Cytomegalovirus-Infected Primary Endothelial Cells Trigger NKG2C⁺ Natural Killer Cells

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Key Words

NKG2C · Human leukocyte antigen E · Natural killer cells · Endothelial cells · Cytomegalovirus

Abstract

Among innate cells, natural killer (NK) cells play a crucial role in the defense against cytomegalovirus (CMV). In some individuals, CMV infection induces the expansion of NKG2C⁺ NK cells that persist after control of the infection. We have previously shown that KIR2DL⁺ NK cells, in contrast to NKG2C⁺ NK cells, contribute to controlling CMV infection using a CMV-infected monocyte-derived dendritic cell (MDDC) model. However, the nature of CMV-infected cells contributing to the expansion of the NKG2C⁺ NK cell subset remains unclear. To gain more insight into this question, we investigated the contribution of NKG2C⁺ NK cell activation by CMV-infected primary human aortic endothelial cells (EC) isolated from kidney transplant donors, which constitutively express the human leukocyte antigen (HLA)-E molecule. Here, we show

that, although classic HLA class I expression was drastically downregulated, nonclassic HLA-E expression was maintained in CMV-infected EC. By comparing HLA expression patterns in CMV-infected EC, fibroblasts and MDDC, we demonstrate a cell-dependent modulation of HLA-E expression by CMV infection. NKG2C⁺ NK cell degranulation was significantly triggered by CMV-infected EC regardless of the nature of the HLA-E allele product. EC, predominantly present in vessels, may constitute a privileged site for CMV infection that drives a 'memory' NKG2C⁺ NK cell subset.

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Introduction

Human cytomegalovirus (CMV) infection is highly prevalent worldwide. In immunocompetent individuals, CMV infection is usually asymptomatic and immune cells rapidly control its multiplication. Among the different cellular immune components, natural killer (NK)

cells constitute a major effector of the innate immune system in controlling viral infections. Indeed, it has been reported that some rare individuals who are selectively deficient in NK cells are susceptible to herpesviruses, including CMV [1, 2]. Like all herpesviruses, CMV remains latent in the host after the primary infection, and persists for life. CMV adapts to maintain a replicatively dormant state at infected sites, and is controlled by host immune surveillance, resulting in host/virus coexistence. This common virus causes major damaging effects in newborns and immunocompromised individuals such as organ- or cell-transplanted recipients as well as HIV patients. Moreover, this virus has recently been linked to immunosenescence [3].

CMV is a large, double-stranded DNA virus encoding more than 200 immunogenic proteins. To escape innate and adaptive cell immune recognition, it displays a wide arsenal of evasion strategies, notably targeting the down-regulation of human leukocyte antigen (HLA) protein expression. Indeed, CMV peptide-loaded classic HLA class I molecules are directly recognized by CMV-specific T lymphocytes. Several CMV-derived viral immunoevasins (e.g. the US2 and US11 regions) counteract HLA class I molecule trafficking, inducing their cell surface downregulation on CMV-infected cells, thus rendering them resistant to T cell lysis [4]. However, HLA class I molecules constitute the primary signature of innate immune self-restriction, and are recognized by NK cells via inhibitory receptors known as KIR. As a consequence, decreased expression of HLA class I molecules on CMV-infected cells limits the engagement of inhibitory KIR, triggering NK cell function. CMV-infected cells express a late UL18 protein that is stably associated at the cell surface with $\beta 2$ microglobulin. This allows subsequent engagement with the inhibitory immunoglobulin-like transcript 2 (ILT2) receptor [5], which counters the absence of KIR engagement. Moreover, nonclassic HLA class I molecules that normally engage with functional NK receptors are particularly targeted by CMV evasion. The downregulation of HLA class I molecule expression impacts directly on HLA-E expression, which is dependent on the presentation of HLA class I leader sequences. HLA-E is recognized by the lectin-like CD94/NKG2A heterodimer receptor and inhibits NK cell responses [6]. UL40 is a late CMV protein which contains a signal peptide similar in its sequence to the signal peptide of HLA class I molecules and can restore cell surface HLA-E expression [7]. Moreover, two CMV proteins, UL142 [8] and UL16 [9], respectively, retain the MHC class I-related chain A (MICA) and chain B (MICB) proteins in the cytoplasm of CMV-infected

cells, thus limiting NKG2D⁺ NK cell attack. In addition, US18 and US20 target MICA for lysosomal degradation [10]. Finally, the viral miRNA miR-UL112 acts in synergy with cellular miRNAs to inhibit MICB expression in CMV-infected cells [11], and MICA expression is regulated by at least two RNA-binding proteins [12].

