Title:
Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts.

Short title: CD94/NKG2C+ NK cells and cytomegalovirus infection

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ABSTRACT
CD94/NKG2C+ NK cells are increased in healthy individuals infected with human cytomegalovirus (HCMV), suggesting that HCMV infection may shape the NK cell receptor repertoire. To address this question, we analysed the distribution of NK cell subsets in PBL co-cultured with HCMV-infected fibroblasts. A substantial increase of NK cells was detected by day 10 in samples from a group of HCMV+ donors, and CD94/NKG2C+ cells outnumbered the CD94/NKG2A+ subset. Fibroblast infection was required to induce the preferential expansion of CD94/NKG2C+ NK cells, that was comparable with allogeneic or autologous fibroblasts, and different virus strains. A CD94-specific mAb abrogated the effect, supporting an involvement of the lectin-like receptor. Purified CD56+ populations stimulated with HCMV-infected cells did not proliferate, but the expansion of the CD94/NKG2C+ subset was detected in the presence of IL-15. Experiments with HCMV deletion mutants indicated that the response of CD94/NKG2C+ NK cells was independent of the UL16, UL18 and UL40 HCMV genes, but was impaired when cells were infected with a mutant lacking the US2-11 gene region. Taken together the data support that the interaction of CD94/NKG2C with HCMV-infected fibroblasts, concomitant to the inhibition of HLA class I expression, promotes an outgrowth of CD94/NKG2C+ NK cells.
INTRODUCTION

Human cytomegalovirus (HCMV) infection generally follows a subclinical course but may lead to severe disorders in immunocompromised individuals and is a main cause of infectious congenital diseases. HCMV remains latent in immunocompetent hosts, undergoing occasional reactivation\(^1\). Studies in murine models revealed that an effective defence against CMV requires the participation of NK and T cells\(^2\;\,^3\). Detection of antibodies and CD8\(^+\) T lymphocytes specific for HCMV antigens allow to assess the adaptive immune response to the pathogen\(^4\;\,^5\). To escape from CD8\(^+\) T cells, HCMV inhibits the expression of HLA class I molecules and interferes with antigen presentation using a set of glycoproteins (US2, US3, US6, US10 and US11) whose genes are clustered within the unique short (US) region of the virus genome\(^6\;\,^8\). The loss of HLA class I molecules in HCMV-infected cells impairs the engagement of inhibitory receptors and prompts the activation of NK cell effector functions; reciprocally the virus has developed several strategies to evade NK-mediated recognition\(^9\).

The nature of receptor-ligand interactions involved in the NK cell response to CMV-infected cells is incompletely understood. In strains of mice expressing the Ly49H receptor, NK cell functions are triggered upon recognition of the m157 mouse CMV (MCMV) glycoprotein, becoming essential to control replication\(^10\;\,^11\); by contrast, human activating NK cell receptors (NKR) specific for HCMV molecules have not been identified. The involvement of activating killer Ig-like receptors (KIR) and natural cytotoxicity receptors (NCR) (i.e. NKp46, NKp30 and NKp44) in the response to HCMV is uncertain. The interaction of the pp65 HCMV tegument protein with NKp30 has been reported to inhibit rather than to activate NK cell functions\(^12\). The ability of the UL16 HCMV molecule to interfere with the surface expression of NKG2D ligands\(^13\;\,^15\), and the evidence that similar evasion mechanisms operate in MCMV infection\(^16\;\,^17\), support an important role for this killer lectin-like receptor (KLR) in the anti-viral defence\(^18\).
Recently, the UL141 HCMV molecule has been shown to inhibit the expression in infected cells of CD155, a ligand for the DNAM-1 stimulating receptor\textsuperscript{19}.

HCMV may also escape NK-mediated surveillance keeping inhibitory receptors for HLA class I molecules engaged. The viral UL18 molecule binds with high affinity to the ILT2 (CD85j) inhibitory receptor\textsuperscript{20; 21}, though its role in immune evasion has not been precisely elucidated\textsuperscript{9}. On the other hand, HLA-E appears constitutively resistant to the action of US2 and US11\textsuperscript{22}, and it becomes as well refractory to the action of US6 when bound to a peptide from the leader sequence of the HCMV UL40 protein\textsuperscript{23; 24}. Thus, the HLA class Ib molecule may be preserved in infected cells and interfere with the NK cell response by engaging the inhibitory CD94/NKG2A KLR\textsuperscript{25}.

We recently reported\textsuperscript{26} that healthy HCMV-seropositive individuals displayed increased proportions of NK and T cells that expressed the triggering CD94/NKG2C KLR, which binds HLA-E with a lower affinity than CD94/NKG2A.\textsuperscript{27; 28} Increased numbers of circulating CD85j+ T lymphocytes were also significantly associated to HCMV infection. Taken together, the data supported that the challenge exerted by HCMV on the innate immune system might shape the NKR repertoire.

