Influence of human cytomegalovirus infection on the NK cell receptor repertoire in children

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Human cytomegalovirus (hCMV) infection is usually asymptomatic but may cause disease in immunocompromised hosts. It has been reported that hCMV infection may shape the NK cell receptor (NKR) repertoire in adult individuals, promoting a variable expansion of the CD94/NKG2C$^+$ NK cell subset. We explored the possible relationship between this viral infection and the expression pattern of different NKR including CD94/NKG2C, CD94/NKG2A, immunoglobulin-like transcript 2 (ILT2, CD85j), KIR2DL1/2DS1, KIR3DL1, and CD161 in peripheral blood lymphocytes from healthy children, seropositive ($n = 21$) and seronegative ($n = 20$) for hCMV. Consistent with previous observations in adults, a positive serology for hCMV was associated with increased numbers of NKG2C$^+$ NK and T cells as well as with ILT2$^+$ T lymphocytes. Moreover, the proportions of CD161$^+$ and NKG2C$^+$CD56$^-$CD3$^-$ NK cells also tended to be increased in hCMV$^+$ individuals. Excretion of the virus was associated with higher proportions of NKG2C$^+$ NK cells. Altogether, these data reveal that hCMV may have a profound influence on the NKR repertoire in early childhood.

Key words: C-type lectins · Human cytomegalovirus · Killer immunoglobulin-like receptor (KIR) · NK cell · T cell

Introduction

Activation and function of NK cells is regulated by several membrane receptors, including killer immunoglobulin-like receptors (KIR), CD94/NKG2 lectin-like receptors, and the immunoglobulin-like transcript 2 (ILT2). Although these molecules are commonly designated as NK cell receptors (NKR), their expression is not restricted to NK cells [1–3]. KIR genes are clustered within the leukocyte receptor complex, and a subset of these receptors (e.g. KIR2DL, KIR3DL) bear ITIM which, by recruiting tyrosine phosphatases, exert an inhibitory role on different intracellular signaling pathways [4–6]. In contrast, other KIR (e.g. KIR2DS, KIR3DS) possess shorter intracytoplasmic domains, do not bear ITIM, and are able to interact with the activating adaptor molecule KARAP/DAP12. KIR, which display a variegated distribution, are

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expressed by NK cells as well as by T-cell subsets, and their known ligands are HLA class I molecules [3].

ILT2, also termed CD85j, leukocyte immunoglobulin-like receptor 1(LIR1), or leukocyte immunoglobulin-like receptor B1 (LILRB1), belongs to a family of inhibitory and activating leukocyte receptors mainly expressed by myeloid cells [7–9]. ILT2 is detected in monocytes, B cells, NK, and T-cell subsets, and functions as an inhibitory receptor by recruiting the SHP-1 phosphatase through its intracytoplasmic ITIM [10, 11]. ILT2 specifically interacts with classical and non-classical class I HLA molecules as well as with the UL18 glycoprotein of human cytomegalovirus (hCMV) [8, 9, 12].

NKG2 molecules are C-type lectins encoded at the NK gene complex in human chromosome 12 [13]. Surface expression of NKG2A and NKG2C requires their covalent assembly with CD94. The CD94/NKGA heterodimer is an inhibitory receptor that recruits the SHP1 tyrosine phosphatase through the ITIM of the NKG2A subunit [14, 15]. On the other hand, CD94/NKG2C functions as an activating receptor associated with DAP12 through the transmembrane lysine residue of NKG2C [16].

CD94/NKG2 receptors recognize the non-classical HLA class I molecule HLA-E [17], which mainly presents peptides derived from the leader sequence of other HLA class I molecules, thus, allowing NK cells to indirectly monitor their biosynthesis [17–19]. CD94/NKG2A is mainly detected in cytotoxic lymphocytes such as NK cells, and $\gamma\delta$ or $\alpha\beta$ $CD8^+$ T cells. A constant recycling of this receptor between the plasma membrane and intracellular compartments preserves its expression on the cell surface [20]. CD94/NKG2A expression is inducible by different cytokines, including IL-15 and IL-12 [21, 22].

The immune mechanisms of resistance against viral infections involve a complex interplay between host effector cells and virus-infected cells. In this regard, viruses have evolved mechanisms to evade the innate and adaptive immune responses. hCMV infection is usually asymptomatic but may cause congenital disorders and a severe disease in immunocompromised hosts [23]. To evade T-cell-mediated recognition, hCMV induces, through the independent effects of several proteins (US2, US3, US6, and US11), a downregulation of HLA class I expression [23]. However, this process facilitates NK cell-mediated lysis of infected cells. In this regard, hCMV has developed different strategies to evade NK cell recognition [24]. Among them, it has been described that UL18 is a high affinity ligand for ILT2 [9, 25] and that UL40 contributes to stabilize HLA-E expression and the engagement of CD94/NKG2A [26, 27]. On the other hand, different mechanisms prevent the surface expression of NKG2D ligands. The hCMV protein UL16 intracellularly retains MICB, ULBP1 and ULBP2, which are induced in the course of hCMV infection [28, 29]. Furthermore, the hCMV UL142 protein interferes with the expression of MICA and the hCMV miR-UL112 downregulates MICB [30, 31]. All the above may result in the protection of hCMV-infected cells from NK cell-mediated effector functions.