Recent studies have shown an expansion of NK cells expressing the activating NKG2C receptor at high levels in CMV-seropositive but not CMV-seronegative individuals [13–16]. NKG2C associates with CD94 and recognizes HLA-E as its inhibitory counterpart NKG2A. The interaction of the CD94/NKG2C receptor with HLA-E leads to NK cell activation [17]. We have previously shown that these amplified NKG2C⁺ NK cells preferentially express KIR2DL receptors that contribute to NK cell activation in CMV infection [18]. However, the role played by NKG2C receptors in the immune responses against CMV infection remains an open issue. Recently, it has been shown that CMV induces HLA-E on infected fibroblasts and triggers CD14⁺ monocytes to secrete IL-12 [19]. The authors suggested that, in concert, these factors drive the expansion of NKG2C⁺ NK cell subset in CMV infection. This observation based on CMV-infected fibroblasts seems to be limited to this type of cells since CMV does not induce HLA-E on infected monocyte-derived dendritic cells (MDDC), known to produce IL-12.

Endothelial cells (EC) are ubiquitously present at the internal surface of blood vessels. They are a site of CMV replication, a potential site of latency [20], and are continually exposed to circulating NK cells. Moreover, EC constitutively express the non-classical HLA-E molecule and may constitute pivotal target cells in driving NKG2C⁺ NK cell expansion. In a previous study, we showed that CMV-infected MDDC, which express very low levels of HLA-E weakly triggered NKG2C⁺ NK cell degranulation in contrast to the KIR2D⁺ NK cell counterpart [18]. In the light of this, we have established an *in vitro* model using CMV-infected primary human aortic EC, and measured the changes in HLA class I and HLA-E molecule expression. Furthermore, we sought to determine whether NKG2C⁺ NK cells can mount effector responses against these CMV-infected cells.

Materials and Methods

Cells

Peripheral blood mononuclear cells (PBMC) were isolated from citrate-phosphate-dextrose blood from healthy adult volunteers by gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Axis-Shield, PoC AS, Oslo, Norway). All blood donors were re-

cruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France) and informed consent was obtained from all individuals. Human CMV serological status was determined using the chemiluminescent immunoassay LIAISON® CMV IgG II (DiaSorin, Saluggia, Italy). Primary EC, isolated from kidney transplant donors, were characterized and cultured as previously described [21, 22]. Briefly, primary EC cultures are prospectively isolated at the time of transplant surgery from deceased transplant donors (French Health Minister project No. 02G55). Cells are isolated from small vascular pieces by collagenase digestion and purification using anti-CD31-coated magnetic beads. For each donor, initial cell culture is grown up for 10 days to reach passage 1 and to allow the long-term storage in liquid nitrogen of several samples by donor at an early passage. Phenotyping of the cell culture is routinely performed before freezing to ensure both the endothelial phenotype and the ability of ECs to respond to cytokine stimulation by the regulation of a set of activation markers including CD31, E-selectin, ICAM-1, VCAM-1 and HLA class I molecules. Freezing the EC cultures at a distance from the isolation step also avoids immediate changes due to cell death and the harvesting process. Our experiments were performed using at least 4 independent cell cultures.

These cells were used between passages 2 and 5. HLA-E*01:01 (EC101), HLA-E*01:03 EC (EC103) were characterized as previously described [22]. MRC5 fibroblasts (RD-Biotech, Besançon, France) were cultured in MEM medium containing penicillin-streptomycin and supplemented with 10% FBS. The HLA class I-deficient 721.221 (221) cell line and the HLA-E-transfected 221 cell line (kindly provided by Dr V. Braud, CNRS/UNSA UMR 7275, Valbonne, France) were cultured in RPMI 1640 medium containing glutamine and penicillin-streptomycin, and supplemented with 10% FBS (all from Life Technologies, Paisley, UK). The HLA-E-transfected 221 cell line expresses a chimeric molecule (GLS-B*5801) containing the HLA-G leader sequence, extracellular transmembrane and cytoplasmic domains of the HLA-B*5801 molecule as previously described [6]. Mycoplasma tests performed by PCR were negative for all cell lines.

Isolation of Monocytes and Generation of Infected MDDC

Monocytes were separated from lymphocytes by using a high-density hyper-osmotic Percoll density gradient (Sigma) [23] and cultured for 6 days in RPMI 1640 medium containing glutamine, penicillin-streptomycin and supplemented with 10% human AB serum (all from Gibco, Paisley, UK), interleukin 4 (IL-4; 200 U/ml, CellGenix) and granulocyte-macrophage colony-stimulating factor (GM-CSF; 1,000 U/ml, CellGenix).