Signalling by NCR and KIR may control the proliferation and/or survival of NK cells\textsuperscript{29; 30}; we recently reported that CD94/NKG2C+ NK and T cell subsets divided in response to stimulation with an HLA class I-deficient tumour cell line transfected with HLA-E\textsuperscript{31}. On that basis, we hypothesized that CD94/NKG2C-mediated recognition of virus-infected cells might promote the expansion of NKG2C+ cell subsets, as described for Ly49H+ cells in MCMV-infected mice\textsuperscript{32}. To address this issue we analysed the NKR repertoire in PBL co-cultured with HCMV-infected fibroblasts; little information is available on the NK cell response in this system, widely used to study CTL specific for viral antigens. Our results indicate that stimulation of PBL from HCMV+ donors with virus-infected fibroblasts elicited a preferential expansion of CD94/NKG2C+ NK
cells, and that a cognate interaction of the activating KLR with HCMV-infected cells may contribute to drive the proliferation of this lymphocyte subset.
MATERIALS AND METHODS

Subjects.

Heparinized blood samples were obtained from 15 healthy adult individuals (age: 23-51 years); written informed consent was obtained, and the study protocol was approved by the institutional Ethics Committee (CEIC-IMAS). Standard clinical diagnostic tests were used to analyse serum samples for circulating IgG antibodies against CMV (Abbot Laboratories, Abbot Park, IL); four donors were seronegative (HCMV-) and 11 seropositive (HCMV+).

Cell lines

MRC-5 fetal human lung fibroblasts and human foreskin fibroblasts (HFF) cell lines were obtained from the American Type Culture Collection (Manassa, VA) and grown in Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin and streptomycin (referred to as complete medium). Primary fibroblast lines were established from skin biopsies obtained from two subjects following standard procedures; briefly, tissue samples were minced, treated with 0.2% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany), washed and grown in complete medium.

Cell lines were screened and shown to be negative for Mycoplasma contamination by PCR using primers as described\(^{33}\). Fibroblast lines used were between passages 8 and 15 (HFF), 20 and 30 (MRC-5 cells) and 4 and 6 (primary fibroblast lines).

HCMV preparations and infection of fibroblasts

Stocks of HCMV strains AD169, Towne, and Toledo, and HB5-derived mutants were prepared, and titers of infectious virus determined by standard procedures on HFF or MRC-5 cells. Viral preparations were screened for Mycoplasma as indicated above. The
Towne strain of HCMV was purified as described with slight modifications. UV-inactivation of virus was performed using a 30 W Phillips lamp (model G13) and confirmed by monitoring expression of the IE1 protein on MRC-5 cells by indirect immunofluorescence. Recombinant HCMV genomes were generated in E. coli. The AD169-derived HCMV bacterial artificial chromosome plasmid (BACmid) HB5, which lacks the US2-6 gene region was used to generate all HCMV mutants described in the study.

The HB5-ΔUS2-11 mutant was constructed by homologous recombination, as described, with some modifications. Briefly, 5':

TCACACATACCTTTGTGCATACGGTTTATATATGA
CCATCCACGCTTATAACGAACCTACGATTTATTCAACAAGCCACG and 3':
TGCTATAAGACAGCCTTTGAGTCTAGACAGGGTAACACAGCTTCC
CTTGTAAAGCCAGTGTAAACAATTTAAACC, PCR primers were used to amplify the kanamycin gene, adding sequences homologous to regions upstream of US7 and downstream of US11, respectively. The 5'-primer corresponds to nucleotides (nt) 197237 to 197296 of the coding strand, and the 3'-primer to nt 201356 to 201297 of the antisense strand of AD169 (GenBank # NC_001347). The PCR product was transformed into E. coli carrying the HB5 BACmid and expressing the Red recombinase system from the plasmid pkD46. Recombinant clones were selected at 43°C under chloramphenicol and kanamycin selection, and correct mutagenesis was confirmed by restriction digest analysis, PCR and southern blot analysis of isolated BACmid DNA.

Reconstitution of the virus mutant was carried out by transfecting 5 μg of BACmid into MRC-5 cells using superfect (Quiagen, Hilden, Germany). Human CMV recombinants HB5-ΔUL14-20 and HB5-ΔUL40-42 have been previously described.

Human fibroblasts in 24 or 96-well plates at 70% confluence were mock- or HCMV-infected (moi=1). After a 2-h adsorption period, the inoculum was removed, cells were
washed with PBS and incubated in complete medium; in some experiments infection was carried out at a different moi (0.01-5).

**Lymphocyte cultures**

PBL were obtained by centrifugation of heparinized blood on Ficoll-Hypaque (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway). PBL were incubated in 24-well (2x10^6/well) or 96-well (2x10^5/well) plates in complete medium in the presence of mock- or HCMV-infected fibroblasts; in some experiments PBL were stimulated with free HCMV (0.05 infectious particles/cell). PBL were added to infected fibroblasts immediately after the 2-h adsorption period; in time-course experiments co-culture was delayed 24-72 hours post-infection. All cultures were supplemented with IL-2 (10 U/ml) (Eurocetus) at day 3, and in some experiments with IL-15 (10 U/ml) (Peprotech Ec, London, UK). Cells were incubated for 10-12 days, fed every 3 days and eventually split. Some experiments were set up in a Transwell permeable support (0.4 μ) (Corning Inc, NY); infected fibroblasts were cultured with and without PBL, and the phenotype of PBL incubated separately in the upper chamber was analysed. CD94/NKG2C+ NK cell clones were derived as previously described.