It has been shown that $CD94/NKG2C^+$ NK cells and $ILT2^+$ T lymphocytes are increased in healthy hCMV-seropositive individuals, suggesting that the viral infection may shape the NKR repertoire [32]. An expansion of $CD94/NKG2C^+$ cells has also been observed in $hCMV^+$ individuals infected by HIV-1 [33, 34]. In this regard, it has been shown that the interaction of PBMC with hCMV-infected fibroblasts, which downregulate HLA class I expression, promotes an expansion of $CD94/NKG2C^+$ NK cells [35]. As primary hCMV infection often occurs during childhood, we decided to explore its possible impact on the NKR repertoire in a pediatric population. Our results support that hCMV infection may have a profound effect on the NKR repertoire since early childhood.

Results

CD94/NKG2C expression is increased in hCMV seropositive children

Based on the levels of hCMV-specific IgG and IgM antibodies, children included in this study were divided in two groups: without evidence of CMV infection (seronegative, $n = 20$), and with past or current CMV infection (seropositive, $n = 21$). We identified two donors with evidence of recent infection (positive serology for IgM) by hCMV. An active excretion of the virus was detected in 12 individuals in urine ($n = 6$), in saliva ($n = 1$), or in both urine and saliva ($n = 6$).

By flow cytometry analysis, four different peripheral blood lymphocyte (PBL) subsets were defined on the basis of CD3 and CD56 expression: $CD3^-CD56^+$ NK cells, $CD3^+CD56^-$ T cells, $CD3^+CD56^+$ T cells, and $CD3^-CD56^-$ cells (Fig. 1A). In agreement with the observations reported by Gumá et al. in adult blood donors [32], the proportions of $CD94/NKG2C^+$ cells were quite variable and a heterogeneous staining intensity of $NKG2C^+$ cells was observed in samples from children (Fig. 1B and C). It is of note that Gumá et al. [32] restricted their analysis to the $NKG2C^{bright}$ cell subset, whereas in this study, all $NKG2C^+$ lymphocytes were considered. As observed in adults, the group of children with a positive serology for hCMV showed significantly increased proportions of $NKG2C^+$ NK and T lymphocytes compared with seronegative individuals ($p < 0.05$ in all cases, Fig. 1B and C). Both groups showed similar proportions of total $CD3^- CD56^+$ NK cells ($10.98 \pm 1.23\%$ and $9.7 \pm 1.29\%$, mean and SEM for seropositive and seronegative donors). In addition, the percentages of $CD94/NKG2C^+$ NK cells positively correlated with the levels of IgG specific for hCMV ($r = 0.516$, $p < 0.01$), thus supporting the influence of the viral infection. However, no significant correlation between the levels of hCMV-specific IgG and the proportions of $NKG2C^+$ cells was found when the analysis was restricted to seropositive donors (data not shown). Remarkably, samples from $hCMV^+$ individuals also displayed increased proportions of $NKG2C^+$ cells within the $CD3^-CD56^-$ lymphocyte population (Fig. 1B). Though the latter mainly corresponds to B lymphocytes, it may contain a population of
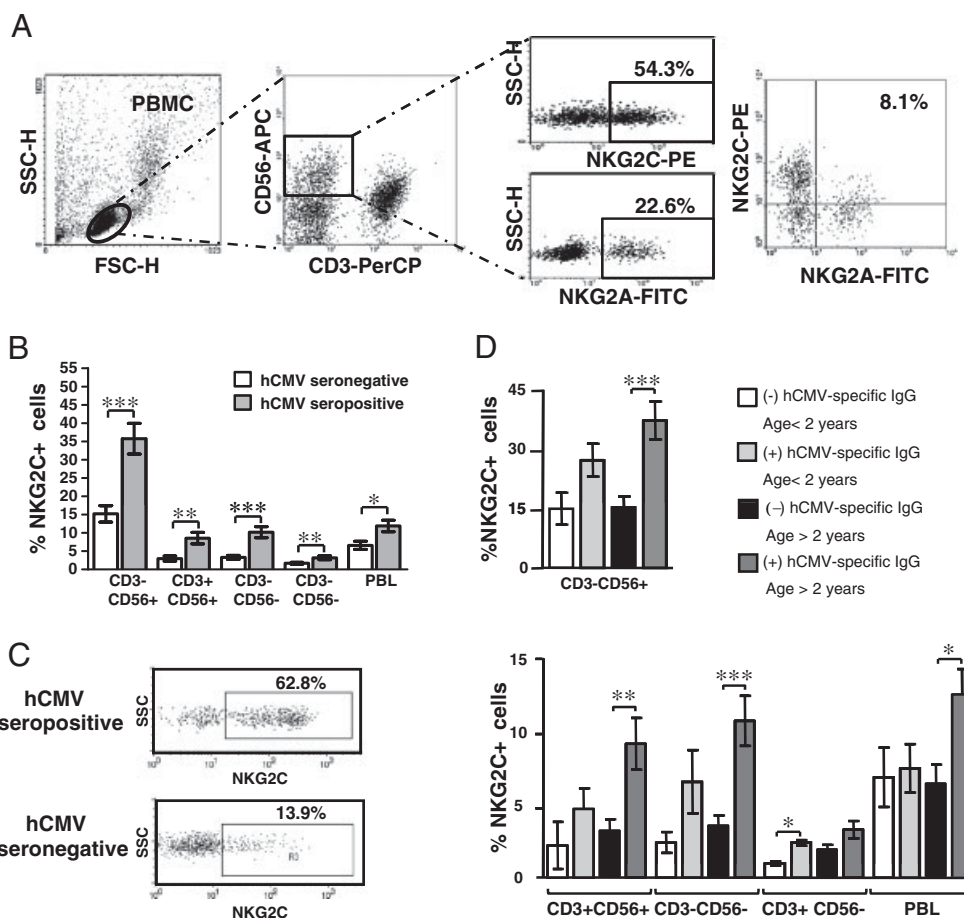
Figure 1. Increased CD94/NKG2C expression in lymphocytes from hCMV-seropositive children. PBL from children included in the study were immunostained for NKG2C, NKG2A, CD56, and CD3, and cells were analyzed by four-color flow cytometry; different cell subsets were gated according to the expression of CD3 and CD56. (A) Flow cytometry gating strategy for the analysis of NKG2C and NKG2A expression in the CD3$^-$CD56$^+$ cell subset is shown. The data in (B) and (D), and dot plots displayed in (C) were generated based on similar gating strategies. Cut-off for background fluorescence was defined based on isotype negative controls. (B) Percentages of NKG2C$^+$ cells were calculated for CD3$^-$CD56$^+$, CD3$^+$CD56$^+$, CD3$^-$CD56$^-$, and CD3$^+$CD56$^-$ cells as well as for total PBL. Representative dot plots from hCMV-seropositive and hCMV-seronegative children are shown. Numbers indicate the percentage of NKG2C$^+$CD3$^-$CD56$^+$ cells. (D) Analysis of NKG2C expression by the indicated cell subsets in children according to age (less, $n = 9$, or more than 2 years old, $n = 32$), and serological status (negative, $n = 20$, and positive for anti-hCMV antibodies, $n = 21$). Data correspond to the mean $\pm$ SEM. Statistical analysis was performed as described in *Materials and methods* (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$).

