Altered $CD4^+T$ cell immunity in nurses occupationally exposed to viral pathogens

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Title: Altered CD4$^+$ T-Cell Immunity in Nurses Occupationally Exposed to Viral Pathogens

Short title: Viral Exposure Alters CD4$^+$ T-Cells in Nurses

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Author Contributions:

BO conceived the study; GE, ES, VVT, BO designed the study; GE, AS, SH, HJ, BO performed the experiments; GE, AS, VVT, BO analysed and interpreted the data; PB, PVD, ES, PT, VVT, BO supervised the study; GE wrote the first draft and all other authors contributed to the final version.

Keywords:

T-cell, varicella zoster virus, cytomegalovirus, immunosenescence, immune signatures

Abbreviations

CM	**central memory**
CMV	**Cytomegalovirus**
DMSO	**Dimethyl Sulfoxide**
EBV	**Epstein–Barr virus**

EM	**effector memory**
EGF	**Epidermal growth factor**
FACS	**Fluorescence-activated cell sorting**
FGF2	**fibroblast growth factor 2**
Flt3.Ligand	**FMS-like tyrosine kinase 3 ligand**
GCSF	**granulocyte colony-stimulating factor**
GMCSF	**granulocyte-monocyte colony-stimulating factor**
GRO	**growth-regulated oncogene**
HSV	**Herpes Simplex virus**
IFN-γ	**interferon-γ**
IL-2	**Interleukin-2**
IP10	**INF-γ inducible protein 10**
MCP1	**monocyte chemoattractant protein-1**
MCP3	**monocyte chemoattractant protein-3**
MDC	**macrophage-derived chemokine**
MIP-1α	**macrophage inflammatory protein-1α**
MIP-1β	**macrophage inflammatory protein-1β**
NICU	**neonatal intensive care unit**
PED	**paediatric**
PHA	**Phytohemagglutinin**
sCD40L	**soluble CD40 ligand**
EMRA	**terminally differentiated effector memory**
TGFa	**transforming growth factor alpha**
TNFa	**tumor necrosis factor-α**
TNFb	**tumor necrosis factor-β**
TT	**tetanus toxoid**

VEGF	**vascular endothelial growth factor**
VZV	**Varicella zoster virus**
VZV gE	**VZV glycoprotein E**
VZV IE62	**VZV intermediate-early protein 62**
VZV IE63	**VZV intermediate-early protein 63**

Summary

Pathogen exposure, including but not limited to herpesviruses, molds the shape of the immune system, both at a basal state and in response to immune challenge. However, little is known about the impact of high exposure to other viruses on baseline immune signatures, and how the immune system copes with repetitive exposures to maintain a balanced functionality. Here we investigated baseline immune signatures, including detailed T-cell phenotyping, antigen-specific $CD4^+$ and $CD8^+$ T-cell responses and cytokine profile in paediatric (PED) nurses, who have a high occupational exposure to viral pathogens including varicella zoster virus (VZV) and respiratory viruses, and in neonatal intensive care unit (NICU) nurses, as a control group with infrequent occupational exposure.

Our results show a lower $CD4^+$ T-cell response to two VZV proteins (IE62 and gE), and to tetanus toxoid (TT) in PED nurses who are CMV-seronegative, as compared to CMV-seronegative NICU nurses, and that the decline might be more pronounced the more sustained the exposure is. This decline might be due to an attrition of VZV and TT-specific T-cells as a result of the continuous pressure on the $CD4^+$ T-cell compartment. Moreover, our data suggests that the distinct T-cell phenotypes known to be associated with CMV-seropositivity might be less prominent in PED nurses compared to NICU nurses, implying a plausible attenuating effect of occupational exposure on CMV-associated immunosenescence. Overall,

this pilot study reveals an impact of occupational exposure to viral pathogens on $CD4^+$ T-cell immunity and supports further investigation in a larger cohort.

Introduction

First exposure and re-exposure to pathogens mold the shape of the human immune system, whether by natural encounter or through vaccination [1]. It is thought that the memory T-cell compartment is limited in size and T-cells with a high diversity and rapid dynamics must coexist within a contested space [2, 3]. This paradigm suggests that repetitive exposure to pathogens might result in spatial competition between the newly expanded and the pre-existing memory T-cells, wearing down pre-existing immunity. However, more recent work suggests that memory T-cell compartment might be highly expandable and able to accommodate continuous recruitment of memory T-cells without memory attrition [4, 5].

It was recently demonstrated that paediatricians who showed signatures of T-cell inflationary-like expansion as a result of repetitive exposure to different pathogens within a clinical setting were still capable of mounting two to four fold higher immune responses to VZV than their matched controls [6]. The question thus remains how the immune system copes with repetitive exposures to a wide array of pathogens to maintain a balanced functionality [7].