**Antibodies. Immunofluorescence and flow cytometry analysis.**

Monoclonal antibodies (mAbs) specific for CD56, CD94, NKG2A, KIR, ILT2, NCR and NKG2D have been previously detailed. HP-1F7 anti-HLA-class I was generated in our laboratory; 3D12 anti-HLA-E was provided by Dr. D. Geraghty (Fred Hutchinson Cancer Research Center, Seattle, WA); anti-NKG2C (MAB1381) and anti-NKG2C-PE were from R&D Systems Inc (Minneapolis, MN).

For immunofluorescence staining, cells were pretreated with human aggregated Ig (10 μg/ml) to block FcR, and subsequently labeled with the different mAb and analyzed by
flow cytometry (FACScan, Becton Dickinson, Mountain View, CA), as described. In
experiments where blocking mAbs (CD94 or CD56) were used, harvested cells were
stained with anti CD3-PerCP, anti NKG2A-FITC, or anti NKG2C-PE. In some
experiments, cells were sorted (FACSVantage, Becton Dickinson, Mountain View, CA)
after labeling with CD56-PE and NKG2C-PE.
Detection of HCMV IE1 was performed by indirect immunofluorescence with MAb
8130 (Chemicon, Temecula, CA) and an FITC-labeled anti-mouse IgG (Sigma, St.
Louis, MO).

**Proliferation and cytotoxicity assays**

As described, PBL were resuspended in RPMI-1640 (10^7/ml) and incubated for 10
min at 37°C with the intracellular fluorescent dye 5(6)-carboxyfluorescein diacetate
succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) (2 µM). After two washes,
CFSE-labeled PBL were cultured on mock- and HCMV- infected MRC-5. FACS
analysis was performed at 7 days, after staining cells with anti NKG2C or anti NKG2A
mAbs by indirect immunofluorescence with PE-tagged F(ab’)2 rabbit anti-mouse Ig
(Dakopatts, Glostrup, Denmark). The proportions of dividing cells within each subset
were calculated as described.

CD94/NKG2C+ NK cell clones were tested in a 4h ^51Cr-release assay against mock-
and HCMV (Towne)-infected (24 or 72 h) autologous or HFF fibroblasts, at different
effector/target ratios. Cells were treated with Trypsin-EDTA (Invitrogen Gibco, Grand
Island, NY) labeled with ^51Cr and used in standard cytotoxicity assays. In parallel
samples, effector cells were pre-incubated with CD94 or CD56- specific mAbs for 30
min before adding targets. All assays were set up in triplicate and specific lysis was
calculated as described.
Statistical analysis

Statistical analysis was performed by the Mann-Whitney $U$ test, using the SPSS 9.0 software (SPSS, Chicago, IL). Results were considered significant at the 2-sided $P$ level of 0.05.
RESULTS

Preferential expansion of NKG2C+ NK cells in PBL stimulated with HCMV-infected fibroblasts.

We previously reported that increased proportions of CD94/NKG2C+ NK cells and ILT2+ (CD85j+) T lymphocytes were detectable in PBL from HCMV seropositive (HCMV+) donors, indicating that the viral infection might shape the NK cell receptor repertoire\(^2\). To address whether HCMV could promote the expansion of CD94/NKG2C+ lymphocytes, PBL from HCMV+ donors were co-cultured either with mock- or HCMV (Towne)-infected MRC-5 fibroblasts, in the presence of an exogenous supply of IL-2 (10 U/ml); at different time-points the proportions and phenotype of NK cells were comparatively assessed. In vitro stimulation of PBL with HCMV-infected fibroblasts has been extensively used to analyse CTL specific for viral antigens, but NK cells have not been systematically studied in this system.

A proliferative response of PBL to HCMV-infected cells, was observed by phase-contrast microscopy. Cell recovery after 10-12 days, referred to the input, was 1.5-5 fold in cultures with HCMV-infected fibroblasts and 0.8-1.5 fold with mock-infected cells in different experiments (n=25). Studies carried out with CFSE-labelled PBL also showed that cell proliferation stimulated by HCMV-infected MRC-5 cells exceeded that induced by mock-infected cultures, allowing a phenotypic analysis of the dividing cells (Figure 1A). As compared to NKG2A+ lymphocytes, NKG2C+ cells mainly divided upon stimulation by HCMV-infected MRC-5 cells; yet, only a fraction proliferated.

