD4+ CD27- late TEM and TEMRA subsets were expanded in CMV+ children at 6 months, but of these only the expansion of the most highly differentiated TEMRA subsets expressing CD57, KLRG1 and CX3CR1 at high levels persisted at 6 years.

3.2. Early life CMV infection causes transient alterations within CD27+ early effector memory T cells but more persistent changes within CD27- late effector memory T cells

To further study the phenotypical changes in memory CD8+ and CD4+ T cells at early (6 months) and latent (6 years) stages of early life CMV-infection, we next analyzed the less differentiated CD27+ early TEM and highly differentiated CD27- late TEM memory T-cell subsets separately. Interestingly, several markers (CD38, CD57, TIGIT, and CX3CR1) were expressed at higher levels and CD28 at lower levels within CD27+ early TEM CD8+ T cells in CMV+ compared to CMV- children at 6 months (Fig. 2A). However, none of these changes were detected at the age of 6 years. Similar changes, together with decreased PD-1 expression, were also detected within CD27- late TEM CD8+ T cells in CMV+ children at 6 months (Fig. 2B). Of these, only the increased expression of CD57 and decreased expression of CD28 and PD-1 could be observed at 6 years.

Similar to CD8+ T cells, CD57 expression was increased and CD28 expression was decreased both within CD27+ and CD27- early and late TEM CD4+ T cells in CMV+ children at 6 months, and only the changes within late TEM CD4+ T cells were detected at 6 years. Moreover, increased CD38 and CX3CR1 was also detected within late TEM CD4+ T cells in CMV+ children at 6 months and the increase of CX3CR1 was also observed at 6 years. Interestingly, some differences between CD8+ and CD4+ memory T cells were also noted. Namely, in contrast to late TEM CD8+ T cells, the expression of PD-1 and KLRG1 were increased in late TEM memory CD4+ T cells but TIGIT expression was unaffected in CMV+ children at 6 months. The increase in KLRG1 expression was also detectable in late TEM memory CD4+ T cells at 6 years. (Suppl. Fig. 7).

Taken together, after primary CMV-infection alterations were detected within the less differentiated CD27+ early TEM CD8+ and CD4+ T cells at the age of 6 months, but these disappeared with time. Moreover, alterations within CD27- late TEM cells were also more profound at the age of 6 months compared to the analysis at 6 years. In particular, the expression of the activation marker CD38 was only increased at 6 months. Finally, some alterations caused by early life CMV-infection appeared to differ between the CD8+ and CD4+ memory T cells.