Pathogen exposure influences the composition of the T-cell compartment as determined by expression of CD45 isoforms and lymph node homing receptors CCR7 and CD62L [8]. Moreover, CD28 can be used to assess the differentiation status of T-cells [9]. CD28 was the first described costimulatory molecule in the context of the two signals model of T-cell activation, and binds B7 on antigen-presenting cells (APCs), ensuring initiation of a T-cell response only upon B7 upregulation on properly activated APC. Repeated antigen stimulations of primed T-cells result in a gradual loss of CD28 towards a late-

differentiated phenotype, and numerous studies have shown that ageing is associated with an overall loss of CD28 [10–12]. CD26 is another costimulatory molecule on T-cells and has dipeptidyl peptidase 4 (DPP4) enzymatic activity. It is upregulated on activated T-cells and was shown to be highly expressed on memory T-cells [13, 14]. $CD4^+$ T-cells with high expression of CD26 are enriched for T helper type 1 (T_H1) and 17 (T_H17) cells [15], whereas $CD8^+$ T-cells with high CD26 expression were mostly long-term memory T-cells specific for acute viral infections such as influenza [16]. CD26, as a costimulatory molecule, has not been fully characterized in the context of T-cell differentiation and senescence. Co-stimulation serves as signal 2 during T-cell priming and alterations in co-stimulatory molecule expression could have important implications for the magnitude and quality of T-cell responses; thus, it is important to understand the extent to which repetitive exposure to pathogens contributes to variation in co-stimulation.

Furthermore, cytokines including interferons, tumor necrosis factors, interleukins and chemokines, are instrumental in regulating inflammation, immunity and immunosenescence. Cytokines serve as signal 3 [17] during T-cell priming and altered cytokine levels may affect the threshold or degree of activation of a T-cell. Moreover, dysregulation of normal physiology of those cytokines is associated with various diseases [18–20]. It is plausible that a systemic inflammatory response is induced in highly exposed individuals, and may alter the cytokine profile.

Generally, there are two sources of repetitive antigenic exposure, exogenous and endogenous. Exogenous exposure refers to external encounter of pathogens, such as that encountered by healthcare professionals when caring for ill patients. Endogenous exposure refers to chronic pathogens that co-exist with the host, such as herpesviruses. When considering the effects of exogenous exposures, as determined by occupational exposure, it is important to account for the effects of herpesviruses, as they have been shown to alter immune phenotype at a basal state and in response to immune challenge [21,

22]. Cytomegalovirus (CMV) is known to establish latency upon infection and the persistent production of viral antigens continues to stimulate T-cells, resulting in the accumulation of highly differentiated CMV-specific $CD8^+$ T-cells and to a lesser extent, $CD4^+$ T-cells [23]. Other persistent sources of antigen include Epstein–Barr virus (EBV) and herpes simplex virus (HSV). A history of CMV, EBV or HSV exposure might have an impact on the immune system [24].

Neonatal intensive care unit (NICU) and paediatric nurses (PED) are occupationally exposed to different levels of infectious challenge, with the latter thought to have a higher exposure to viral pathogens, including varicella zoster virus and respiratory viruses. In this exploratory study, we sought to examine the impact of high viral exposure on basal immune signatures, in terms of T-cell functionality and differentiation and cytokine levels, taking into consideration CMV, EBV and HSV serostatus.

Methods

Subjects

Eighteen neonatal intensive care unit (NICU) and 20 paediatric (PED) nurses, representing low-exposure and high-exposure groups, respectively, were recruited in the period between October and December 2013. Only 4 out of the 38 nurses were males, and all worked in a paediatric unit. In order to minimize any bias by gender we excluded all males from the analysis. The age of participants ranged from 22 to 53 years (average 39.4 ± 9.9 years). Number of years working at the respective unit, referred to here as occupational exposure duration (OED), ranged from 1 to 32 years (average 14.1 ± 9.8 years). Peripheral blood (45 mL) was collected into heparinised tubes (Vacutainer®, Lithium heparin) and K2EDTA tubes (Becton Dickinson, NJ, USA) and serum tubes (Terumo, NJ, USA).

This study was approved by the ethics board of the Antwerp University Hospital, Antwerp, Belgium. Written informed consent was obtained from all study participants.

Isolation of peripheral blood mononuclear cells

Within 8 hours after peripheral blood collection, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque Plus gradient separation (Amersham Biosciences, Uppsala, Sweden) and washed three times with phosphate-buffered saline (PBS).