Analysis at different time-points of PBL co-cultured with HCMV-infected fibroblasts indicated that T cells were predominant during the first week (Figure 1B), but a shift in the distribution of lymphocyte populations was observed by day 10-12. Remarkably, NK cells substantially increased (Figure 1B), becoming up to 35-80 % of the population in different experiments (n=25), and NKG2C+ lymphocytes outnumbered the NKG2A+
subset (Figure 2A); the proportions of cells co-expressing both KLR were negligible (data not shown). The NKG2C+ cell recovery in cultures with HCMV-infected cells was 5-25 fold that obtained with mock-infected fibroblasts; the NKG2C+/NKG2A+ ratios were 0.3-1.3 and 2.2-6.7 in mock- and HCMV-infected cultures, respectively. Together with a majority of NKG2C+ NK cells, small proportions of CD3+ NKG2C+ and NKG2A+ T lymphocytes were also identified (Figure 2A). By contrast, the phenotype of PBL from HCMV-seronegative donors (n=4) was comparable upon incubation with virus- or mock-infected MRC-5 cells (Figure 2A). It is of note that the late outgrowth of NKG2C+ cells was not perceived in samples from a group of HCMV+ donors (5 out of 11), in which T cells remained the predominant population all over the culture. As compared to responders (R), fresh PBL from the non-responder (NR) group displayed significantly lower proportions of NKG2C+ cells (NR=2.2±1.5 vs R=10.1±7.3; p=0.03) and reduced NKG2C/NKG2A ratios (NR=0.3±0.3 vs R=1.7±1.1; p=0.02).

The expansion of NKG2C+ cells was detectable when PBL were co-cultured with HCMV-infected HFF (data not shown) or autologous skin fibroblasts (Figure 2B). Together with the limited NK cell proliferation in response to mock-infected MRC-5 cells, these results ruled out alloreactivity as the main stimulus responsible for driving the proliferation of NKG2C+ NK cells, and strongly supported a central role for the virus. The effect was comparable upon infection with different HCMV strains (i.e. AD169 and Toledo), as well as with a purified Towne preparation, thus excluding a role for cellular products present in crude virus stocks (not shown); subsequent experiments were performed using either Towne or AD169, as specified in every case.

**Distribution of NKR and NCR in PBL stimulated with HCMV-infected fibroblasts.**
The expression of other NKR (i.e. KIR2D, KIR3D, NKG2D, ILT2) and NCR (i.e. NKp46 and NKp30) was studied in NK and T cells stimulated with HCMV-infected fibroblasts, as previously done in PBL from HCMV+ donors. Expression of NKG2D, NKp46 and NKp30 was similar in mock- and HCMV-infected cultures (Figure 3), except for a slight reduction of NKp46 and NKp30 in the latter; it is of note that NKG2C+ NK cells were reported to express lower levels of these receptors than the NKG2A+ subset\textsuperscript{26}. Some differences in the distribution of KIR were also noticed, but did not follow a reproducible pattern in samples from different donors. By contrast, the proportions of ILT2+ T lymphocytes were found to be significantly increased (p=0.04) upon stimulation with HCMV-infected fibroblasts (mean±SD=51±47; 30-82; n=6) as compared to mock-infected cultures (mean±SD=18±12; range= 8-40).

**Fibroblast infection is essential to promote the expansion of NKG2C+ cells.**

An additional set of experiments was carried out to define the conditions required for HCMV to promote the expansion of NKG2C+ cells, that was dependent on the infectious dose used (Figure 4A). Treatment of MRC-5 with UV-inactivated HCMV (Figure 4B) abolished the effect. On the other hand, incubation of PBL with the virus in the absence of fibroblasts induced a proliferative response of T lymphocytes, including the ILT2+ subset, but did not expand NK cells (Figure 4B). Infected fibroblasts, either alone or with PBL, did not stimulate NK cell proliferation in PBL samples cultured separately in a transwell system (not shown). Taken together, these findings indicate that a direct interaction between PBL and HCMV-infected fibroblasts is required to induce the proliferation of NKG2C+ cells.

Time-course experiments were carried out culturing PBL with MRC-5 cells at different stages after HCMV-infection. As shown in Figure 4C, the expansion of NKG2C+ cells was optimal upon stimulation of PBL at day 1, but was reduced when the co-culture
was delayed to day 3 post-infection, revealing the existence of an optimal temporal window for the cellular interactions required to stimulate the NKG2C+ subset.

The expansion of NKG2C+ NK cells involves the CD94/NKG2C receptor and is enhanced by IL-15.

Depletion of NKG2C+ lymphocytes by cell sorting abolished the effect, pointing out that the responding cells constitutively express the activating KLR (not shown); positive selection of NKG2C+ cells was not considered reliable due to the potential agonistic/antagonistic effects mediated by the receptor-specific mAb. Thus, to further dissect the process, CD56+ and CD56- populations were sorted and incubated with HCMV-infected fibroblasts. Remarkably, the CD56+ population, that included both NKG2C+ and NKG2A+ subsets, did not divide despite the presence of exogenous IL-2 (10 U/ml); in fact, IL-2Rα (CD25) expression remained negative after 48-72 h (not shown). By contrast, cell proliferation was observed among the CD56- population, and the proportions of CD56+ NKG2C+ cells increased, becoming detectable (not shown). These findings indicated that additional signals, missing in the CD56+ population, were required to stimulate the proliferation of NK cells in response to HCMV-infected fibroblasts. Such complementary stimuli, provided by cells contained within the CD56- fraction, likely accounted for the expansion of residual NKG2C+ CD56+ cells (<1-2 %) after sorting.