CMV Preparation and Cell Infection

Stock suspensions of CMV strain VHL/E (provided by Prof. James Waldman, Columbus, Ohio, USA) were produced as previously described [18, 24]. CMV VHL/E was first inoculated onto confluent and low-passaged human umbilical vein EC (HUVEC; PromoCell, Heidelberg, Germany) monolayers in 25-cm² gelatin-coated culture flasks. Cells were cultured in EC growth medium supplemented with 10% FBS and appropriate EC supplements (EGM-2 BulletKit Medium; Lonza, Basel, Switzerland). When 50% of HUVEC exhibited a cytopathic effect, they were harvested, washed and frozen in EGM + 20% FBS. MRC-5 fibroblasts were grown to 80% confluence in 25-cm² flasks in MEM medium containing 10% FBS. They were then inoculated with VHL/E-infected

HUVEC diluted with serum-free MEM. After 12 h, fresh medium supplemented with 10% FBS was added. When a cytopathic effect of 80% was reached, the MRC-5 fibroblasts were washed, trypsinized, suspended in serum-free MEM and inoculated into PBS-washed MRC-5 monolayers in 75-cm² flasks. After 12 h, fresh medium supplemented with 10% FBS was added. Cell cultures were incubated for 6–8 days and frozen when a 90% cytopathic effect was observed. CMV VHL/E was then harvested from the MRC5-supernatant after a freeze-thaw step and ultracentrifugation (2 h at 28,000 rpm) in a sucrose density gradient. Pellets containing virions were resuspended in serum-free RPMI medium and stored at –80°C.

Infectivity was checked and titrated on the infected MDDC, primary EC and fibroblasts (MRC-5) grown to confluence in 6-well plates. Cells were washed and inoculated (or not) with increasing volumes of CMV VHL/E stock in serum-free medium. After 12 h, cells were washed and fresh medium containing 10% FBS was added. Two days after infection, cells were harvested and stained with immediate-early 1 (IE1) specific antibody (8B1.2; Millipore) to assess the percentage of infected cells and to determine the optimal volume of CMV suspension to use in all designed experiments. An optimal multiplicity of infection (MOI) of 0.2–0.3 was determined, based on VHL-E-infected fibroblast cultures.

Functional Assays and Flow Cytometry

PBMC were preincubated with anti-CD107a (H4A3; BD Biosciences, San Jose, Calif., USA). NK cell degranulation was assessed after incubation for 5 h alone (negative control) with target cells.

PBMC were stained with antibodies (Abs) against CD3 (SK7), ILT2 (GHI/75), DNAM-1 (DX11), CD161 (DX12) (BD Biosciences), NKG2C (134591; R&D Systems), NKG2A (Z199), KIR2DL1/S1 (EB6), KIR2DL2/3/2DS2 (GL183) and KIR3DL1/S1 (Z27) (Beckman Coulter), NKp46 (9E2), 2B4 (2-69), CD16 (NKP15) (Becton Dickinson, Franklin Lakes, N.J., USA) and KIR2DL1/2/3/S1/S2 (1A6) [25]. EC were stained with Abs against HLA-A, HLA-B, HLA-C (NaM41-1E3; EFS) [26] and HLA-E molecules (3D12; MiltenyiBiotec). Flow cytometry was performed using a FACS-Calibur apparatus with CellQuest software (BD Biosciences) and analyzed using FlowJo 7.6.1 software (Tree Star, Ashland, Oreg., USA).

HLA-E Genotyping

Total RNA was isolated from 1×10^6 CMV⁺ PBMC using a Nucleospin® RNA kit (Macherey Nagel GmbH, Düren, Germany). Primers used to amplify the HLA-E gene were: 5'-GGCTGGGATCATGGTAGATG-3' (forward) and 5'-GAAGCCAGAGTCCCCTAAGA-3' (reverse). HLA-E cDNA sequencing was performed by the Sequencing Core Facility (SFR François Bonamy, Nantes, France) using an ABI 3730 automatic system (Applied Biosystems, Carlsbad, Calif., USA). HLA-E cDNA sequence alignment was performed using a Codon Code Aligner. The sequence was then aligned against the reference HLA-E sequences.

VHL/E UL40 Sequencing

Total RNA was purified from VHL/E-infected MDDC using the NucleoSpin RNA kit RNA. Primers to amplify the UL40 gene were designed using the sequences for human herpesvirus 5 strain AD169 and wild-type strain Merlin as previously described [27] and were 5'-GGCTCTGTCTCGTCGTCATT-3' (forward) and 5'-CGACACCGATCGATTTTCTT-3' (reverse) (Geneworks,

Hindmarsh, S.A., Australia). UL40 cDNA sequencing was performed as previously described for HLA-E genotyping. The sequence was then aligned against the human herpesvirus 5 strains, AD169, Toledo, Town and Merlin.