In vitro stimulation

One million PBMC were resuspended in 1 mL of Iscove's modified Dulbecco's medium (IMDM) (Gibco Life Technologies, Ghent, Belgium) plus 10% FBS in polypropylene tubes. PBMC from each subject were stimulated overnight in the presence of GolgiPlug (BD Biosciences), a protein transport inhibitor, with 1 µg/mL of each of the following: (i) VZV intermediate-early protein 62 (IE62) PepMix (JPT, Berlin, Germany), (ii) VZV IE63 PepMix (JPT), (iii) VZV glycoprotein E (gE) PepMix (JPT), (iv) tetanus toxin (TT) mix (PANATecs, Heilbronn, Germany) and (v) cytomegalovirus (CMV) pp65 peptide mix (NIH, Bethesda, MD). Mixtures of overlapping peptides had an optimal peptide size (15 amino acids) and overlap to simultaneously stimulate $CD4^+$ and $CD8^+$ T-cells [25, 26]. Phytohemagglutinin (PHA) at 1 µg/mL was used as a positive control, and an unstimulated condition was used as a negative control.

Flow cytometric analysis

Following overnight stimulation, cells were washed and stained using antibodies as described in Table 1. Due to limitations imposed by the number of channels available on the flow cytometer, CD26 and CD28

were stained using antibodies conjugated to the same fluorochrome in separate panels. LIVE/DEAD fixable violet (Carlsbad, CA, USA) was used to identify viable cells before fixation and permeabilization.

Cells were stained for 15 min at room temperature in the dark. After washing, fixation, and permeabilization (FACS™ Lysing Solution, BD FACS™ Permeabilizing Solution 2; BD Pharmingen) according to the manufacturer's protocol, cells were stained intracellularly to detect interferon γ (IFNγ) and interleukin-2 (IL2). Cells were acquired on a FACSAria II flow cytometer (BD Biosciences) equipped for detection of nine fluorophores. When possible, 1 million events were collected for each sample. Data was analysed using FlowJo software version 10.0.3 (Tree Star, Inc., Ashland, OR). Upon gating on $CD4^+$ and $CD8^+$ T-cells, CD45RA and CCR7 expression was used for further characterization. Those markers separate both $CD4^+$ and $CD8^+$ T-cells into four phenotypically distinct subsets: naive ($T_{N)}$ ($CD45RA^+CCR7^+$), central memory (T_{CM}) ($CD45RA^-CCR7^+$), effector memory (T_{EM}) ($CD45RA^-CCR7^-$) and terminally differentiated effector (T_{EMRA}) ($CD45RA^+CCR7^-$) populations (gating strategy is shown in Fig. S1).

Intracellular staining of IFNγ was used as a functional readout for antigen-specific T-cells following *in vitro* stimulation. It was decided to exclude IL2 from downstream analysis due to low responses. To account for the background release of IFNγ, responses to the negative control were subtracted from the responses to peptide mixtures.

Serology

Serum was separated and immediately stored at -80°C for batch analysis. Serum IgG antibodies to CMV, EBV-VCA, HSV-1 and 2 and VZV were determined using commercially available sandwich ELISA kits in accordance with the manufacturer's instructions.

Elecsys CMV IgG kit was purchased from Roche (Basel, Switzerland), EBV-VCA ELISA kit and Herpes Simplex Virus 1+2 (HSV) IgG Mixed Antigens kit were purchased from GenWay Biotech (San Diego, CA, USA) and Liaison VZV IgG kit from DiaSorin (Saluggia, Italy)

Multiplex bead-based immunoassay

The following cytokines and chemokines were simultaneously analysed in duplicate in serum samples from NICU and PED nurses using commercially-available multiplex bead-based sandwich immunoassay kit (HCYTMAG-60K-PX41, Millipore, Billerica, MA) and measured on a Luminex 200 analyzer (Luminex, Austin, TX, USA) as per the manufacturer's instructions: epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), eotaxin, transforming growth factor alpha (TGF-α), granulocyte colony-stimulating factor (G-CSF), granulocyte-monocyte colony-stimulating factor (GM-CSF), fractalkine, IFNγ, IFN-α2, tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β), growth-regulated oncogene (GRO), macrophage-derived chemokine (MDC or CCL22), soluble CD40 ligand (sCD40L), IFNγ inducible protein 10 (IP10 or CXCL10), monocyte chemoattractant protein-1 (MCP1), monocyte chemoattractant protein-3 (MCP3 or CCL7), macrophage inflammatory protein-1α (MIP-1α or CCL3), macrophage inflammatory protein-1β (MIP-1β or CCL4), FMS-like tyrosine kinase 3 ligand (Flt-3L), IL1a, IL1b, IL1RA, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL12p40, IL12p70, IL13, IL15 and IL17A.

Statistical analysis: Statistics were calculated with Graphpad prism 5 software (GraphPad Software, San Diego, CA). Two-sided Fisher's exact test and two-sided Mann-Whitney test were applied for comparison of two groups. Spearman's rank correlation coefficient was used for correlation analysis. All data in figures are given as median with a range.

Results:

No differences in age, white blood cell counts or IgG titres against CMV, EBV or HSV between NICU and PED groups

No differences were observed in age between NICU and PED groups (medians of 44.3 vs. 37.9 years, P = 0.38), but NICU nurses tended to have higher occupational exposure duration (OED) at their respective unit than PED nurses (P = 0.051) with medians of 19.5 and 5.5 years, respectively (Fig. S2).