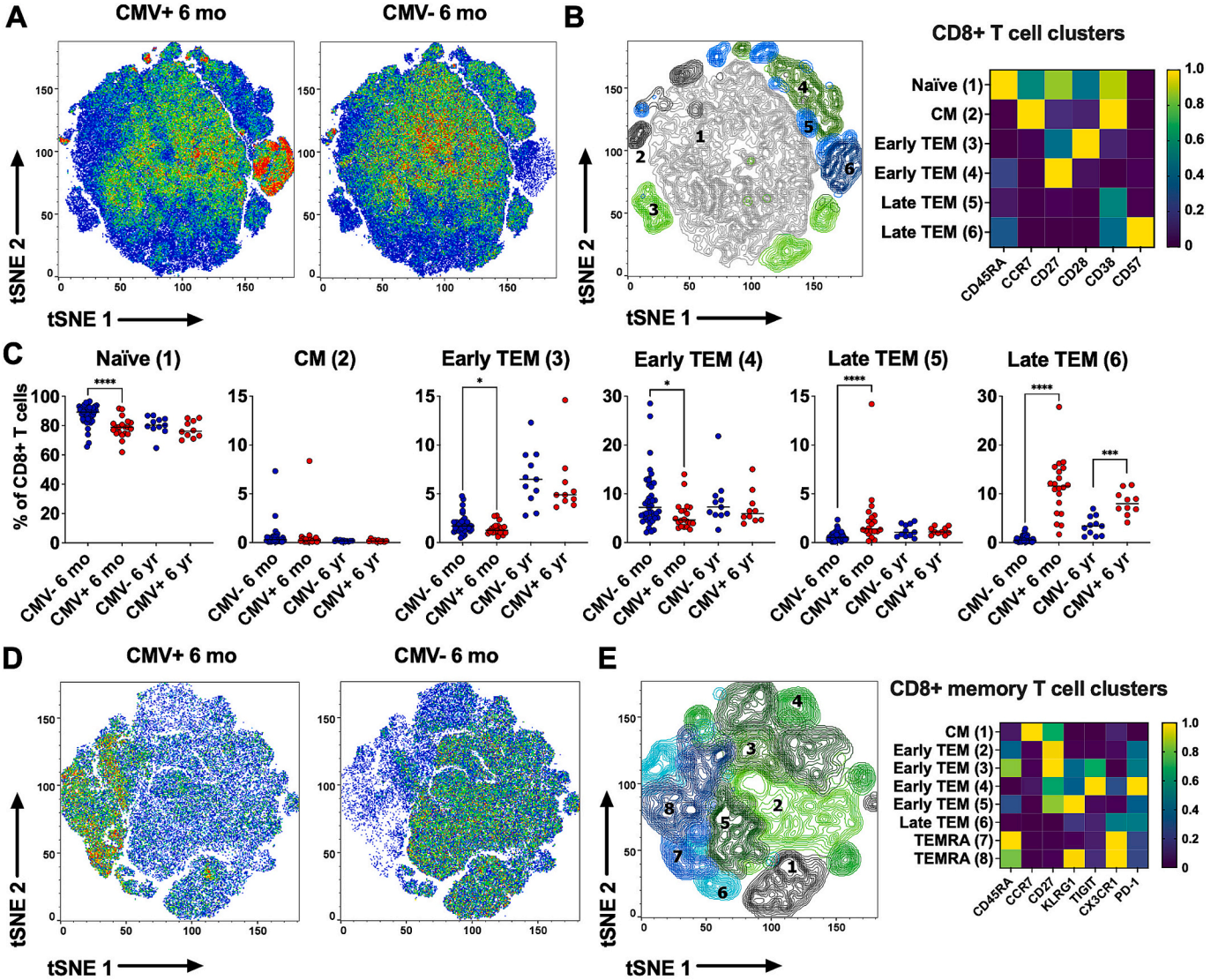

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Fig. 1. High-dimensional flow cytometry analysis of CD8+ T-cell differentiation status in CMV+ and CMV- children. (A) tSNE projection of CD8+ T-cells from concatenated samples (20,000 CD8+ T cells per sample) from CMV+ (left) and CMV- (right) children at 6 months of age (staining panel 1). (B) tSNE projection of FlowSOM clusters and normalized mean fluorescence intensity (MFI) of surface marker expression within the different clusters. (C) Frequency of CD8+ T cells within each cluster at the ages of 6 months and 6 years, blue dots presenting CMV- and red dots CMV+ children. (D) tSNE projection of memory CD8+ T-cells from concatenated samples (3000 memory CD8+ T cells per sample) from CMV+ (left) and CMV- (right) children at 6 months of age (staining panel 2). (E) tSNE projection of FlowSOM clusters and normalized mean fluorescence intensity (MFI) of surface marker expression within the different clusters. (F) Frequency of memory CD8+ T cells within each cluster at the ages of 6 months and 6 years, blue dots presenting CMV- and red dots CMV+ children. Frequencies were compared between the groups using the Mann-Whitney *U* test and the horizontal lines indicate median values. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