IL-15 plays a primordial role in regulating NK cell proliferation and differentiation, contributing to the accumulation of NK cells in CMV-infected mice. Purified CD56+ populations, containing NK and T cells, were cultured either with HCMV- or mock-infected MRC-5 fibroblasts, and replicate samples were supplemented with IL-15 at different time points. Experiments with CFSE-labelled cells indicated that, in both cases, CD56+ cells divided by day 8 in response to IL-15 (Figure 5A). Yet, the cytokine
promoted a preferential expansion of the NKG2C+ subset in response to HCMV-infected MRC-5 when cultures were supplemented by day 4 (Figure 5B) but not earlier (day 0). Similar results were obtained with sorted CD3-CD56+ NK cell populations (data not shown). Altogether, these observations supported that the interaction of NKG2C+ cells with HCMV-infected fibroblasts enhances their responsiveness to IL-15. Further studies are required to dissect the influence of the cytokine network in the response of NKG2C+ cells to HCMV.

To approach whether the CD94/NKG2C receptor participated in the response to HCMV-infected cells, experiments were carried out in the presence of an F(ab’)2 anti-CD94 mAb (HP-3B1). As compared to the control (anti-CD56), the HP-3B1 mAb hampered the expansion of NKG2C+ cells when added early (days 0-1) after stimulating PBL with HCMV-infected fibroblasts (Figure 6A). Interestingly, the inhibitory effect vanished if the mAb supply was delayed to days 3-4 (Figure 6B), when infected fibroblasts co-cultured with PBL were no longer detectable (not shown), further arguing against a non-specific effect of the reagent. These results were also consistent with the existence of a temporal window in which cell interactions with infected fibroblasts, required to drive the expansion of NKG2C+ cells, take place and likely involve the CD94/NKG2C receptor.

By contrast, the cytolytic activity of NKG2C+ NK clones in response to HCMV-infected MRC-5 or autologous fibroblasts was not consistently inhibited by the anti-CD94 mAb, thus indicating that the KLR did not play a dominant role in triggering NK effector functions against virus-infected fibroblasts (data not shown).

**Response of CD94/NKG2C+ cells to fibroblasts infected with HCMV deletion mutants.**
The CD94/NKG2C receptor specifically interacts with HLA-E bound to peptides derived from the leader sequences of other class I molecules, though with a lower affinity than NKG2A\textsuperscript{27,28}. It has been proposed that HLA-E may be selectively preserved during HCMV infection, impairing the response of CD94/NKG2A\textsuperscript{+} NK cells\textsuperscript{23,24}. Thus, the HLA class Ib molecule was considered a first candidate ligand for CD94/NKG2C in HCMV-infected cells. To address whether UL40 was required for the expansion of CD94/NKG2C\textsuperscript{+} cells, a targeted deletion mutant of HCMV missing the UL40-42 genes\textsuperscript{36} was studied. The HB5\textsubscript{Δ}UL40-42 mutant was constructed from a HCMV BACmid pHB5, that lacks the \textit{US2-US6} gene region. Thus, HB5 and HB5\textsubscript{Δ}UL40-42 viruses retain only US11 to inhibit HLA class I expression. In these experiments, PBL were stimulated with MRC-5 cells infected with either AD169, HB5, HB5\textsubscript{Δ}UL40-42, or another HB5-derived targeted deletion mutant (HB5\textsubscript{Δ}UL14-20) missing the UL16 and UL18 genes\textsuperscript{36}. In line with a previous report\textsuperscript{23}, surface HLA-E was undetectable by flow cytometry in both mock- and HCMV-infected fibroblasts (Figure 7A). As compared to the marked inhibition of class I expression exerted by AD169 in MRC-5 cells, infection with either HB5, HB5 \textsubscript{Δ}UL40-42 or HB5\textsubscript{Δ}UL14-20 had only a modest effect, consistent with the exclusive use of US11 to inhibit class I molecules (Figure 7A, panel c). Albeit reaching a slightly lower yield compared with AD169, the late outgrowth of NKG2C\textsuperscript{+} NK cells was observed in every case, indicating that it was independent of UL40, and excluding as well a critical involvement of UL18 or UL16 (Figure 7B).

By contrast, the expansion of NKG2C\textsuperscript{+} cells was impaired in response to another HCMV mutant that lacked all MHC I downregulating genes, i.e. \textit{US2} through \textit{US11} (HB5\textsubscript{Δ}US2-11) (Figure 7B) which, as expected, did not alter the expression of HLA
class I in infected fibroblasts (Figure 7A). Similar results were obtained with another independently derived HB5ΔUS2-US11 mutant (Ana Angulo, unpublished).