CD3$^-$CD56$^-$ NK cells, as originally described in HIV-infected patients [36], that are also detectable in some healthy adult individuals (N. Romo *et al.*, unpublished results).

No correlation between donor age and the proportions of NKG2C$^+$ cells (data not shown) was found. Yet, the increment in the percentages of NKG2C$^+$ cells reached statistical significance only when hCMV$^+$ children older than 2 years were analyzed, whereas a higher expression of this activating receptor in CD56$^-$ T cells was perceived in children under 2 years of age (Fig. 1D).

CD94/NKG2A expression is not related to hCMV serological status

As reported in adults [32], no significant differences for the expression of the CD94/NKG2A inhibitory receptor were detected

between seropositive and seronegative children (Fig. 2A). On the other hand, in agreement with a recent report [21], a fraction of NK cells co-expressing CD94/NKG2C and CD94/NKG2A was identified (Fig. 2B); it is of note that the proportions of this double-positive subset appeared to be unrelated to hCMV serology (Fig. 2B).

Expression of CD161 by lymphocytes from hCMV seropositive children

The expression of other NKR was also explored. In agreement with previous reports in adult donors [37, 38], the NKRP1A (CD161) C-type lectin-like receptor was expressed by NK and T-cell subsets (Fig. 3). Remarkably, CD161 was detected in a significant proportion of CD3$^-$CD56$^-$ cells (Fig. 3A), further confirming the presence of NK cells into this population. The
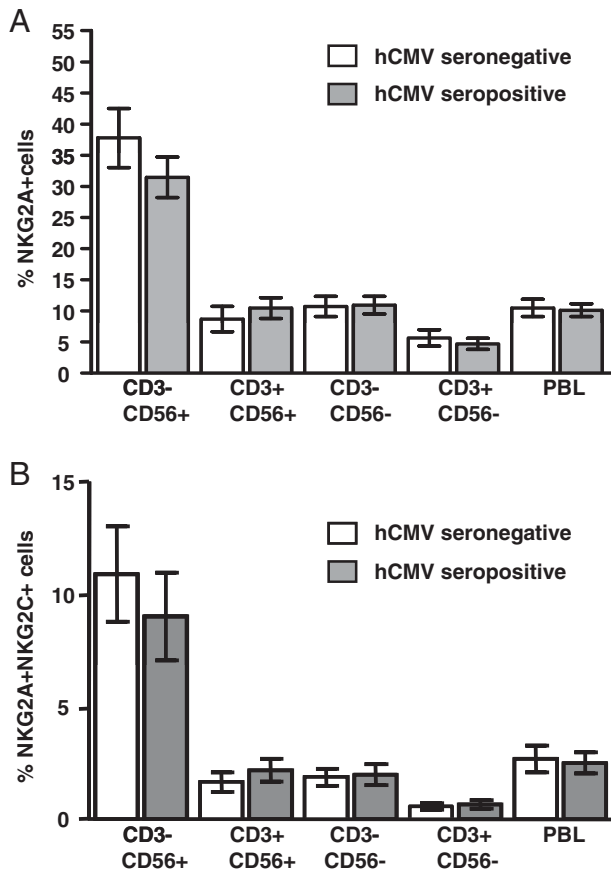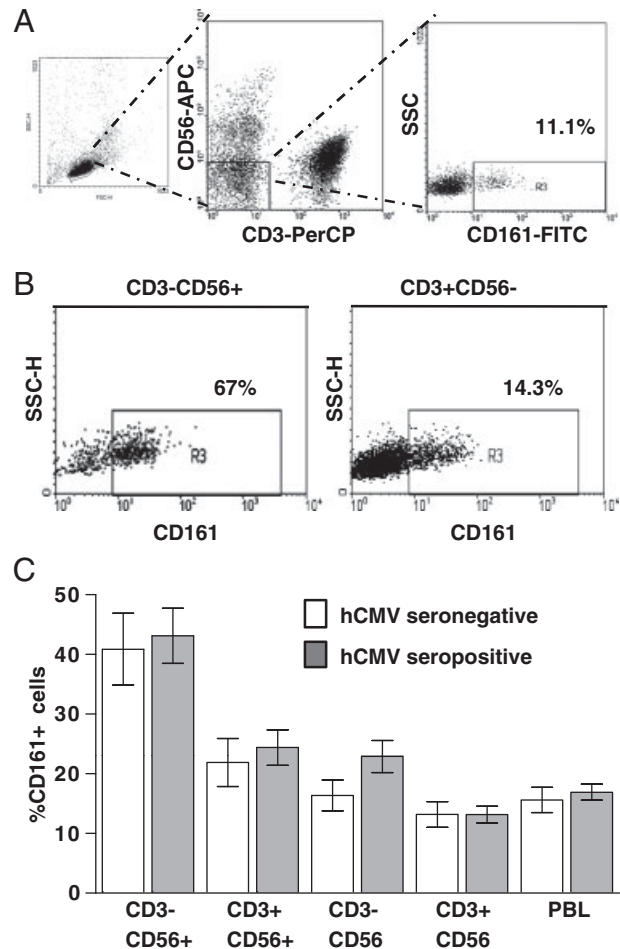
Figure 2. Expression of CD94/NKG2A in lymphocytes from hCMV-seropositive and hCMV-seronegative children. PBL from hCMV-seropositive, $(n = 21)$ and hCMV-seronegative $(n = 20)$ children were immunostained for NKG2A, NKG2C, CD56, and CD3 and analyzed by flow cytometry. Percentages of (A) NKG2A$^+$ and (B) NKG2A$^+$NKG2C$^+$ double-positive cells were calculated for CD3$^-$CD56$^+$, CD3$^+$CD56$^+$, CD3$^-$CD56$^-$, and CD3$^+$CD56$^-$ cells, as well as for total PBL. Data correspond to the mean$\pm$SEM.