Statistical Analysis

All statistical analyses were performed with GraphPad Prism v6.0 (GraphPad software). Intergroup comparisons were assessed with the one-way analysis of variance (ANOVA) test from all independent groups of experiments. $p < 0.05$ was considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

Results

CMV Fails to Downregulate HLA-E on EC

Because EC constitutively express higher levels of HLA-E compared to conventional antigen-presenting cells and since CMV immunoevasins interfere with HLA molecule trafficking, we evaluated the modulation of both HLA class I and HLA-E molecules on EC infected with CMV, 48 h after infection in comparison to MDDC and fibroblasts by flow cytometry. Infection was performed with the endotheliotropic VHL/E strain. CMV-infected cells express the nuclear IE1 viral protein and a low level of HLA class I molecules compared to mock-infected cells (fig. 1a). Thus, CMV-infected cells can be selected based on the fact that they express lower levels of HLA class I compared to mock-infected cells. $43 \pm 19\%$ of EC were IE1⁺ 48 h after infection with VHL-E. Similar rates of infection were observed for the MDDC ($41 \pm 6.5\%$) [18] and fibroblasts ($63 \pm 5.3\%$) using the same protocol of CMV infection.

The constitutive HLA class I expression was not significantly higher on EC compared to MDDC (fold change = 0.81) and fibroblasts (fold change = 1.85) but was significantly higher on MDDC compared to fibroblasts (fold change = 2.28; a mean of 479 ± 49 on MDDC and 210 ± 54 on fibroblasts; $p = 0.02$; fig. 1c, d).

Interestingly, HLA class I expression was strongly decreased on EC (fold change = 4.18; a mean of 387 ± 48 on mock-infected EC and 93 ± 19 on CMV⁺ EC; $p < 0.0001$) after CMV infection (fig. 1c, d). In contrast, the effect was less pronounced on fibroblasts that express a lower level of HLA class I on mock-infected cells (fold change = 2.78; a mean of 210 ± 54 on mock-infected fibroblasts and 75.4 ± 36 on CMV⁺ fibroblasts; $p = \text{n.s.}$), and even less pronounced in MDDC (fold change = 1.64; a mean of 479 ± 49 on mock-infected MDDC and 292 ± 72 on CMV⁺ MDDC; $p = \text{n.s.}$; fig. 1c, d). After CMV infection, the expression level of HLA class I molecule was higher on

Table 1. UL40-encoded HLA-E binding peptide from VHL/E CMV strain

CMV strain	P1	P2	P3	P4	P5	P6	P7	P8	P9
AD169	V	M	A	P	R	T	L	I	L
Merlin	V	M	A	P	R	T	L	I	L
Toledo	V	M	A	P	R	T	L	V	L
Towne	V	M	A	P	R	T	L	L	L
VHL/E	V	M	A	P	R	T	L	L	L

MDDC than EC (292 ± 72 on CMV⁺ MDDC and 93 ± 19 on CMV⁺ EC; $p = 0.0053$) or fibroblasts (fig. 1c, d). Neither the nature of the HLA class I molecule nor the KIR ligand HLA-C subtype (C1 or C2) impacted the HLA class I expression pattern (data not shown). However, although HLA-E expression was significantly decreased on MDDC (mean of 5.3 ± 0.88 on mock-infected MDDC and 2.2 ± 0.14 on CMV⁺ MDDC; $p = 0.02$), little or no change in HLA-E level expression was observed on EC (a mean of 7.4 ± 0.69 on mock-infected EC and 6.5 ± 0.95 on CMV⁺ EC; n.s.) and seemed to be slightly higher on fibroblasts (a mean of 3.5 ± 0.84 on mock-infected fibroblasts and 4.78 ± 0.24 on CMV⁺ fibroblasts; $p = \text{n.s.}$; fig. 1d, e). Thus, the modulation of HLA-E expression after CMV infection seems to depend not only on the constitutive level of HLA-E but also on the nature of the target cells. Although the HLA class I expression was drastically decreased on CMV-infected EC, the HLA-E expression level was unaltered.

Identification of UL40-Encoded HLA-E Binding Peptide from VHL/E CMV Strain

The sharp downregulation of HLA class I molecule expression on CMV-infected EC should impact directly on HLA-E expression which is dependent on the presentation of HLA class I leader sequences. Only some CMV strains provide UL40 peptides that stabilize HLA-E on the cell surface and are involved in the interaction with the heterodimers CD94/NKG2A and CD94/NKG2C. To test the hypothesis that the maintenance of HLA-E expression on infected EC could be due to the loading of the UL40 peptide on HLA-E molecule, we sequenced the UL40 gene from VHL/E-infected cells. Indeed, an amino acid sequence alignment of VHL/E UL40 peptide (VMAPRTLIL) with previously published ones [27] highlighted a full homology between the VHL/E and Towne strains (table 1). It has been published that the UL40 peptide from the Towne strain leads to HLA-E expression and its functional engagement with CD94/