These results suggested that normal levels of HLA class I may dampen the stimulation of CD94/NKG2C+ cells, presumably engaging the inhibitory receptors (i.e. KIR and/or ILT2) expressed by this cell subset. Further studies are required to determine whether HLA-E in HCMV-infected fibroblasts participates in engaging CD94/NKG2C and inducing the response.
DISCUSSION

The detection of increased proportions of CD94/NKG2C+ cells in PBL from healthy HCMV+ donors suggested that the viral infection might shape the NKR repertoire\textsuperscript{26}. In the present report we provide direct in vitro evidence supporting that the CD94/NKG2C+ NK cell subset preferentially expands upon interaction with HCMV-infected fibroblasts, and that the KLR itself may participate in driving the proliferation. The requirements for HCMV-infection to promote this effect have been defined, and its complexity partially dissected.

To assess the influence of HCMV on the NKR repertoire, PBL were re-stimulated with infected fibroblasts, an experimental system conventionally employed to study virus-specific CTL\textsuperscript{43}. T lymphocytes were predominant during the first week, but a substantial increase of NK cells was detected by day 10-12. NKG2C+ cells systematically outnumbered the NKG2A+ subset and a minor proportion of NKG2C+ T lymphocytes was also identified. The proliferation of NKG2C+ cells was confirmed by two-colour analysis of CFSE-labelled samples. This pattern of response observed in PBL samples from a group of HCMV+ donors, but not in HCMV- individuals, was similar regardless of the origin of fibroblasts (allogeneic vs autologous) and the virus strains tested (i.e. Toledo, Towne and AD169). Fibroblast infection was essential for the expansion of NKG2C+ NK cells, as it was dependent on the infectious dose and was not substantiated with UV-inactivated HCMV, nor when PBL were incubated with the virus alone. The unresponsiveness of PBL from HCMV-seronegative donors is presumably due to the absence of virus-specific memory T lymphocytes and the low/undetectable proportions of CD94/NKG2C+ cells\textsuperscript{26}.

Anti CD94 mAb F(ab’\textsuperscript{2}) markedly inhibited the response of NKG2C+ cells, supporting that signalling by the KLR was required. An antagonistic effect of NKG2C-specific mAbs would lend stronger support to this possibility; yet, such experiments were not
feasible due to the difficulties to generate F(ab’)_2 fragments from the commercially available unconjugated anti NKG2C mAb (IgG2b). Nevertheless, purified CD56+ populations, containing NKG2C+ and NKG2A+ cells, did not proliferate in response to HCMV-infected fibroblasts despite the presence of exogenous IL-2, pointing out the requirement of additional signals. The outgrowth of NKG2C+ cells became evident when the CD56+ subset was stimulated with HCMV-infected cells in the presence of exogenous IL-15, added at day 4 after the culture onset. IL-15 has been reported to be required for the accumulation of NK cells in MCMV infection\(^42;\)\(^44\). Altogether the observations are consistent with the view that signalling by the CD94/NKG2C receptor, upon interaction with infected fibroblasts, enhances the responsiveness to IL-15 and promotes the proliferation of the corresponding subset. DAP12 signalling has been shown to enhance NK cell responsiveness to IL-15 (communicated by A.R. French and W.M. Yokoyama; 21\(^{st}\) International NK cell Workshop, Kawai, November 2005). The molecular basis for this effect deserves further attention, and the contribution of other signals in the expansion of NKG2C+cells is not excluded. More extensive studies are required to dissect how HCMV-induced changes in the cytokine network may contribute to the expansion of NKG2C+ cells and the role of endogenous IL-15.

The nature of the putative ligand(s) expressed by HCMV-infected fibroblasts responsible for engaging CD94/NKG2C is a key open question. The observed phenomenon was reminiscent of the activation of NKG2C+ NK and T cells in response to a transfectant of the 721.221 HLA class I-deficient cell line overexpressing HLA-E+ (.221-AEH)\(^31\). This class Ib molecule was reported to be refractory to the action of US6 when bound to a peptide from the leader sequence of the HCMV UL40 protein, and appeared constitutively resistant to the action of US2 and US11\(^22\)-\(^24\). On that basis it was proposed that surface HLA-E expression may be preserved in infected cells protecting them against CD94/NKG2A+ NK cells; a CD8+ T cell subset that specifically recognizes HLA-E via
the TcR might counteract this evasion mechanism. In another report, downregulation of MHC class I by the HCMV US2-11 gene region was reported to dominate UL40 mediated effects in infected fibroblasts attacked by NK cells.

The analysis of HCMV deletion mutants provided a valuable information. First, the response to HB5ΔUL40-42 unequivocally demonstrated that UL40 was not required to promote the expansion of NKG2C+ cells. Moreover, cells infected with the HB5ΔUL14-20 elicited a response of NKG2C+ cells comparable to that observed with wild-type HCMV, also excluding a critical role of UL16 and UL18. This is relevant considering that most NKG2C+ cells express the ILT2 (CD85j) receptor which binds with high affinity to UL18. Moreover, 19 viral genes, present in the UL/b' region of HCMV strain Toledo but missing in AD169 and Towne, are obviously dispensable for the expansion of CD94/NKG2C cells.