expression of CD161 in T lymphocytes and CD3$^-$CD56$^+$ NK cells appeared unrelated to hCMV infection (Fig. 3C). However, although the difference did not reach statistical significance, hCMV IgG-seropositive children tended to display higher proportions of CD161$^+$CD3$^-$CD56$^-$ NK cells than seronegative children (Fig. 3C). Nevertheless, a significant correlation between the percentages of CD161$^+$CD3$^-$CD56$^-$ cells and the levels of hCMV-specific IgG ($r = 0.36$, $p<0.05$) was found when data from all donors were pooled (data not shown), thus supporting that the relative numbers of CD3$^-$CD56$^-$ NK cells tend to increase in hCMV$^+$ children, as shown above for NKG2C$^+$ cells (Fig. 1).

Excretion of hCMV is associated with increased expression of NKG2C

To evaluate the influence of the status of hCMV infection on the expression of NKG2C, we compared the immunophenotype of donors stratified according to the detection of virus excretion.


Figure 3. Analysis of CD161 expression by lymphocytes from hCMV-seropositive and hCMV-seronegative children. PBL from hCMV-seropositive $(n = 21)$ and hCMV-seronegative $(n = 20)$ donors were immunostained for CD3, CD56, and CD161 and cells were analyzed by flow cytometry; CD161 was assessed in the different cell subsets gated according to CD3 and CD56 expression. (A) Flow cytometry gating strategy for the analysis of CD161 expression in the CD3$^-$CD56$^-$ cell population (R3). The number indicates the percentage of CD3$^-$CD56$^-$ CD161$^+$ cells. Data displayed in (B) and (C) were generated based on similar gating strategies. (B) Representative dot plots of samples from a seropositive donor analyzed as described above. (C) Percentages of CD161$^+$ cells were calculated for CD3$^-$CD56$^+$, CD3$^+$CD56$^+$, CD3$^-$CD56$^-$, and CD3$^+$CD56$^-$ cells as well as for total PBL. Data correspond to the mean$\pm$SEM.