Fig. 1. CMV fails to downregulate HLA-E on EC. **a** FACS analysis of HLA class I expression on mock and CMV-infected EC. CMV-infected cells were targeted following a lower HLA class I expression pattern after checking that these cells expressed nuclear IE1 viral protein. EC, MDDC and fibroblasts were infected for 48 h with VHL/E CMV strain. **b** Histogram representing HLA class I expression (HLA-I) on mock-infected EC, CMV-infected EC and CMV-exposed but not infected EC after 48 h of CMV infection. **c** Density plots illustrating the phenotype of CMV-infected cells

(EC, MDDC and fibroblasts) using HLA-E- and HLA-I-specific monoclonal Abs (staining performed by flow cytometry). **d** FACS analysis comparing HLA-I (left) and HLA-E (right) expression at the cell surface (EC, MDDC and fibroblasts) on mock-infected and CMV-infected (for 48 h) cells. Results are expressed as a quantitative analysis of HLA-I or HLA-E geometric mean fluorescent intensity (MFI) minus IgG control geometric MFI for 7 experiments for EC, 3 for MDDC and 3 for fibroblasts. Histograms represent the mean $\pm$ SEM for each condition.

NKG2A and CD94/NKG2C receptors [27]. Thus, in accordance with these published results, we suggest that the UL40 signal peptide from VHL/E is processed and loaded specifically in the groove of HLA-E, maintaining its membrane expression.

The NKG2C⁺ NK Cell Subset Is Triggered by CMV-Infected EC

We have previously shown [18] that in accordance with the low expression of HLA-E on CMV-infected MDDC, CMV-infected MDDC weakly triggered NKG2C⁺ NK cell degranulation, in contrast to its KIR2D⁺ NK cell counterpart. We therefore evaluated the degranu-

Fig. 2. CMV-infected EC triggered the degranulation of the NKG2C⁺ NK cell subset. **a** Density plot illustrating NKG2C⁺ NKG2A⁻ and NKG2C⁻ NKG2A⁺ NK cell subsets targeted with a combination of NKG2C- and NKG2A-specific monoclonal Abs in CD3⁻ cell population from ex vivo PBMC of CMV⁺ individuals. **b** Frequency of NKG2A⁺ and NKG2C⁺CD3⁻ cell subsets expressing CD107a after 5 h of incubation with mock- or CMV-infected EC at an effector:target ratio of 50:1. Results are shown as mean $\pm$ SEM of 4 independent experiments. **c** Histograms showing an

overlay of HLA class I (HLA-I, left) and HLA-E (right) expression on 221 and 221-E cell lines determined by flow cytometry using HLA-I- and HLA-E-specific monoclonal Abs. **d** Dot representation of CD107a⁺CD3⁻ cell frequencies in NKG2C⁺ NKG2A⁻ and NKG2C⁻ NKG2A⁺ cell subsets after incubation with medium, 221 and HLA-E⁺ 221 cells for 6 independent experiments. Statistical significance (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) between both groups was determined using ANOVA.

lation of NKG2C⁺ NK after stimulation with CMV-infected EC on which HLA-E expression is maintained after 48 h of CMV infection. For this, using flow cytometry, we gated on NKG2C⁺ NKG2A⁻ and NKG2C⁻ NKG2A⁺ NK cell subsets from the PBMC of patients presenting an expansion of NKG2C⁺ NK cells in the peripheral blood (fig. 2a). Indeed, NKG2C associated with CD94 recog-

nizes HLA-E, as does its inhibitory counterpart NKG2A. Thus, the discrimination between NKG2A and NKG2C allows us to compare their impact on NK cell response to stimulation with HLA-E⁺ target cells. Of note, we observed an exclusive expression of NKG2A or NKG2C on NK cells and a very small frequency of NK cells expressing both markers. The degranulation of both NKG2A⁺ and

NKG2C⁺ NK cell subsets was determined by measuring the frequency of CD107a⁺ cells after incubation with mock- or CMV-infected EC. CMV-infected EC significantly triggered NKG2C⁺ NK cell subset (a mean of $7.25 \pm 1.4\%$ with EC and $30.1 \pm 6.3\%$ with CMV⁺ EC; $p = 0.01$) and triggered, to a lesser extent, the NKG2A⁺ NK cell subset (a mean of $11.35 \pm 1.6\%$ with EC and $18.25 \pm 3.3\%$ with CMV⁺ EC; n.s.; fig. 2b). In order to validate the assumption that NKG2C⁺ and NKG2A⁺ NK cell subsets are respectively activated and inhibited by HLA-E⁺ target cells, we evaluated their degranulation against the HLA class I-deficient cell line, 721.221 (221) and the HLA-E-expressing 221 cell line (221-E) (fig. 2c). In this setup, the degranulation of NKG2C⁺ NK cells was significantly enhanced by HLA-E expression on 221-E target cells, underlying the functional impact of this triggering interaction (fig. 2d). In parallel, HLA-E expression on 221-E target cells weakly inhibited NKG2A⁺ NK cell degranulation. Thus, the CMV-infected EC which maintained an expression level of membrane HLA-E after 48 h of CMV infection significantly triggered NKG2C⁺(NKG2A⁻) NK cells.