No differences were observed in total white blood cell counts, or frequency of lymphocytes, monocytes or granulocytes between the two groups (data not shown).

CMV was seropositive in 7/18 (38.9%) NICU nurses and 10/16 (62.5%) PED nurses (P = 0.3 using Fisher's exact test). No differences were detected in the CMV IgG titre between NICU and PED CMV-seropositives (data not shown). HSV was seropositive in 10/18 (55.6%) NICU nurses and 6/16 (37.5%) PED nurses (P = 0.3 using Fisher's exact test). No differences were detected in the HSV IgG titre between NICU and PED HSV-seropositives. Moreover, all participants but one were EBV-seropositive, with no statistically significant difference in titres between NICU and PED groups (data not shown).

All participants but one were VZV IgG seropositive, with no statistically significant difference in titres between NICU and PED groups (Fig. S3).

CMV-seropositivity but not occupational exposure is associated with distinct T-cell phenotypes

No differences were observed in the composition of $CD4^+$ and $CD8^+$ compartments of T-cells between NICU and PED groups. CMV-seropositivity, on the other hand, was associated with a lower frequency of

$CD8^+$ T_N cells (P = 0.0047) and a higher frequency of $CD8^+$ T_{EMRA} cells (Fig. 1b) (P= 0.0019), with no differences observed in subsets of $CD4^+$ T-cells (Fig. 1a). Moreover, CMV-seropositivity was associated with a significantly higher frequency of $CD28^-$ $CD4^+$ and $CD28^-$ $CD8^+$ T-cell subsets (Fig. 1c and d). Most of the loss of CD28 expression observed in $CD4^+$ T-cells was confined to T_{EM} and T_{EMRA} subsets (Fig. S4a) (P = 0.008 and P = 0.01, respectively), while for $CD8^+$ T-cells, the loss is only significant in the T_{EM} (P = 0.033) but not T_{EMRA} subset (Fig. S4b). No distinct T-cell phenotypes were found to be associated with HSV-seropositivity, and since the majority of nurses were EBV-seropositive, it was not possible to examine the impact of EBV serostatus.

Considering the impact of CMV-seropositivity and in order to discern the effect of high exposure to viruses other than CMV, NICU and PED nurses were compared in the context of CMV serostatus. Although no significant differences were obtained for $CD4^+$ or $CD8^+$ T-cell subsets between NICU and PED groups, a trend toward higher frequency of $CD4^+$ T_{EMRA} cells in PED nurses was observed in CMV-seronegative (p = 0. 0.098) but not in CMV-seropositive nurses (p = 0.962) (Fig. 2a).

Worth noting here that within the $CD4^+$ T-cell compartment, a trend toward higher frequency of T_{EMRA} cells was associated with CMV-seropositivity in NICU (p = 0.069) but not in PED group (p = 0.792) (Fig. 2a), and within $CD8^+$ compartment, a lower and higher frequencies of T_N and T_{EMRA} cells, respectively, were associated with CMV-seropositivity in NICU (P = 0.014 and 0.0113, respectively) but not in PED nurses (P = 0.117 and 0.117, respectively) (Fig. 2b).

VZV responders show an expanded T_{EM} compartment

As most nurses were VZV-seropositive, it was not possible to examine the impact of VZV serostatus on immune signatures. To circumvent this, we divided the nurses based on antigen-specific $CD4^+$ T-cell responses in *ex vivo* assays towards three VZV proteins, IE62, IE63 and gE, into those who showed responses against all proteins (N = 10), hereby called VZV responders (R), and those who showed no response to any of them (N = 8), hereby called VZV non-responders (NR). VZV(R) group had a tendency

to higher frequency of $CD4^+$ T_{EM} and significantly higher $CD8^+$ T_{EM} cells than VZV(NR) group (Fig. 3a and b) (P = 0.067 and 0.014, respectively). Interestingly, the VZV IgG titre tended to be lower in VZV(R) group (P = 0.083) (Fig. 3c).

$CD4^+$ T-cell immune responses to two VZV proteins differ significantly between the two groups of nurses within the CMV-seronegative subcohort

The only significant difference observed when comparing the magnitude of antigen-specific T-cell responses between NICU and PED nurses was against VZV IE62, where the NICU group showed a higher $CD4^+$ T-cell response (P = 0.013) (Fig. S5). No significant differences were observed for $CD8^+$ T-cell responses. Because of the observed impact of CMV-seropositivity on T-cell subsets, we examined the differences in cell-mediated immunity to VZV between the two groups in the context of CMV serostatus. Significantly higher $CD4^+$ T-cell responses against VZV IE62 (P = 0.014), VZV gE (P = 0.044) and TT (P = 0.039) were observed in CMV-seronegative NICU nurses compared to CMV-seronegative PED nurses (Fig. 4a). Notably, no differences were observed within CMV-seropositive nurses.