Interestingly, individuals with active hCMV excretion tended to display higher percentages of both NKG2C$^+$CD3$^-$CD56$^+$ and CD3$^-$CD56$^-$ NK cells (Fig. 4A and B).
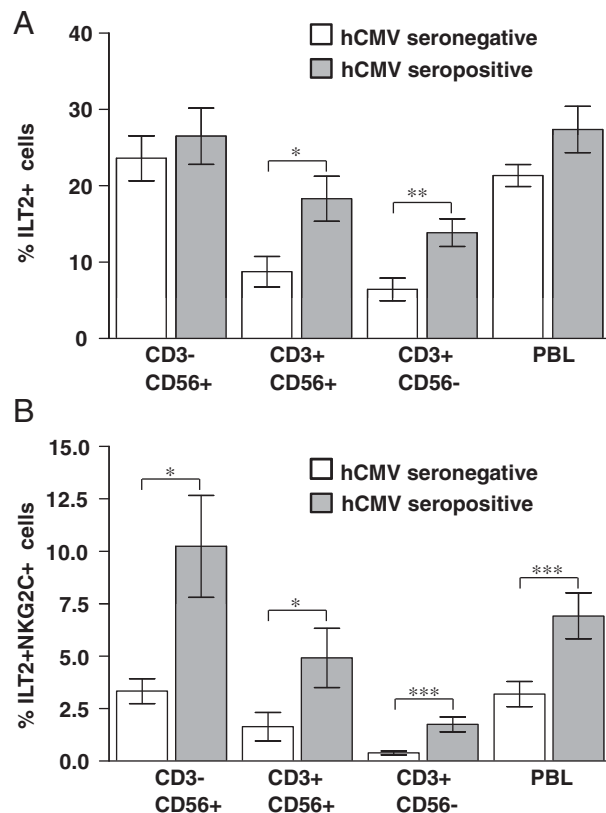
Expression of ILT2 is increased in hCMV seropositive children

Previous studies in adult blood donors revealed a significant association between positive serology for hCMV and expression of the ILT2 leukocyte inhibitory receptor by T cells [32]. Despite the observation that CD3$^+$CD56$^+$ and CD3$^+$CD56$^-$ subsets contained
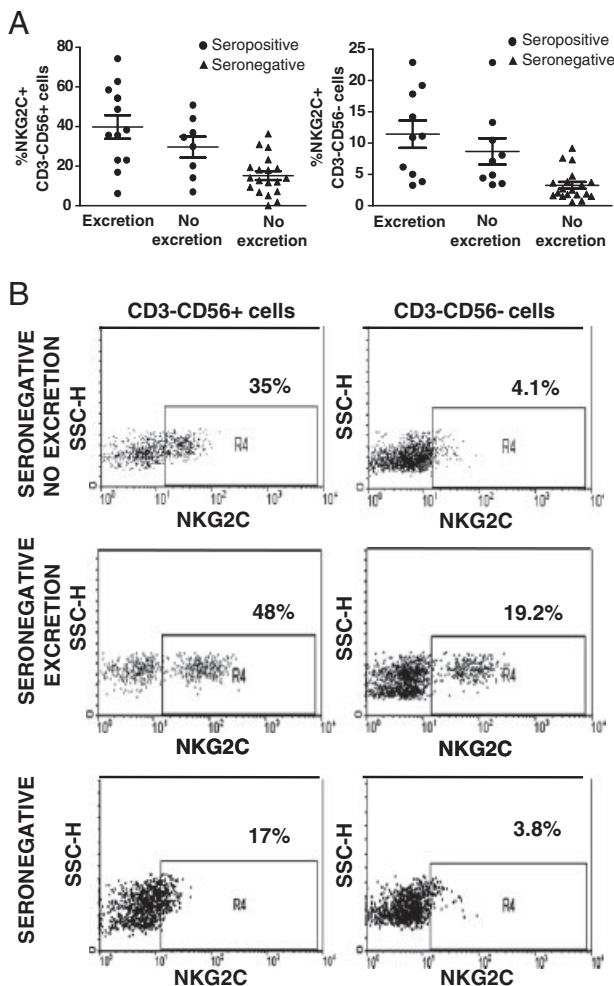
Figure 4. Expression of NKG2C in children with active excretion of hCMV. (A) Peripheral blood lymphocytes (PBL) from hCMV-seropositive children, with or without active excretion of CMV, and seronegative children were analyzed by flow cytometry for NKG2C expression by CD3$^-$CD56$^+$ and CD3$^-$CD56$^-$ cells as described in Fig. 1. Data correspond to the mean±SEM. (B) Cells were gated on the CD3$^-$CD56$^+$ or CD3$^-$CD56$^-$ populations and further analyzed for NKG2C expression (R4). Numbers correspond to the % NKG2C$^+$ cells. Representative dot plots of CD3$^-$CD56$^+$ and CD3$^-$CD56$^-$ cells from two seropositive children with or without active CMV secretion (middle and upper panels, respectively) and a seronegative donor (lower panels) are displayed.

Figure 5. ILT2 expression is increased in T cells from hCMV-seropositive children. (A) PBL from hCMV-seropositive and hCMV-seronegative children were immunostained for ILT2, CD3, and CD56, and cells were analyzed by flow cytometry. Percentages of ILT2$^+$ cells were calculated for CD3$^-$CD56$^+$, CD3$^+$CD56$^+$ and CD3$^+$CD56$^-$ cells as well as for total PBL. Mean and SEM of the percentage of positive cells are shown. (B) PBL were immunostained for NKG2C and ILT2, and the percentage of double-positive cells were calculated for the same lymphocyte subsets analyzed in (A) by four-color flow cytometry; $^*p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$.

differences in the expression levels of these receptors were detected between hCMV-seropositive and hCMV-seronegative children (data not shown).