HCMV mutants derived from AD169 (HB5) lack the US2-US6 genes, and inhibit HLA class Ia expression using only US11, which was reported to preserve the constitutive expression of HLA-E. The interaction of CD94/NKG2C with HLA-E, concomitant to the inhibition of HLA class Ia expression, might contribute to drive the proliferation of NKG2C+ cells. In our hands, surface expression of HLA-E was virtually undetectable by flow cytometry in mock- and HCMV-infected fibroblasts using a bona fide specific mAb (3D12). Tomasec et al. reported similar difficulties to detect the class Ib molecule in fibroblasts employing a mAb cross-reactive with HLA-E and HLA-C. Thus, if HLA-E bound to class I-derived nonamers is indeed the ligand responsible for engaging CD94/NKG2C in infected fibroblasts, it should be effective despite its limiting expression levels and low affinity for the activating KLR. This is conceivable if signalling by inhibitory receptors is prevented by a reduction of HLA class Ia expression, even minimal as in cells infected with the HB5-derived mutants. The inability to expand NKG2C+ cells upon stimulation with cells infected with HB5ΔUS2-
11, which fully preserved HLA class I molecules, supported this view; yet, a direct role for other gene(s) in the US7-11 region is not excluded. Alternatively, it cannot be ruled out that infected fibroblasts may display HLA-E/peptide complexes with high affinity for CD94/NKG2C or a viral ligand capable of directly engaging the receptor.

CD94 mAb did not consistently block cytotoxicity of NKG2C+ NK clones against HCMV-infected fibroblasts, questioning a dominant role of the KLR in triggering this NK effector function. Nevertheless, these observations do not rule out a relevant participation of CD94/NKG2C+ cells in the response to HCMV-infected cells. First, this NK subset is not susceptible to HLA-E-mediated inhibition. Moreover, the KLR may act in concert with other NKR (i.e. NKG2D, DNAM-1) and/or NCR; in this regard, it has been reported that the NK response to some tumor cells could not be inhibited by blocking individual receptor-ligand pairs.

Experiments with CFSE-labeled PBL indicated that only a fraction of NKG2C+ cells proliferated. Moreover, the expansion was not perceived in PBL from a group of HCMV+ donors that displayed lower proportions of NKG2C+ cells. Thus, it is conceivable that this NK cell subset may be heterogeneous in terms of its replication capacity, and that the proliferating “progenitor” pool responsible for the expansion may be variably represented in different individuals. Lower proportions of this subset, together with the intrinsic complexity of the co-culture system where T lymphocytes also respond, may render undetectable the proliferation of NKG2C+ cells accounting for the “non-responder” phenotype. The possibility that other signals contributing to the expansion of NKG2C+ cells may be defective in these samples, and/or that regulatory mechanisms interfere with their response, are not ruled out. Preliminary experiments indicate that the expansion of NKG2C+ cells may be perceived stimulating CD56+ populations from “non-responders” with HCMV-infected cells in the presence of IL-15.
Together with the skewed distribution of NKG2C+ cells, an expansion of ILT2+ T lymphocytes was observed in PBL from HCMV+ donors stimulated with infected fibroblasts. ILT2+ T cells were also significantly increased in fresh PBL from HCMV+ individuals, and in transplant patients undergoing HCMV infections.

The impact of HCMV infection on the CD94/NKG2C+ subset is reminiscent of the oligoclonal expansion of CTL specific for viral antigens, that display an effector/memory phenotype and tend to substantially increase in elderly individuals. The basis for the variability in the numbers of NKG2C+ cells observed in HCMV+ donors is uncertain, but likely reflects the challenge exerted by the virus on the innate immune system. The frequency of reactivation episodes, the extension of latent infection, and/or genetic differences in HCMV clinical isolates may be relevant in this context. The analysis of NKG2C+ cells becomes a potentially useful novel parameter to monitor the host-pathogen relationship.

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FIGURE LEGENDS

Figure 1. NK cell proliferation in response to HCMV-infected fibroblasts.

(A) CFSE-labeled PBL from an HCMV+ donor were co-cultured with mock- and Towne-infected (moi=1) MRC-5 cells as described in Methods. Flow cytometry analysis was carried out at day 7, after staining cells with anti NKG2C or NKG2A mAbs; numbers correspond to the proportions of dividing and non-dividing cells in NKG2C+ and NKG2A+ populations, calculated as described in Methods. The experiment is representative of 6 performed. (B) PBL were stimulated with Towne-infected fibroblasts as described above, and flow cytometry analysis was carried out at different time points with anti CD3-PerCP and anti CD56-PE, counting the numbers of recovered cells. The % and the calculated numbers of NK and T cell populations are represented.

Figure 2. Preferential expansion of CD94/NKG2C+ NK cells upon stimulation with HCMV-infected autologous or allogeneic fibroblasts.