KIR Inhibition of NKG2C⁺ NK Cell Degranulation against CMV-Infected EC

Like other study groups [13, 28], we have shown that NKG2C is mainly coexpressed with inhibitory KIR on NK cells [18]. Indeed, KIR2D (KIR2DL1/S1, KIR2DL2/3/2DS2) are mainly coexpressed with NKG2C, and slightly with NKG2A on NK cells from CMV⁺ individuals with peripheral blood NKG2C⁺ NK cell expansion (fig. 3a) as illustrated for 1 representative CMV⁺ individual (fig. 3b). To further evaluate the impact of KIR on NKG2C⁺ NK cells in our CMV⁺ EC model, we focused on the NKG2C⁺KIR2D/3DL1/S1/NKG2A⁻ (named NKG2C⁺KIR/NKG2A⁻) NK cell subset in comparison to NKG2C⁺KIR/NKG2A⁺ NK cell subsets from ex vivo CMV⁺ PBMC (fig. 3c). Of note, the NKG2C⁺KIR/NKG2A⁺ population is mainly made up of the NKG2C⁺KIR⁺ NK cell subset as NKG2C and NKG2A were not coexpressed in all studied individuals (fig. 2a). Although both NK cell subsets present a similar degranulation pattern against K562 (data not shown) and mock-infected EC, KIR expression on NKG2C⁺ NK cells significantly limited the degranulation against CMV⁺ EC (mean of 13 ± 1.4) in comparison to the NKG2C⁺KIR/NKG2A⁻ NK cell subset (a mean of 22 ± 4 ; $p = 0.03$; fig. 3d). Of note, HLA class I expression decreased on CMV-infected EC, but remaining levels were high enough to ensure the functional engagement of inhibitory KIR. Moreover, HLA class I expression increased on CMV-exposed but

uninfected EC (IE1⁻) (fig. 1b). As around 50% of EC were infected in our culture, the increased expression of HLA class I molecules on CMV-exposed EC (IE1⁻) may contribute to inhibit NK cell activation via HLA-specific inhibitory KIR. The NKG2C⁺KIR/NKG2A⁻ NK cell degranulation was significantly increased by CMV-infected EC compared to mock-infected cells (a respective mean of 22 ± 4 and $5.3 \pm 1.6\%$, $p < 0.0001$) at an effector:target ratio of 50:1, and remained significantly increased at 10:1 ($p = 0.0002$) (fig. 3e). Finally, we studied the phenotype of the NKG2C⁺ NK cell subset in comparison to the NKG2A⁺ NK cell subset in focusing on inhibitory ILT2 and activating NK receptors like DNAM-1 (DNAX accessory molecule-1), NKR1A (CD161), 2B4 (CD244) and CD16, known to modulate NK cell functions (online suppl. fig. 1; www.karger.com/doi/10.1159/000445320). All activating DNAM-1, CD161, 2B4 and CD16 were similarly expressed on the NKG2C⁺ and NKG2A⁺ NK cell subsets. However, inhibitory ILT2 was expressed on all NKG2C⁺ NK cell subsets but not on the NKG2A⁺ NK cell subset. CMV escapes to ILT2⁺ NK cell recognition via the UL18 protein which mimics HLA class I molecules [5]. Depending on the time point during the CMV replicative cycle, ILT2 interaction with UL18 protein may contribute to inhibiting NKG2C⁺ NK cell functions. Overall, KIR expressed on a high proportion of NKG2C⁺ NK cells modulates NKG2C⁺ NK cell functional abilities against CMV-infected EC.

EC Triggered NKG2C⁺ NK Cell Degranulation

Regardless of the Nature of the HLA-E Allele Product

HLA-E, as with other unclassic HLA class I molecules, is characterized by a poor allelic polymorphism. Indeed, only 17 alleles and 6 corresponding proteins have been reported (<http://www.ebi.ac.uk/ipd/imgt/hla>) [29]. However, it has been previously described that the membrane stability of the HLA-E molecule can depend on the nature of the HLA-E allele [30, 31]. Moreover, the impact of the HLA-E polymorphism on effector cell function expressing the HLA-E receptor is poorly documented. Thus, we evaluated the expression of the more frequent HLA-E*01:01 and HLA-E*01:03 allele products (<http://www.allelefreqencies.net>) [32] on mock- and CMV-infected EC. On CMV-infected cells (HLA^{low}), despite a strong downregulation of HLA class I expression, this was maintained at a similar level to those observed on mock-infected cells, regardless of the nature of HLA-E allele product (fig. 4a). Interestingly, the expression of HLA-E and classic HLA class I was upregulated on CMV-exposed but uninfected EC (HLA^{high}) (fig. 4b) as previ-