To further deduce whether the observed differences in $CD4^+$ T-cell immunity are due to occupational exposure, we arbitrarily divided the two group of nurses further - based on the global median of 17 years of OED into nurses with shorter (<17y) and longer OED (>17y). We observed that the differences in $CD4^+$ T-cell immune responses were significantly different between PED and NICU nurses with longer OED against VZV IE62 (P = 0.025), VZV gE (P = 0.031) and TT (P = 0.04), but we were not able to detect significant differences between the two groups with shorter OED (Fig. 4b).

Expression of CD26 on T-cells is altered in CMV-seropositive subcohort and correlate with CD28 expression

CD26 expression on $CD8^+$ T-cells can be divided into three categories: low ($CD26^-$), intermediate ($CD26^{int}$) and high ($CD26^{high}$).

$CD26^-$ $CD8^+$ T-cells were more abundant in CMV-seropositive nurses (Fig. 5a) ($P < 0.0001$) with the loss observed in all subsets of $CD8^+$ T-cells, including naïve $CD8^+$ T-cells (Fig. S6). On the contrary, $CD26^{int}$ $CD8^+$ T-cells were less abundant in CMV-seropositive nurses (Fig.5b) ($P = 0.0042$). While frequency of $CD26^{high}$ $CD8^+$ T-cells was lower in CMV-seropositive nurses, consistent with a lower frequency of early effector memory $CD8^+$ T-cells, it did not reach statistical significance ($P = 0.09$) (Fig. 5c). As both subsets of $CD26^-$ and $CD28^-$ $CD8^+$ T-cells were substantially higher in CMV-seropositive nurses, we examined their correlation. Frequency of $CD28^-$ $CD8^+$ T-cells positively correlated with frequency of $CD26^-$ $CD8^+$ T-cells (Fig. 6a).

No differences were observed in the frequency of $CD26^-$ $CD4^+$ T-cells between CMV-seronegative and seropositive nurses. However, CMV-seropositivity was associated with higher frequencies of $CD26^-$ T_{EM} and $CD26^-$ T_{EMRA} cells but did not reach statistical significance ($P = 0.06$ and 0.076) (Fig. S7). Moreover, as for $CD8^+$ T-cells, frequency of $CD28^-$ $CD4^+$ T-cells positively correlated with frequency of $CD26^-$ $CD4^+$ T-cells (Fig. 6b).

Serum MCP1, sCD40L and IL17A are elevated in CMV-seropositive subcohort

When comparing serum concentrations of cytokines between the CMV-seronegative and seropositive nurses, a significantly higher level of MCP1, sCD40L and IL17A was associated with CMV-seropositivity (Fig. 7a, b and c). Of the three markers, only MCP1 showed a significant correlation with age (Fig. 7d).

Moreover, EGF was significantly higher in NICU compared to PED group within CMV-seronegative but not CMV-seropositive nurses (Fig. S8a). In addition, there was a trend (P = 0.055) toward a lower concentration of GRO in NICU compared to PED group within CMV-seropositive but not CMV-seronegative nurses (Fig. S8b). Interestingly, of all cytokines tested, only GRO showed a significantly negative correlation with VZV IgG titre (Fig. S8c). No significant differences in cytokine concentrations were detected between HSV-seronegative and seropositive nurses (data not shown).

Discussion

This pilot study was designed to explore the impact of occupational exposure to viral pathogens on basal immune signatures in healthy adults. Paediatric nurses (PED) and neonatal intensive care unit nurses (NICU) offer an opportunity to investigate such an impact, as the PED group is known to have a high occupational exposure to viral pathogens, including varicella zoster virus (VZV) and respiratory viruses, compared to the NICU group. It is possible that any alterations in basal immune signatures are masked by the overpowering impact of CMV, hence the CMV-seronegative subcohorts offer an opportunity to examine effects of occupational exposure to viral pathogens without the confounding effects of CMV on the immune system.

Our results show lower $CD4^+$ T-cell responses to two VZV proteins (IE62 and gE), and to tetanus toxoid (TT) in PED nurses who are CMV-seronegative, , as compared to CMV-seronegative NICU nurses, accompanied with an expansion, although not significant, of $CD4^+$ T_{EMRA} cells. Moreover, our data indicates a plausible further deterioration in antigen-specific $CD4^+$ T-cell responses with longer occupational exposure duration. The significantly higher $CD4^+$ T-cell responses detected in NICU against two VZV proteins, IE62 and gE, within CMV-seronegative nurses was not expected, particularly considering that varicella complications are still an important cause for paediatric admissions in Belgium