Discussion

hCMV infection is highly prevalent and occurs in approximately 1% of neonates, worldwide and in Mexico [23, 41]. Congenital CMV infection is symptomatic in about 10% of infected neonates and is associated with neurological complications in almost 50% of them. In addition, neurological disease will eventually develop in 8–13% of neonates with asymptomatic infection [23]. Among women with a primary infection during pregnancy, the rate of fetal infection is approximately 40% [42]. Postnatal transmission is established mainly through contact with secretions and may occur early after birth through breast feeding. In most individuals CMV establishes a life-long latent infection undergoing occasionally subclinical reactivation. Primary infection or reactivation is

relatively low proportions of ILT2$^+$ cells in children (Fig. 5A), the hCMV-seropositive group displayed an increased expression of this receptor in both T lymphocyte subsets ($p<0.05$ in both cases, Fig. 5A). Furthermore, when co-expression of ILT2 and NKG2C was analyzed, a higher proportion of double-positive cells was also detected in the CD3$^+$CD56$^+$, CD3$^+$CD56$^-$, and CD3$^-$CD56$^+$ populations from seropositive donors (Fig. 5B). Since most B lymphocytes are ILT2$^+$, the expression of this receptor by CD3$^-$CD56$^-$ cells was not evaluated. As it has been reported that expression of certain KIR may be related to the course of some viral infections [39, 40], the distribution of KIR2DL1/KIR2DS1 and KIR3DL1 was analyzed. Yet, no significant

associated to an important morbidity in immunosupressed and immunodeficient individuals [43].

An effective defense against CMV requires the participation of both NK cells and T cells [24]. It has been shown that hCMV may shape the NKR repertoire, leading to a variable expansion of NKG2C$^+$ NK and T-cell subsets in adult healthy individuals [32], and in aviremic HIV-1-infected patients [33, 34]. Moreover, NKG2C$^+$ cells were observed to expand *in vitro* upon co-culture of PBMC from seropositive individuals with hCMV-infected fibroblasts [35]. In that report, we hypothesized that a cognate interaction between the activating CD94/NKG2C receptor and a ligand expressed by hCMV-infected cells might drive the process [35]. However, the molecular basis underlying the impact of hCMV infection on the NKR repertoire still remains unsolved. Interestingly, an NKG2C$^+$ NK cell lymphocytosis was detected during an acute hCMV infection in a patient with a primary T-cell immunodeficiency, apparently achieving a reduction of viremia despite the absence of T cells [44], thus suggesting that NKG2C$^+$ NK cells may actively participate in the control of hCMV infection. These observations are reminiscent of the expansion in mCMV-infected mice of Ly49H$^+$ NK cells, which contribute to control the infection and confer a "memory" response pattern [45]. As previously reported [32], the NKG2C$^+$ NK cell subset does express lower levels of NKp30 and NKp46 NCR, but contains higher proportions of ILT2$^+$ and KIR$^+$ cells than the NKG2A$^+$ subset. Thus, it is conceivable that the impact of hCMV infection on the NKR repertoire may influence the quality of the NK cell-mediated innate responses.

Although the magnitude of the increase of circulating NKG2C$^+$ cells is quite variable in seropositive adult blood donors [32], longitudinal studies indicate that the phenotype tends to remain rather stable (Romo and López-Botet, unpublished). Whether the high levels of NKG2C$^+$ cells maintained in some hCMV$^+$ individuals result from a cumulative process secondary to a higher subclinical hCMV reactivation rate, as indirectly supported by studies in HIV-1$^+$ patients [33, 34], or may also depend on other factors (*e.g.* time of primary infection), remains as an open issue. On that basis, we considered with interest the assessment the possible relationship between hCMV infection and the NKR repertoire in childhood.

In this study, we have observed that children infected with hCMV exhibited higher proportions of NKG2C$^+$ NK and T cells than non-infected individuals; moreover, this phenotypic feature was particularly prominent in individuals with viral excretion. These data are in agreement with a previous report in adults in which increased numbers of NKG2C$^+$ cells were also detected in healthy hCMV-seropositive individuals [32]. Whether the marked impact of hCMV infection on the NKR repertoire observed in some children is transient or tends to persist throughout adult life is a relevant question that warrants long-term longitudinal studies. In the present study, all NKG2C$^+$ cells were analyzed, whereas Gumá *et al.* restricted their analysis to NKG2C^{bright} cells, as explained in their original report [32]. Therefore, despite that similar conclusions were reached regarding the influence of hCMV on the distribution of the NKG2C$^+$ subset in seropositive

and seronegative donors, the relative numbers of NKG2C$^+$ cells reported by both studies are not comparable. In this regard, further phenotypic and functional analyses are required to define the heterogeneity of the NKG2C$^+$ population in the context of the response to hCMV. It is of note that the proportions of double-positive NKG2C$^+$NKG2A$^+$ NK cells appeared unrelated to hCMV serology in children. As previously discussed [21], whether the NKG2C$^+$NKG2A$^+$ subset corresponds to a discrete NK cell subset or, alternatively, to NKG2C$^+$ cells that transiently acquire the NKG2A inhibitory receptor under the influence of cytokines (*e.g.* IL-12) remains as an interesting point to be explored. The first option would be indirectly supported by the observation that double-positive cells tend to display an NKG2A^{bright} NKG2C^{dim} phenotype (data not shown). Thus, it can be hypothesized that the inhibitory function of CD94/NKG2A might prevail, hampering the response of this cell subset to hCMV infection.