(A) PBL from HCMV+ and HCMV- donors were co-cultured with mock- and AD169-infected (moi=1) MRC-5 cells. Two-colour analysis was carried out at day 10 with an anti CD3 mAb combined to anti NKG2C or NKG2A mAbs. The results are representative of the patterns of response observed in 6 out of 11 HCMV+ individuals, and in 4 HCMV- donors. (B) PBL from an HCMV+ donor were co-cultured with mock- and AD169-infected allogeneic (MRC-5) or autologous fibroblasts, and samples were analysed at day 10 as described in (A). Similar results were obtained in 6 different experiments with PBL from two different individuals.
**Figure 3.** NKR and NCR distribution in PBL stimulated with HCMV-infected fibroblasts.

PBL from an HCMV+ donor were co-cultured with mock- and Towne-infected (moi=1) MRC-5 cells. Cells harvested at day 11 were stained by indirect immunofluorescence with mAbs specific for different NKR or NCR, followed by labelling with anti CD56-PE and CD3-PerCP mAbs. Three-colour flow cytometry analysis was carried out gating on CD3- CD56+ (A) and CD3+ (B) populations. The data are representative of four different experiments.

**Figure 4.** Expansion of NKG2C+ NK cells is dependent on the HCMV-infection of fibroblasts and on the time of their interaction.

(A) The distribution of NKG2C+ cells in response to HCMV-infected MRC-5 cells at different moi was analysed. The % of NKG2C at moi 0.01 was comparable to mock-infected samples (not shown). (B) PBL from HCMV+ donors were co-cultured with mock- or Towne-infected MRC-5 fibroblasts; in parallel, PBL were incubated with the virus alone (HCMV) or fibroblasts infected with UV-inactivated Towne (iHCMV). Two-colour flow cytometry analysis was performed at day 10 with anti CD3, NKG2C and ILT2 mAbs. Data are representative of 5 different experiments. (C) PBL were incubated with Towne-infected MRC-5 cells at different time points post-infection (days 0, 1 and 3). At day 10, cells were harvested, counted and analysed by flow cytometry; the numbers of NKG2C+ and NKG2A+ cells recovered are shown. A similar pattern of response was observed in 3 different experiments.

**Figure 5.** IL-15 promotes the preferential expansion of NKG2C+ cells in CD56+ populations stimulated with HCMV-infected cells.
CD56+ populations sorted from PBL of HCMV+ donors were cultured with mock- or HCMV (AD169)-infected MRC-5 cells, infected 24 h before, in the presence of IL-2 (10 U/ml). (A) Proliferation of CFSE-labelled CD56+ cells cultured for 8 days with mock- or HCMV-infected cells supplemented with IL-15 at day 4. (B) Distribution of NKG2A+ and NKG2C+ subsets in CD56+ lymphocytes co-cultured for 8 days with mock- or HCMV-infected cells in the presence of IL-15, added at days 1 or 4. The data are representative of 6 experiments performed.

**Figure 6.** Anti CD94 mAb blocks the expansion of NKG2C+ NK cells stimulated by HCMV-infected fibroblasts.

(A) PBL from an HCMV+ donor were cultured with mock- and Towne-infected (moi=1) MRC-5 cells. Either F(ab’)2 anti CD94 or CD56 mAbs (10 μg/ml) were added at day 0. Flow cytometry analysis with anti CD3, NKG2C and NKG2A mAbs was carried out at day 10. (B) The differential effects in the numbers of recovered cells in the presence of the anti CD94 mAb added at day 0 or day 4 are compared. The data are representative of 5 experiments performed.

**Figure 7.** Expansion of NKG2C+ NK cells in response to fibroblasts infected with HCMV deletion mutants.

(A) (a) The expression of total class I molecules (HLA-I) and HLA-E was analysed by flow cytometry in MRC-5 fibroblasts. The expression of HLA-E was also undetectable in AD169-infected MRC-5 cells (not shown). (b) Staining of an HLA-E+ cell line (.221-AEH) is shown for comparison. (c) Expression of HLA-I in MRC-5 cells infected for 72 with either AD169, HB5 (ΔUS2-6) or HB5-ΔUS2-11 mutants; HLA expression in cells infected with HB5-ΔUS40-42 or HB5-ΔUS14-20 was comparable to HB5 (not shown). Production of type I IFN upon HCMV infection upregulates HLA class I expression\(^{52}\).
In this experiment, the presence of a fraction of bystander non-infected cells among AD169-treated cells provides a suitable internal control. Numbers correspond to the MFI of the histograms.

(B) PBL from an HCMV+ donor were cultured in parallel with MRC-5 cells infected (moi=1) with the wild type AD169 HCMV strain or different deletion mutants generated from the HB5 BACmid clone (ΔUS2-6). Two-colour flow cytometry analysis was performed by day 10. Data are representative of 6 different experiments. The proportion of NKG2C+ cells in mock-infected cultures (not shown) was 10.5%.
Figure 1A

Figure 1B
Figure 2A
Figure 2B
Figure 3
Figure 4A-B
Figure 4C
Figure 5
Figure 6
Figure 7A
Figure 7B
Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts

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