[27, 28]. We have shown previously that repetitive exposures to VZV in paediatricians induce a persistently higher level of IFNγ^+ T-cells against, at least, three VZV antigens (gE, IE62 and IE63) than in controls [6] and that paediatricians have frequent monthly exposures to VZV (in contrast to the annual occupational VZV exposure in PED nurses). The exogenous boosting hypothesis, proposed in 1965 by Hope-Simpson, postulates that re-exposure to VZV can boost specific immune responses, leading in the long-term to a reduced risk of VZV reactivation and herpes zoster [29]. This exogenous boosting hypothesis has caused a delay in introducing a universal childhood chickenpox vaccination in several countries [30]. It is tempting to speculate that a repetitive and sustained encounter with multiple pathogens might result in an attrition of VZV and TT-specific T-cells, however, interrogation of T-cell immunity against a wider panel of pathogens in a larger cohort is necessary to corroborate our findings.

Moreover, we show that a high IFNγ $CD4^+$ T-cell response to VZV is associated with an expansion of $CD4^+$ and $CD8^+$ T_{EM} compartments, and that this cell-mediated response is not necessarily associated with higher VZV titres. A primary VZV infection manifests as varicella (or chickenpox) and occurs usually early in life. The virus establishes itself in sensory ganglia in a dormant state, from which it can reactivate later in life, causing herpes zoster (or shingles) [31]. Memory VZV-specific T-cells have central and effector memory phenotypes with higher frequencies observed in the $CD4^+$ than $CD8^+$ T-cell compartment [32]. Our observations add to the sparse data on the relationship between VZV and T-cell differentiation. It should be noted, however, that using IFNγ secretion following ex vivo stimulation as a surrogate of antigen-specific T-cells tends to overrepresent cells of effector memory phenotype over those of central memory phenotype [33]. Moreover, the three proteins addressed in our study do not cover the highly complex composition of the viral particle and hence cannot identify the full repertoire of VZV-specific T-cell responses [34].

Furthermore, we show that CMV-seropositive nurses have a decreased frequency of $CD8^+$ T_N cells accompanied with an increased frequency of $CD8^+$ T_{EMRA} cells, and also increased frequencies of both $CD28^-CD4^+$ and $CD28^-CD8^+$ T-cells, likely due to the accumulation of CMV-specific memory T-cells.

Interestingly, those alterations might be more pronounced in NICU compared to PED group, implying a plausible attenuating effect of occupational exposure on CMV-associated immunosenescence, although a larger sample size with a broader age range is evidently required to derive conclusions with higher certainty. CMV establishes a life-long latency, where the virus is sporadically reactivated, inducing expansion of CMV-specific T-cells that accumulate overtime and occupy a substantial portion of the T-cell repertoire, resulting in a decline in overall T-cell diversity [35], with the effects more prominent in $CD8^+$ than $CD4^+$ T-cells [36–38]. The higher frequency of $CD4^+CD28^-$ T-cells observed in CMV-seropositive nurses was described before [39]. This subset shows a cytotoxic potential and its expansion correlates with the activity of a range of cardiovascular and autoimmune disorders [40–42]. Similarly, $CD8^+$ T-cells with $CD28^-$ phenotype have been extensively studied and are known to accumulate with age and due to chronic infections, particularly CMV, and to a much larger extent than $CD4^+$ T-cells. $CD28^-$ $CD8^+$ T-cells have been characterized as oligoclonal in nature, implying that a high expansion might lower the diversity of the total $CD8^+$ T-cell repertoire, and subsequently compromises cell-mediated immunity as was recently reported in transplant patients upon CMV reactivation [43].

The increase in the frequency of $CD26^-$ $CD8^+$ T-cells as well as the decline in the frequency of $CD26^{int}$ $CD8^+$ T-cells observed in CMV-seropositive nurses have not been described before, but are consistent with a $CD26^-$ subset having terminally differentiated effector phenotype and a $CD26^{int}$ subset having a naïve phenotype in the $CD8^+$ T-cell compartment as was described by Ibegbu et al [16]. Furthermore, loss of CD26 on $CD8^+$ T-cells positively correlated with the loss of CD28. Hatano et al [44] showed that $CD8^+$ T-cells that lack CD28 also lack CD26, whilst the opposite is not true. In addition, they reported an enhanced effector function following CD26 co-stimulation compared to CD28 co-stimulation, in the context of the second signal for TCR activation.