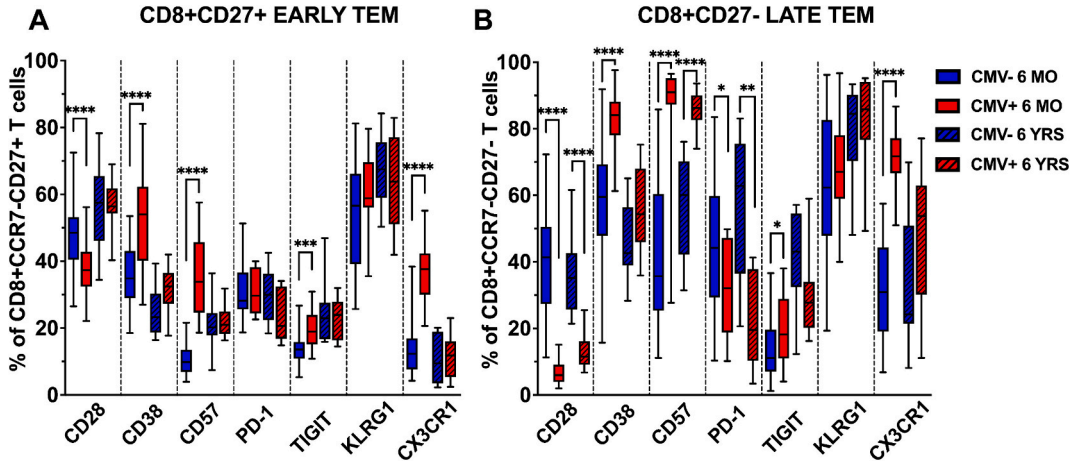

Fig. 2. CMV-associated changes in CD27+ early and CD27- late TEM CD8+ T cells. Marker expression within CD27+ early (A) and CD27- late CD8+ TEM (B) subsets among CMV+ (red) and CMV- (blue) children at the ages of 6 months (solid bars) and 6 years (striped bars). Mann-Whitney U test was used for statistical analyses. Median values are presented as horizontal lines and interquartile range as boxes with whiskers from min to max. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Early life CMV infection causes a persistently increased production of proinflammatory cytokines in CD27- late TEM CD8+ T cells

Next, we analyzed the functionality of CD8+ and CD4+ T cells in CMV+ and CMV- children. For this, PBMCs were stimulated with PMA and ionomycin, and the production of proinflammatory cytokines (IFN-γ, TNF-α, GM-CSF and IL-2), as well as the expression of the cytotoxic degranulation marker CD107a were assessed. Again, we analyzed the CD27+ early TEM and CD27- late TEM subsets separately at the 6 months and 6 years time points. An increased frequency of CD27+ early