Fig. 3. KIR inhibition of NKG2C⁺ NK cell degranulation against CMV-infected EC. **a** Histograms representing the frequency of NKG2A⁺ and NKG2C⁺ NK cell subsets in NK cells (CD3⁺CD56⁺) from 7 CMV⁺ donors (D1–D7) with peripheral blood NKG2C⁺ NK cell expansion. The frequencies of KIR2D⁺NKG2A⁺ and KIR2D⁺NKG2C⁺ NK cells for all studied donors are shown in black. **b** Density plots illustrating KIR2D (KIR2DL1/S1, KIR2DL2/3/2DS2) expression on NKG2A⁺ and NKG2C⁺ NK cell subsets from a representative CMV⁺ donor (D5). **c** Density plot illustrating NKG2C⁺KIR/NKG2A⁺, NKG2C⁺KIR/NKG2A⁺ and NKG2C⁺KIR/NKG2A⁺ NK cell subsets. A combination of NKG2C-specific monoclonal Ab and a combination of KIR-

(KIR2DL1/2/3/2DS1/2-specific 1A6 and KIR3DL1/3DS1-specific Z27) and NKG2A-specific monoclonal Abs in CD3⁺ cell population from ex vivo PBMC of CMV⁺ individuals. **d** Histograms summarizing the frequency of both NK cell subsets expressing CD107a after 5 h of incubation with EC or CMV-infected EC at an effector:target ratio of 50:1 for 6 experiments. **e** Dot graph representing the frequency of the NKG2C⁺KIR/NKG2A⁺ NK cell subset expressing CD107a after 5 h of incubation with mock-infected EC (white circle) or CMV-infected EC at an effector:target ratio of 50:1 (grey circle) and 10:1 (black circle), respectively, for 6 and 8 experiments performed. Statistical significance (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$) between groups was determined using ANOVA.

Fig. 4. NKG2C⁺ NK cell degranulation is triggered by CMV-infected EC regardless of the HLA-E allele product. **a** Density plots illustrating the staining of mock- and CMV-infected EC with HLA class I (HLA-I)-specific and HLA-E-specific monoclonal Abs. **b** HLA expression was determined on mock- (heavy black line) and CMV-infected HLA-E*01:01 homozygous EC (EC101) and HLA-E*01:03 homozygous EC (EC103). Mock-infected (black line) and CMV+ EC (filled grey) were targeted, respectively, following their high and low HLA-I level expression (defined in fig. 1a). Isotype IgG control was included (grey line). **c** Density plot

illustrating NKG2A⁺ and NKG2C⁺ NK cell degranulation (CD107a %) after stimulation with mock and CMV-infected EC101 or EC103 cell lines. **d** CD107a⁺ NK cell frequency fold induction (percentage of CD107a⁺ NK cells observed against CMV+ EC/percentage of CD107a⁺ NK cells observed against mock-infected EC) determined for 6 experiments. **e** Nature of HLA-E alleles in 6 CMV+ individuals with or without NKG2C⁺ NK cell expansion. The percentage of NKG2C⁺ NK cells without an NK cell population (CD3⁺CD56⁺) is indicated.

ously described [18, 33] regardless of the nature of the HLA-E allele product. Cytokines produced by CMV-infected cells such as type 1 IFN seems to contribute to this effect. In accordance with the HLA-E phenotype of CMV-infected EC (EC101 and EC103), NKG2C⁺ NK cell degranulation was strongly triggered by both CMV-infected primary EC (EC101 and EC103) (fig. 4c: a repre-

sentative experiment; fig. 4d: a summary) with the fold-increased ratio of CD107a⁺ NK cells observed with CMV-infected EC. Lastly, the CMV+ individuals with an NKG2C⁺ NK expansion in their NK cell repertoire did not harbor a specific HLA-E allele product as documented in 6 CMV seropositive individuals with or without NKG2C⁺ NK cell expansion (fig. 4e).

Discussion

EC constitutively express the nonclassic HLA-E molecule, are ubiquitously present in the blood circulatory system and are therefore continually exposed to circulating NK cells. We set up an *in vitro* model using CMV-infected primary EC isolated from kidney transplant donors. We then explored the modulation of HLA class I molecule expression on CMV-infected EC and sought to determine whether NKG2C⁺ NK cells can mount an effector response against these CMV-infected cells. We showed that HLA class I expression was strongly reduced on EC but that little or no change was observed in HLA-E level expression. Our results corroborate those obtained by Odeberg et al. [34], showing a disparity in HLA class I modulation by CMV infection on macrophages and EC. Moreover, a reduced HLA-E expression can be observed following prolonged infections, which is probably due to miRNA editing which results in the downregulation of HLA-E [35].