In addition to T-cell immune signatures, comparison of the serum concentrations of cytokines, chemokines, and growth factors at baseline showed higher serum levels of MCP1, sCD40L and IL17A in

CMV-seropositive nurses. MCP1 (also known as CCL2) is C-C chemokine produced by many cell types, including endothelial, epithelial and smooth muscle cells, but monocytes and macrophages are the main source of MCP1. MCP1 attracts monocytes and T lymphocytes, both recognized as major inflammatory cells in atheromatous lesions [45]. It is thought that MCP1 release by endothelial cells is a key event in atherosclerosis [46], and it was shown in a mouse model that CMV infection accelerates inflammation in vascular tissue overexpressing MCP1 [47]. sCD40L is believed to contribute to the pathophysiology of atherosclerosis and atherothrombosis by enhancing platelet activation and platelet-leucocyte conjugation and increasing lipid deposition and macrophage foam cell formation [48, 49]. IL17A is one of the six cytokines that constitute the IL17 family, and is the signature proinflammatory cytokine produced by type 17 helper T (Th17) cells that induce protection against extracellular bacterial and fungal infections [50]. Although the role of IL17A in inflammatory disorders is well described [51], its involvement in vascular inflammation and atherosclerosis is less clear with conflicting reports [52–55]. Aforementioned findings may provide an additional mechanism that further implicate CMV in the development of cardiovascular disease [56], and it warrants further investigation in a larger and more inclusive cohort.

In summary, our results show an altered $CD4^+$ T-cell immunity in nurses with a high occupational exposure to viral pathogens that could become more pronounced with time and might be masked or mitigated by CMV-seropositivity. Moreover, our data suggests that the distinct T-cell phenotypes known to be associated with CMV-seropositivity may be less prominent in PED nurses compared to NICU nurses. Furthermore, our data support the notion that CD26 is lost on highly differentiated T-cells and is positively correlated with CD28 loss. Despite the small sample size, several significant insights were made that warrant further investigation. Future work in a larger cohort should examine the impact of high exposure to viral pathogens on basal immune signatures, T-cell repertoire diversity and functional immune responses and the potential effect of CMV-seropositivity on those observations.

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Disclosure

The authors declare no conflicts of interest.

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Table 1 Antibodies used during flow cytometric characterization of the PBMCs.

Target	Fluorochrome	Clone	Company
CD3	PE-Texas Red	7D6	Life Technologies
CD4	APC-H7	RPA-T4	BD
CD8	Pacific Orange	3B5	Life Technologies
CD45RA	PE-Cy7	HI100	BD
CCR7	PE	150503	BD
CD28	PE-Cy5	CD28.2	BD
CD26	PE-Cy5	L272	BD
IFN-γ	FITC	B27	BD
IL-2	APC	5344.111	BD
Viability	LIVE/DEAD Fixable Violet		Life Technologies

PE, phycoerythrin; APC, allophycocyanin; FITC, fluorescein isothiocyanate

Fig. 1

CMV-seropositivity is associated with modified composition of $CD8^+$ but not $CD4^+$ T-cell compartments and lowered CD28 expression in both $CD4^+$ and $CD8^+$ T-cells.

(a) Four $CD4^+$ and **(b)** four $CD8^+$ T-cell subsets were defined according to the expression of CD45RA and CCR7 as naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory (T_{EMRA}) cells and the frequencies were compared between CMV(-) and CMV(+) cohorts. **(c and d)** Comparison of the frequencies of $CD28^-CD4^+$ and $CD28^-CD8^+$ T-cells between CMV(-) and CMV(+) cohorts. Mann-Whitney test with * $P \leq 0.05$ and ** $P \leq 0.01$

Fig. 2

CMV-seropositivity may have a higher impact on NICU than PED nurses.

Due to the high impact of CMV-seropositivity observed, $CD4^+$ and $CD8^+$ T-cell subsets in NICU and PED nurses were compared in the context of CMV status. **(a)** Four $CD4^+$ and **(b)** four $CD8^+$ T-cell subsets were defined according to the expression of CD45RA and CCR7 as naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory (T_{EMRA}) cells and the frequencies were compared between four groups, CMV(-) NICU, CMV(-) PED, CMV(+) NICU and CMV(+) PED. Mann-Whitney test with * $P \leq 0.05$.

Fig. 3

VZV status has an impact on T-cell differentiation.

Based on $CD4^+$ T-cell responses towards three VZV proteins, IE62, IE63 and gE, nurses were divided into VZV non-responders (NR) and VZV responders (R). **(a and b)** Frequency of $CD4^+$ T and $CD8^+$ T-cells with an effector memory phenotype were compared between VZV (NR) and VZV (R). Mann-Whitney test with * $P \leq 0.05$ **(c)** VZV IgG titres were compared between VZV (NR) and VZV (R).

Fig. 4

Lower CD4+ T-cell immune responses to VZV in CMV(-) NICU nurses

Freshly isolated peripheral blood mononuclear cells were stimulated overnight with overlapping VZV intermediate-early protein 62 (IE62), VZV intermediate-early protein 63 (IE63), VZV glycoprotein E (gE) peptide pools and tetanus toxin (TT) protein. Cells were stained intracellularly to detect interferon-γ (IFNγ) and Interleukin-2 (IL2) and acquired on a FACSAria II. *Ex vivo* $IFN\gamma^+$ $CD4^+$ T-cell responses to VZV IE62, VZV gE and TT in four groups, **(a)** CMV(-) NICU, CMV(-) PED, CMV(+) NICU and CMV(+) PED and **(b)** short OED NICU, short OED PED, long OED NICU and long OED PED. Mann-Whitney test with * $P \leq 0.05$

Fig. 5

Expression of CD26 on $CD8^+$ T-cells is highly altered in CMV(+) compared to CMV(-) cohort.