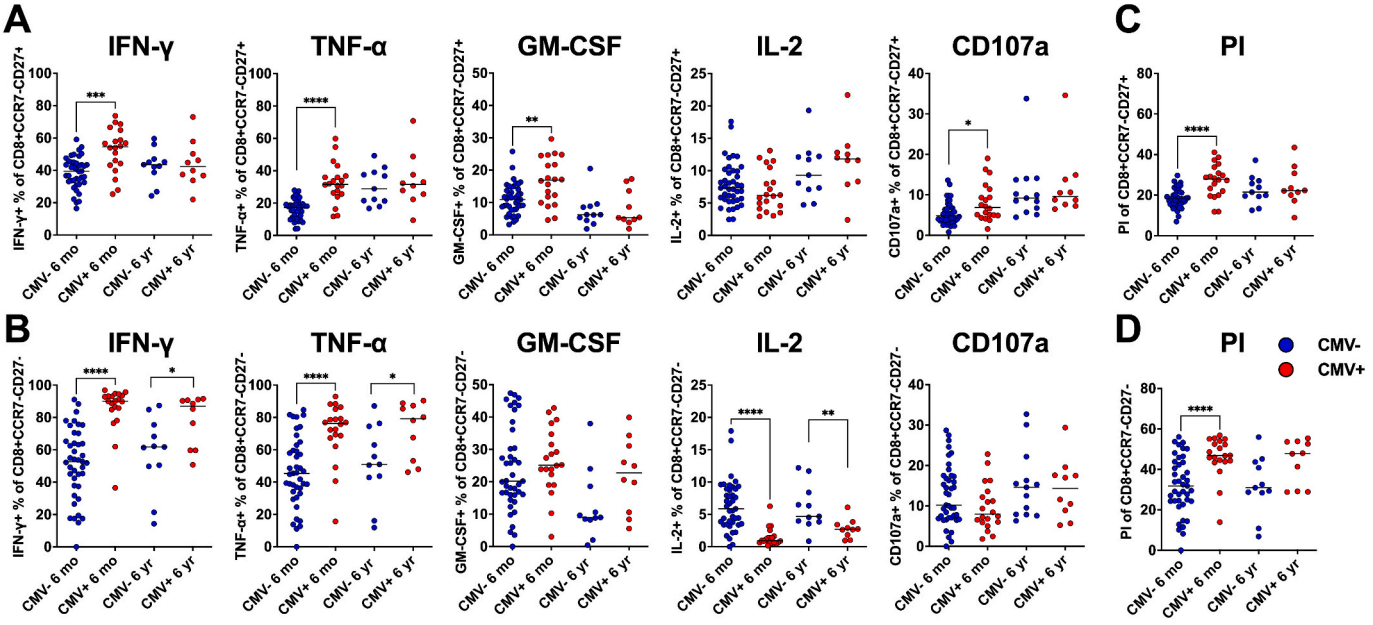

Fig. 3. Polyfunctionality within CD27+ early and CD27- late TEM CD8+ T cells. Cytokine production and CD107a expression by CD27+ (A) and CD27- CD8+ TEM (B) subsets among CMV+ (red) and CMV- (blue) children at the ages of 6 months and 6 years. Calculated polyfunctionality index (PI) in CD27+ early TEM (C) and CD27- late TEM (D) CD8+ T cell subsets. Mann-Whitney U test was used for statistical analyses and the horizontal lines indicate median values. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TEM CD8+ T cells producing IFN-γ, TNF-α and GM-CSF was observed in CMV+ children at 6 months but not at 6 years (Fig. 3A). Within CD27- late TEM CD8+ T cells, an increased frequency of IFN-γ+ and TNF-α+ cells and a decreased frequency of IL-2+ cells were observed both at 6 months and 6 years (Fig. 3B). The capacity of T cells to produce multiple cytokines can be expressed through a calculated polyfunctionality index (PI). PI values were higher in CMV+ children within both CD27+ and CD27- early and late TEM CD8+ T cells at 6 months but not at 6 years (Fig. 3C and D).

Alterations within CD27+ and CD27- early and late TEM CD4+ T cells were less obvious. Within CD27- late TEM CD4+ T cells only the production of IFN- γ was increased and GM-CSF and IL-2 decreased at the age of 6 months but not at 6 years (Suppl. Fig. 8).

In summary, we observed a highly polyfunctional, proinflammatory cytokine production profile after early life CMV infection within both CD27+ and CD27- early and late TEM CD8+ T cells at 6 months. However, again, only the changes within the late TEM subset persisted at 6 years.

3.4. The phenotype of CMV-specific CD8+ T cells corresponds to the changes observed in polyclonal CD8+ T cells after early life CMV infection

To confirm that the observed changes detected in polyclonal CD8+ T cells after early life CMV infection reflect the phenotype of CMV-specific CD8+ T cells in blood, we stained the PBMCs of 8 CMV+ and 8 CMV- children carrying the HLA-A*02 genotype using HLA class I tetramers loaded with the CMV pp65 peptide (Fig. 4A). The frequency of tetramer-positive CD8+ T cells was clearly higher in CMV+ compared to CMV- children at the age of 6 months (mean 0.39% +/− 0.37 SD and mean 0.02% +/− 0.01 SD of CD8+ T cells, respectively; Fig. 4B). Moreover, the tetramer-positive T cells in CMV+ children were predominantly memory T cells, whereas the few tetramer-positive T cells in CMV- children were of naïve phenotype (Fig. 4C). Mapping the tetramer-positive cells to the phenotypic clusters generated earlier for polyclonal CD8+ T cells (Fig. 4D and E) revealed that also the CMV-specific CD8+ T cells were enriched within the highly differentiated CD27- late TEM clusters (clusters 5 and 6 in Fig. 1B and clusters 6–8 in Fig. 1E).