Although we did not detect any significant correlation between the levels of NKG2C$^+$ cells and donor age, as described in adult donors [46], our data indicate that older children (>2 years) showed higher proportions of NKG2C$^+$ cells. Therefore, our study suggests that the NK cell mediated innate immune response to hCMV infection may be influenced by the age of the patients as well as by the time of infection. As both variables may be interrelated but the time of hCMV primary infection cannot be precisely ascertained, a complex prospective sequential analysis in a larger cohort of donors would be required to precisely address this issue.

In agreement with a previous report in adult blood donors [32], an association between a positive serology for hCMV and increased levels of ILT2$^+$ T cells was also detected in children. Moreover, NKG2C$^+$ILT2$^+$ NK cells were significantly increased in hCMV$^+$ donors, whereas this association was not observed for total ILT2$^+$ CD3$^-$CD56$^+$ NK cells. As mentioned above, the NKG2C$^+$ NK cell subset was shown to contain higher proportions of ILT2$^+$ cells than the NKG2A$^+$ population. Although the mechanism(s) that regulate the expression of ILT2 in NK and T cells have not been precisely defined, there is evidence that this leukocyte inhibitory receptor is displayed by virus-specific effector/memory T cells [47, 48]. Thus, it is conceivable that ILT2 may also be acquired by NK cells during their proliferation and late differentiation driven by viral infections. In this regard, it is of note that ILT2$^+$NKG2C$^+$ NK cells are found in the CD56^{dim} subset, whereas CD56^{bright} NK cells appear predominantly NKG2A$^+$ILT2$^-$ (Romo and López-Botet, unpublished results). The implications derived from the high affinity interaction established between the hCMV UL18 glycoprotein and the ILT2 inhibitory receptor in the context of the immune response against the virus remain ill-defined [24, 25, 49].

Human CD161 (NKR-P1A) is a C-type lectin receptor selectively expressed by NK cells and T lymphocyte subsets. Though the precise biological role of this receptor has not been elucidated, it has been reported that upon interaction with its ligand, the lectin-like transcript 1(ILT1), it may inhibit NK cell-mediated cytotoxicity and IFN-γ production [50, 51]. In addition, it has been shown that CD161$^+$ T lymphocytes mainly correspond to memory cells that synthesize IFN-γ and TNF-α [38]. No

differences in the expression of CD161 by $CD3^- CD56^+$ NK cells and T lymphocytes were found between hCMV-seropositive and hCMV-seronegative children, in agreement with results in adult individuals [32].

The unexpected detection among the $CD3^- CD56^-$ population of cells bearing NKG2C, NKG2A, or CD161 molecules, known to be specifically expressed only by NK and T-cell subsets, revealed the presence of $CD3^- CD56^-$ NK cells. Though this atypical NK cell subset was originally described in $HIV-1^+$ individuals [36], it can be also detected in some healthy adult blood donors (Romo and López-Botet, unpublished results). Remarkably, hCMV-seropositive children displayed increased proportions of $NKG2C^+$ $CD3^-CD56^-$ NK cells. Thus, a more precise phenotypic and functional characterization of the $CD3^- CD56^-$ NK cell subset in the context of hCMV infection is warranted.

In summary, our data indicate that hCMV infection may substantially alter the NKR repertoire in childhood, inducing an expansion of $NKG2C^+$NK and T cells as well as of $ILT2^+$ T lymphocytes. Further prospective studies are required to assess the impact of congenital hCMV infection on the distribution of NKR in cord blood lymphocytes.

Materials and methods

Blood donors and samples

We studied 41 clinically healthy children, including 25 female and 16 male, with ages ranging from 9 months to 11 years (median: 5.0 years). Children with malignancies, autoimmune disorders, immunodeficiency, and those with symptoms of infection during the previous 7 days were excluded. In all cases, informed consent was obtained from the donor's parents. Research and Ethics Committee at the Hospital Central "Dr. Ignacio Morones Prieto" approved this study. This work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Peripheral venous blood samples were obtained and collected in heparinized (for flow cytometry analysis) and non-heparinized tubes (for serological analysis). Saliva and urine samples were collected to determine the presence of CMV excretion in all cases. Urine and saliva samples were obtained with the use of a collection bag (for infants) or by clean catch (in older children), and with a cotton tipped swab, respectively.

Antibodies and reagents

The following mAb were used: anti-NKG2A Z199, anti-KIR2DL1/2DS1 HP-MA4, anti-CD161 HP-3G10, and anti-KIR3DL1 DX9, PE-conjugated anti-NKG2C and anti-NKG2A (R&D Systems, Minneapolis, MN, USA), PerCP labeled anti-CD3, APC tagged anti-CD56 (BD Biosciences, San Jose, CA, USA), and biotinylated anti-ILT2 (HP-F1) (R&D Systems). Avidin (Sigma Chemical, St Louis, MO,

USA), and a rabbit anti-mouse IgG antibody (eBioscience, San Diego, CA, USA) labeled with FITC were also employed.