The majority of studies measuring CMV evasion strategies have been determined using transfected cells and CMV-infected immortalized fibroblast strains. Overall, it is difficult to evaluate the hierarchy and kinetics of the different interactions involved in the engagement of CMV-infected target cells and NK cells. This issue seems to be strongly dependent on the nature of the infected cells and their own cellular machinery. Indeed, after 48 h of infection with the endotheliotropic VHL/E strain, we observed some obvious differences in HLA expression patterns (i.e. in MDDC and EC) that were cell type-dependent.

It has been previously shown that HLA-E expression is maintained on CMV-infected fibroblasts, thus facilitating their escape from CD94/NKG2A⁺ NK-mediated cell lysis [36]. Recently, different study groups have highlighted the strong association of NKG2C⁺ NK cells with CMV infection, suggesting an activation of this population during acute CMV infection [15, 37]. Using CMV-infected MDDC, we were not able to demonstrate a contribution of NKG2C⁺ NK cells in controlling CMV infection, due to the weak HLA-E expression on infected MDDC; however, in this MDDC model, we showed that KIR2DL⁺ NK cells are involved in the control of CMV infection [18]. New insights have been provided into the shaping of the NK cell repertoire by CMV infection; nonetheless, many questions remain concerning the cell/CMV location that drives and maintains the 'memory'-like NKG2C⁺ NK cell subset. We demonstrated the contribution of NKG2C⁺ NK cells in measuring their degranulation after stimula-

tion with CMV-infected EC on which HLA-E expression is maintained after 48 h of CMV infection. Interestingly, NKG2C⁺KIR⁻ NK cells were more significantly triggered than NKG2C⁺KIR⁺ NK cells against CMV-infected EC, suggesting an inhibitory impact of KIR on NKG2C⁺ NK cell functional abilities. Of note, our study presents some technical limitations that should be considered for a balanced interpretation of the results. Indeed, the whole population of CMV-exposed EC contained both IE1⁺ and IE1⁻ cells. The nuclear localization of IE1 in CMV-infected EC did not allow us to sort the IE1⁺ cells.

It is probably true that the sum of molecular events, including CMV immunoevasins and UL40 kinetic expression, basal HLA class I level expression, HLA polymorphism (i.e. MICA) and the general cellular machinery, results in different HLA phenotypic patterns of CMV-infected cells. This is very important, since it would explain that the nature of the infected cells can drive the HLA-specific receptor phenotype of NK cells that mount a response to control CMV infection. It explains, in part, the dichotomy observed in CMV⁺ healthy individuals for whom the NK cell repertoire is marked either by an NKG2C phenotype or activating KIR expansion [13]. Recently, Rölle et al. [19] have shown that HLA-E contributes in controlling CMV-driven NKG2C⁺ NK cell expansion. In line with this, we hypothesized that NKG2C⁺ NK cell expansion can be driven preferentially by highly expressed HLA-E⁺ on CMV-infected cells as EC, which are predominantly present in vessels. In contrast, MDDC do not express HLA-E after CMV infection and would be more likely to mount a KIR⁺ NK cell response. Furthermore, both viral and cellular polymorphisms contribute to drive the immune response in CMV⁺ individuals. Thus, it is conceivable that different CMV strain infections may differ in their cytopathic effects based on EC viral genetic determinants [38]. In this study, we showed that in contrast to MDDC, CMV-infected primary EC significantly trigger NKG2C⁺ NK cells regardless of the nature of HLA-E allele product, suggesting that EC may constitute a privileged site for CMV infection that drives a 'memory' NKG2C⁺ NK cell subset. It is conceivable that this 'memory'-like NK cell subset is maintained life-long, due to a continuous EC CMV replication which is balanced by host immune responses. As previously demonstrated, soluble factors like IL-12 appear essential for driving the expansion of an NKG2C⁺ NK cell subset [19, 39]. CMV seems to establish a persistent noncytopathic infection in aortic EC, suggesting that EC can represent a potential infection site for latency [40]. Moreover, the primary EC used in this study were unmanipulated cells iso-

lated from donor grafts. They constitute an interesting CMV cell target, allowing improved knowledge of the NK cell subsets which efficiently control this infection. In the context of kidney transplantation, our data raise the question of whether, in CMV⁺ patients with peripherally expanded NKG2C⁺ NK cells, CMV reactivation could be efficiently controlled in the EC of a kidney graft.

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Disclosure Statement

The authors declare no financial or commercial conflicts of interest.

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