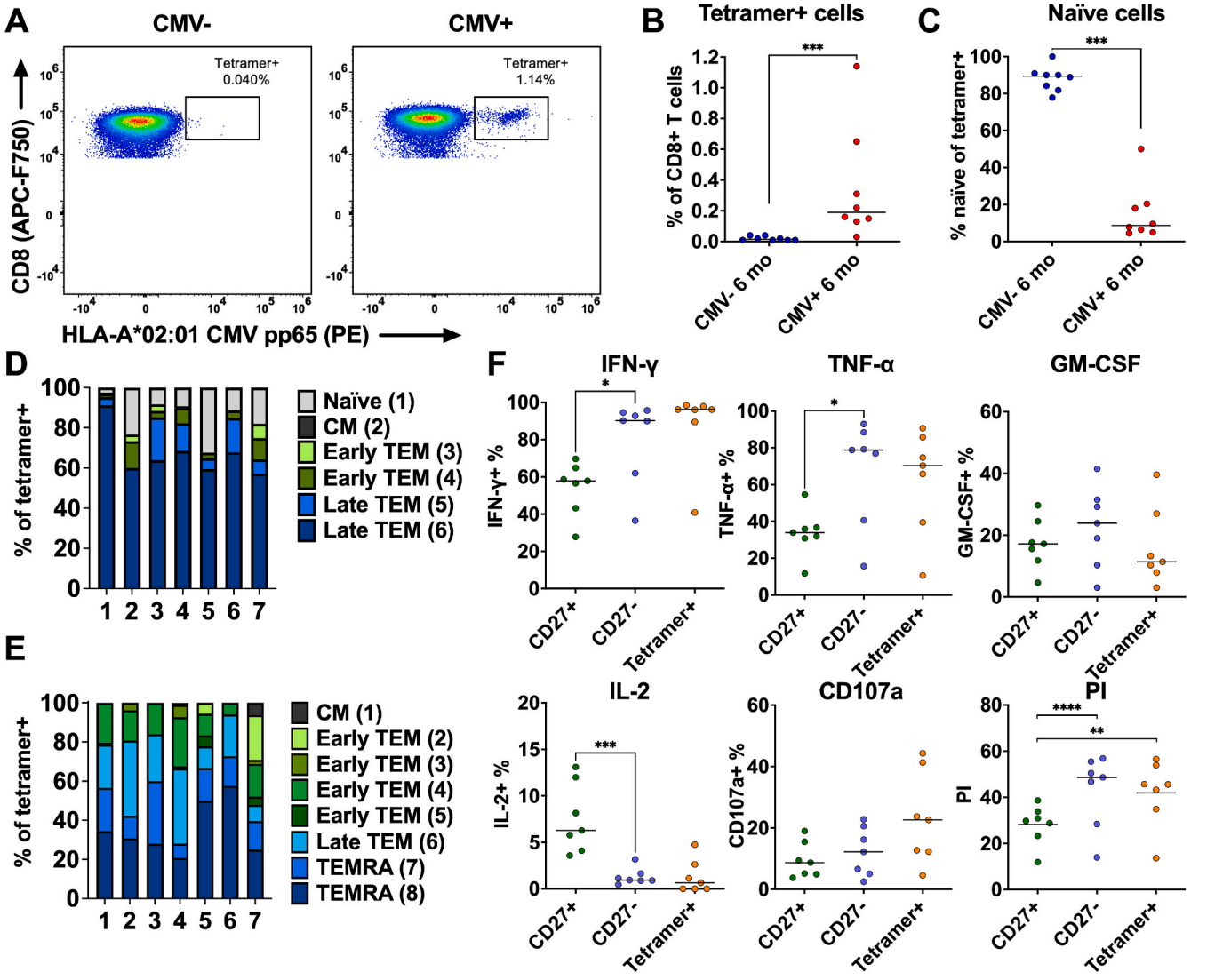

Fig. 4. The phenotype of CMV-specific CD8+ T cells corresponds to the phenotype observed in polyclonal memory CD8+ T cells in CMV-infected children. (A) Representative bivariate plots of CMV HLA class I tetramer staining in CD8+ T cells from a CMV- (left) and a CMV+ child (right). The frequencies of tetramer+ CD8+ T cells (B) and the proportion of naïve T cells within the tetramer+ fraction (C) in children at the age of 6 months. Distribution of tetramer+ T cells from CMV+ children (1–7) within the FlowSOM clusters generated for total (D) and memory (E) CD8+ T cell analyses in Figs. 1B and E. (F) The frequency of cytokine producing and CD107a-expressing tetramer+ CD8+ T cells (orange) compared to that in polyclonal CD27+ (green) and CD27- (blue) CD8+CCR7- memory T cells from the same CMV+ children. Mann-Whitney U test was used for statistical analyses and the horizontal lines indicate median values. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Analysis of cytokine production by CMV-specific CD8+ T cells also corroborated the findings detected within polyclonal CD8+ T cells, as tetramer-positive CD8+ T cells exhibited a highly polyfunctional cytokine secretion profile, with increased expression of IFN-γ and TNF-α together with decreased expression of IL-2 (Fig. 4F).