Immunofluorescence and flow cytometry analysis

PBMC were isolated by Ficoll-Hypaque centrifugation. For surface immunostaning, the following protocol was performed: cells were incubated with the anti-NKG2A Z199 or any of the following anti-KIR2DL1/2DS1, anti-CD161, anti-KIR3DL1, or biotinylated anti-ILT2; subsequently, cells were washed with PBS and incubated with a rabbit anti mouse IgG antibody labeled with FITC. In the case of anti-ILT2, FITC tagged avidin was used. Mouse serum (1:10 dilution) was used to block free binding sites of rabbit anti-mouse IgG antibody. Cells were washed with PBS and finally incubated with an $anti-CD3^-$ PerCP and an $anti-CD56^-$ APC. Finally, cells were also stained with an anti-NKG2C-PE or an anti-NKG2A-PE, followed by washing and fixation in 1% *p*-formaldehyde. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA), using the CellQuest software.

hCMV detection

Standard clinical diagnostic tests were used to analyze serum samples from blood donors for circulating IgG or IgM antibodies against hCMV. The cut-off between negative and positive donors was 22 UI/mL for hCMV-specific IgG and 40 UI/mL for hCMV-specific IgM.

To determine the active excretion of hCMV, the presence of hCMV DNA in urine and saliva samples was determined by PCR assay. For this purpose, DNA was extracted from 200 μL of either urine or saliva by means of a detergent-based DNA extraction technique. Briefly, 500 μL of lysis buffer (Tris-HCL (10 mM, pH 8), EDTA (2 mM, pH 8), and NaCl (400 mM)), 50 μL of 10% sodium dodecyl sulfate, and 20 μL of proteinase K (20 mg/mL) were added to the samples, mixed thoroughly for 30 s and incubated for 90 min at 55°C and at 300 rpm using a thermomixer (Eppendorf AG, Hamburg, Germany). After the incubation step, 199.2 μL of a 5-M NaCl solution was added, gently mixed, and then centrifuged at $16\,000 \times g$ for 15 min. The supernatant was transferred to a sterile tube and 760 μL of ice-cold (4°C) isopropanol was added and kept at −70°C for 1 h to precipitate the DNA. Subsequently, the solution was centrifuged at $16\,000 \times g$ for 10 min and the supernatant discarded. The DNA pellet was allowed to dry at room temperature under a laminar flow hood. Afterwards, the DNA pellet was washed with 500 μL of 70% ethanol, gently mixed, and then centrifuged at $9000 \times g$ for 10 min. The supernatant was discarded, the DNA pellet was once again allowed to dry, and resuspended in 50 μL of nuclease free water. The DNA solution was stored at −70°C until the screening for CMV was performed.

Screening for the presence of CMV relied on a nucleic-acid-based approach using nested PCR using sequence specific oligonucleotide primers. Oligonucleotide primer pairs were designed based on a locally assembled nucleotide sequence

alignment (Multiple Sequence Comparison by Log-Expectation, from the European Bioinformatics Institute Server [52]) for two Human Herpesvirus 5 Immediate Early Protein 2 sequences (C_006273, NC_001347) and six Immediate Early Protein 1 sequences (NC_006273, NC_001347, S71385, AY245900, AY245892, AY245901) retrieved from GenBank. Oligonucleotide primers directed toward conserved regions of the Immediate Early Protein 1 amplify an initial fragment 607 bp long (PCR #1), which is then used as the template for a nested PCR that generates a 380-bp-long fragment. Oligonucleotide primers used are 1st PCR: Forward 5′-GTC-AAA-CAG-ATT-AAG-GTT-CGA-GTG-G-3 (position +284) and Reverse 5′-TGT-ACT-CAT-TAC-ACA-TTG-TTT-CCA-CAC-3′ (position +919) and 2nd PCR: Forward 5′-ACT-GGC-GCC-TTT-AAT-ATG-ATG-GG-3′ (position +394) and Reverse 5′-GAG-CAC-TGA-GGC-AAG-TTC-TGC-3′ (position +777). Both PCR rounds were performed using 12.5 μL final reaction volumes containing 200 nM dNTPs, 2 mM MgSO$_4$, 0.3 IU of *Tfl* DNA polymerase, 0.5 μM of each primer, and 1 μL of DNA (DNA extracted from the clinical specimens for the first round of PCR and PCR product of the first PCR round for the second round). The amplification program included an initial denaturation step of 5 min at 94°C followed by 30 cycles (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C) followed by a final 5-min extension step at 72°C. The amplification products were subjected to 1.2% agarose gel electrophoresis, stained with ethidium bromide, visualized under UV light, and electronically documented.

Statistical analysis

Data were analyzed with the GraphPad InStat 3.06 version program using non-parametric tests. Differences in the levels of expression of NK receptors were evaluated by the Mann–Whitney U test. The analysis of correlations between variables was based on Spearman's rank test. $p < 0.05$ was considered statistically significant.

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