CD26 expression on $CD8^+$ T-cells can be divided into three categories: $CD26^-$, $CD26^{int}$ and $CD26^{high}$. Frequencies of $CD26^-CD8^+$ **(a)** $CD26^{int}CD8^+$ **(b)** and $CD26^{high}CD8^+$ **(c)** T-cells were compared between CMV(-) and CMV(+) cohorts. Mann-Whitney test with * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$

Fig. 6

Loss of CD26 correlate with loss of CD28 T-cells.

Correlation analysis of $CD26^-$CD4+ and $CD28^-CD4^+$ T-cells and $CD26^-CD8^+$ and $CD28^-CD8^+$ T-cells **(a)** Frequency of $CD26^-CD4^+$ T-cells is positively correlated with frequency of $CD28^-$CD4+ T-cells; $P = 0.0003$; $R^2 = 0.4084$. **(b)** Frequency of $CD26^-CD8^+$ T-cells is positively correlated with frequency of $CD28^-CD8^+$ T-cells; $P = 0.0002$; $R^2 = 0.4426$.

Fig. 7

Higher MCP1, sCD40L and IL17A levels in sera of CMV(+) nurses

Serum levels of monocyte chemoattractant protein-1 (MCP1) **(a)**, soluble CD40 ligand (sCD40L) **(b)** and Interleukin 17A (IL17A) **(c)** in CMV(-) and CMV(+) nurses. Mann-Whitney test with * $P \leq 0.05$ and ** $P \leq 0.01$ **(d)** Concentration of MCP1 in serum is positively correlated with the age; $P = 0.0064$; $R^2 = 0.46$

Fig. S1

Gating strategy. A) Identification of the two main T-cell compartments. Aggregates were first excluded followed by a lymphocyte gate. After gating on viable $CD3^+$ cells, $CD4^+$ and $CD8^+$ T-cells were identified. B) Identification of main subsets of $CD4^+$ and $CD8^+$ T-cells based on the expression of CD45RA, CCR7: naive ($T_{N)}$ ($CD45RA^+CCR7^+$), central memory (T_{CM}) ($CD45RA^-CCR7^+$), effector memory (T_{EM}) ($CD45RA^-CCR7^-$) and terminally differentiated effector (T_{EMRA}) ($CD45RA^+CCR7^-$). Moreover, the expression profile of CD26 and CD28 is shown for both $CD4^+$ and $CD8^+$ T-cells. Shown also the gating of cells positive for either IFN-γ or IL-2.

Fig. S2

Comparison of age and occupational exposure duration (OED) in NICU and PED groups. Mann-Whitney test.

Fig. S3

Comparison of VZV IgG titer in CMV (-) NICU, CMV(-) PED, CMV(+) NICU and CMV(+) PED groups

Fig. S4

CMV-associated loss of CD28 on naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated effector memory (T_{EMRA}) subsets of **(a)** $CD4^+$ and **(b)** $CD8^+$ T-cells. Mann-Whitney test with * $P \leq 0.05$ and ** $P \leq 0.01$

Fig. S5

Freshly isolated peripheral blood mononuclear cells were stimulated overnight with overlapping VZV intermediate-early protein 62 (IE62). Cells were stained intracellularly to detect interferon-γ (IFNγ) and

Interleukin-2 (IL2) and acquired on a FACSAria II. *Ex vivo* IFNγ$^+$ CD4$^+$ **(a)** and CD8$^+$ **(b)** T-cell responses to VZV IE62 in the cohort of NICU and PED nurses. Mann-Whitney test with * $P \leq 0.05$

Fig. S6

Impact of CMV-seropositivity on CD26 expression on CD8$^+$ T_N, T_{CM}, T_{EM} and T_{EMRA} cell subsets . Mann-Whitney test with * $P \leq 0.05$

Fig. S7

Impact of CMV-seropositivity on CD26 expression on **(a)** CD4$^+$ T-cells, **(b)** CD4$^+$ T_{EM} cells and **(c)** CD4$^+$ T_{EMRA} cells. Mann-Whitney test with * $P \leq 0.05$

Fig. S8

(a) Serum levels of epidermal growth factor (EGF) in CMV (-) NICU, CMV(-) PED, CMV(+) NICU and CMV(+) PED groups **(b)** Serum levels of GRO in CMV (-) NICU, CMV(-) PED, CMV(+) NICU and CMV(+) PED groups **(c)** Serum level of GRO is negatively correlated with VZV IgG titre; $P = 0.013$; $R^2 = -0.42$