3.5. Early life CMV infection causes minor alterations in other circulating immune cell subsets

Finally, we analyzed whether early life CMV infection influences the frequencies of other innate or adaptive immune cells. We determined the frequencies of total CD3+, CD4+ and CD8+ T cells, as well as CD19+ B cells at the ages of 6 months and 6 years but could not detect differences between CMV+ and CMV- children (Suppl. Fig. 9). Moreover, the frequencies of NK cells, regulatory T cells (Treg), circulating follicular (Tfh) and peripheral helper T cells (Tph), as well as Th1, Th17 and Th2 cells were similar between CMV+ and CMV- children. Interestingly, at the age of 6 months the frequency of classical monocytes (CD14+CD16-) was lower in CMV+ children due to a proportional increase in CD16+ inflammatory monocytes. In line with previous studies [30,43,49], both CD56dimCD16+ NK cells and CD8+ T cells in CMV+ children expressed increased frequencies of the activating NK cell receptor NKG2C. CD8+ T cells in CMV+ children also expressed lower levels of CD127. Of note, CMV+ children harbored increased frequencies of NKT cells (CD3+CD4-CD56+) at the age of 6 years and these cells also displayed an increased expression of NKG2C.

4. Discussion

In this study, we demonstrate that CMV-infection in early life (before 6 months of age) leads to significant changes within both CD8+ and CD4+ memory T cell compartments. The most pronounced alterations occurred shortly after infection, at 6 months of age, affecting both the CD27+ early TEM and CD27- late TEM/TEMRA subsets. However, during the latent phase of the infection, at 6 years of age, only the changes in the late TEM/TEMRA subsets were observed.

Expanded memory CD8+ T cells at the latent phase of CMV infection exhibit a phenotype consistent with the "advanced differentiation phenotype" described extensively in the literature [2,16,17,43,46,49]. These cells display a CD45RA+ TEMRA phenotype, with reduced expression of CD27, CD28, PD-1, and CD127 but increased expression of CD57, KLRG1, CX3CR1, CD56 and NKG2C. All of the upregulated proteins are also expressed by activated NK cells [8,46]. The expression of CD56 and CD57 is associated with high cytotoxic potential on both NK and CD8+ T cells. KLRG1 is a coinhibitory receptor while NKG2C is an activating receptor recognizing the non-classical MHC class I molecule HLA-E. CX3CR1, in turn is a chemokine receptor that mediates the migration of leukocytes to inflamed tissues. This "advanced differentiation phenotype" was detected in this study also early after infection, at 6 months of age, and, interestingly, both within CD27+ early TEM and CD27- late TEM subsets. Additionally, at this early time point, but not at 6 years, there was also increased expression of CD38 across all the TEM/TEMRA subsets. CD38 is expressed by T cells upon activation and is therefore considered to reflect recent activation status [8]. Collectively, these findings suggest a differentiation model in which early life CMV infection leads to an initial expansion of CD27+ early TEM cells, which subsequently differentiate into CD27- TEM and ultimately into TEMRA cells. Of these changes, only the TEMRA cell expansion persists through later life. Importantly, similar T-cell differentiation kinetics have previously been observed between acute and late phases of primary CMV infection in adult lung transplant patients [17].

In contrast to CD8+ T cells, the phenotype of expanded memory CD4+ T cells in the context of CMV infection has been less extensively studied. Here, we demonstrate that expanded memory CD4+ T cells exhibit a largely similar "advanced differentiation phenotype" as CD8+ T cells, a finding reported also in previous studies [35,47]. However, the magnitude of TEM/TEMRA expansions in children was lower in CD4+ T cells, as also previously reported [47]. The reason for this difference between CD4+ and CD8+ T cells is not known, but at least it is not directly explainable by a larger expansion of CMV-specific CD8+ T cells, as the frequencies of CMV-specific T cells within the CD4+ and CD8+ memory T cell compartments have been demonstrated to be largely comparable [38,45]. Interestingly, the expression of PD-1, a marker associated with T-cell activation/exhaustion, appeared to be increased in CD4+ memory T cells, although it was observed not to be increased in CD8+ memory T cells following CMV infection by us and others [16,43],

Using HLA class I tetramers, we demonstrated that on average 0.4% of CD8+ T cells in CMV-infected children were specific to a single CMV epitope at 6 months of age. This finding is consistent with previous studies in both children and adults using the same HLA class I tetramer [20,22]. Importantly, these CMV-specific T cells constitute a significant portion of the total CD8+ memory pool (ranging from 0.6 to 3.7% of memory CD8+ T cells in the children studied) and exhibit a phenotype similar to that of the expanded polyclonal memory CD8+ T cells. Previous studies assaying T-cell responses to multiple CMV epitopes demonstrated that even up to 30–40% of all memory CD8+ and CD4+ T cells can be specific to CMV [38,45]. Collectively, these results suggest that alterations in polyclonal T cells can serve as a reliable proxy for CMV-specific memory T-cell alterations.

A major strength of our study is the analysis of samples obtained from children with confirmed early life CMV infection at two distinct time points, with a time gap of nearly 6 years between the samples. However, a limitation of the study is the lack of precise information on the timing of CMV infection in the children studied. Based on the low frequency of congenital CMV infection in Finland (0.2%, according to [39]), we can reasonably assume that the majority, if not all, of the CMV-positive children (accounting for 14.4% of all children screened at the age of 6 months) acquired the infection postnatally within the first months of life. Nonetheless, this uncertainty regarding the timing of the infection (ranging from 0 to 6 months before sampling) may introduce some variation in our data at the 6-month sampling point. However, previous research has indicated that it can take up to 3 years for CD8+ memory T-cell responses to resemble those observed during the latent phase of CMV infection [11,16]. Therefore, our analysis at 6 months of age should be representative of an early T-cell response to CMV.

The clinical significance of persistent CMV infection has been well established in elderly populations, where it has been associated with increased mortality and cardiovascular disease risk [14,41,42,44]. Mechanistically, this effect is thought to be mediated by increased immunosenescence due to a decline in naïve T cells and an increase in senescent TEMRA cells, as well as by the increased inflammatory response due to persistent CMV infection [21]. The impact of persistent CMV infection on the efficacy of influenza vaccination, however, is still a matter of debate, with conflicting reports of both diminished and enhanced effects on influenza-specific immune responses [9,12,13,27]. The clinical effects of early life CMV infection on the immune responses in children are less well-established, although CMV infection clearly has a profound effect on the T-cell repertoire, as also evidenced in this study. One study of Gambian infants demonstrated a lack of effect by early life CMV infection on antibody responses to vaccination with measles, tetanus toxoid or *Haemophilus influenzae* type B vaccines [28]. There is also some evidence that early life CMV infection may have a beneficial effect on the risk of developing autoimmunity. Our own previous study demonstrated a decreased rate of progression to clinical type 1 diabetes in at-risk children testing positive for autoantibodies [10]. A similar protective effect on the development of celiac disease, another autoimmune disease, has also been reported [19,37]. Additionally, in adults, CMV infection has been associated with a reduced risk of developing multiple sclerosis [3,15]. Whether the T-cell alterations caused by CMV infection directly contribute to suppression of autoimmunity is currently unclear. However, recent research suggests that certain terminally-differentiated CD8+ T-cell phenotypes may be associated with

suppression of autoimmunity [25] or beneficial treatment effect upon immunotherapy [26].

5. Conclusion

In summary, our analysis demonstrates that early life CMV infection appears to lead to both transient and more stable alterations within both CD8+ and CD4+ memory T-cell compartments of young children. Additional studies with larger study cohorts are, however, still needed to validate our results and to establish the potential clinical impact of early life CMV infection and/or CMV-induced immune alterations during childhood and throughout life.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

IE and A-MS designed and performed the experiments. TV was responsible for the CMV antibody analyses. MK and RV were responsible for the analyses of diabetes-associated autoantibodies in the study children. JT provided the clinical samples. JI and JL were responsible for the HLA screening of the study children. IE and TK analyzed the data and drafted the manuscript. All authors contributed to the final version of the manuscript. TK is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

CRediT authorship contribution statement

Ilse Ekman: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Anna-Mari Schroderus:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Tytti Vuorinen:** Writing – review & editing, Methodology. **Mikael Knip:** Writing – review & editing, Resources, Project administration, Methodology. **Riitta Veijola:** Writing – review & editing, Resources, Project administration, Methodology. **Jorma Toppari:** Writing – review & editing, Resources, Project administration. **Jorma Ilonen:** Writing – review & editing, Resources, Project administration, Methodology. **Johanna Lempainen:** Writing – review & editing, Resources, Project administration, Methodology. **Tuure Kinnunen:** Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2024.110330.

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