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Amplified NKG2C+ NK Cells in Cytomegalovirus (CMV) Infection Preferentially Express Killer Cell Ig-like Receptor 2DL: Functional Impact in Controlling CMV-Infected Dendritic Cells

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CMV infection represents a major complication in hematopoietic stem cell transplantation, which compromises graft outcome. Downregulation of HLA class I expression is one mechanism by which CMV evades T cell–mediated immune detection, rendering infected cells vulnerable to killer cell Ig-like receptor (KIR)+ NK cells. In this study, we observed that the amplified NKG2C+ NK cell population observed specifically in CMV seropositive individuals mainly expressed KIR2DL receptors. We have shown that HLA class I expression was downregulated on CMV-infected immature dendritic cells (iDCs), which escape to HLA-A2-pp65–specific T lymphocytes but strongly trigger the degranulation of KIR2D+ NK cells. CMV infection conferred a vulnerability of C2C2+ iDCs to educated KIR2DL1+ and KIR2DL3+ NK cell subsets. Alloreactivity of KIR2DL1+ NK cell subsets against C1C1+ iDCs was maintained independently of CMV infection. Unexpectedly, CMV-infected C1C1+ iDCs did not activate KIR2DL3+ NK cell reactivity, suggesting a potential CMV evasion to KIR2DL3 NK cell recognition. Altogether, the coexpression of KIR and NKG2C on expanded NK cell subsets could be related to a functional contribution of KIR in CMV infection and should be investigated in hematopoietic stem cell transplantation, in which the beneficial impact of CMV infection has been reported on the graft-versus-leukemia effect. The Journal of Immunology, 2013, 191: 000–000.

Human CMV is a β-herpesvirus that establishes a latent infection in healthy individuals but causes a high rate of morbidity in immunocompromised patients and during fetal development. For an effective defense against CMV, both innate and adaptive immune responses are involved (1). Thanks to different inhibitory receptors, NK cells are able to detect the loss of HLA class I molecules from autologous cells, a situation that can occur when cells are perturbed by viral infection or tumor transformation (the “missing self” hypothesis) (2). Indeed, NK cells express different inhibitory receptors to protect against self cells (3). CD94/NKG2A, killer cell Ig-like receptors (KIRs), and ILT2 (LILRB1) are the main receptors engaged by CMV. CD94/NKG2A recognizes leader peptides of HLA-A, -B, -C, and -G molecules presented by the HLA-E molecule (3). Nevertheless, inhibitory KIRs display a more specific recognition of HLA class I ligands. For example, HLA-C allotypes with asparagine at position 80 (C1 ligands) are recognized by KIR2DL2/3 whereas HLA-C allotypes with lysine at position 80 (C2 ligands) are recognized by KIR2DL1 (4). Additionally, ILT2 recognizes all HLA class I molecules. Moreover, the interaction between HLA class I molecules and inhibitory receptors is essential in NK cell acquisition of self-tolerance and functional abilities (5).

Soon after infection, different viral proteins downregulate HLA class I molecule expression on infected cells, leading to escape from CMV-specific T lymphocytes, but they render these infected cells vulnerable to NK cell lysis (6). Furthermore, CMV may also escape NK surveillance by maintaining the expression of nonclassical HLA class I molecules, HLA-E and HLA-G, serving as ligands for the inhibitory receptors CD94/NKG2A and ILT2 (7). Recently, it has been shown that expansion of NK cells expressing the activating NKG2C counterpart is associated with CMV infection (8–12). Furthermore, CMV has developed different strategies to prevent the expression of ligands for some activating NK cell receptors (13, 14). However, no document discusses the involvement of KIR NK cells in controlling CMV infection or the potential CMV evasion to KIR NK cells. The hypothesis of a prospective role of KIR NK cells in controlling CMV infection is reinforced by the knowledge gained from the murine model. In fact, Ly49 receptors, analogous to KIR receptors, are mainly engaged to control murine CMV infections (15).

In allogeneic hematopoietic stem cell transplantation (HSCT), CMV infections are considered a major complication, which may compromise graft outcome. After transplantation, lymphoid T cell reconstitution is delayed, resulting in reactivation of latent viral
infections such as CMV. However, NK cells that rapidly reconstitute hematopoiesis and play a crucial role in mediating the graft-versus-leukemia (GVL) effect constitute the first line of defense against viral infection. In HLA haploidentical HSCT, NK cell alloreactivity is due to the presence in the donor of NK cells expressing KIR that recognize HLA class I allotypes present in the donor but absent in the recipient (16). KIR genotype studies have revealed the beneficial effect of activating KIR genes against CMV infection or reactivation in different graft contexts (16-18). During acute CMV infection, NKG2C+ NK cells expand in HSCT recipients and predominately express KIR (9). However, the contribution of NKG2C in eliminating CMV-infected cells via NK cells has not been demonstrated. It is possible that NKG2C mainly constitutes a CMV infection marker. Thus, the coexpression of NKG2C with KIR on this expanded NK cell population questions the functional KIR contribution to CMV infection control. Moreover, although a recent report suggests the beneficial impact of early CMV infection on the GVL effect in HSCT (19), whether CMV modulates KIR NK cell alloreactivity in an HSCT context has not been investigated. Thus, to address these questions, we have set up a cell culture model based on a CMV-infected immature monocyte-derived dendritic cells (iDCs) as target cells, taking into account the HLA environment, to investigate not only the functional contribution of KIR NK cell subsets in controlling CMV infection but also the impact of CMV in modulating KIR NK cell alloreactivity.

Materials and Methods

PBMCs and cell lines

PBMCs were isolated from citrate-phosphate-dextrose blood from healthy adult volunteers by gradient centrifugation on Ficoll-Hypaque (Lymphoprep; Axis-Shield, Oslo, Norway). All blood donors were recruited at the Blood Transfusion Center (Établissement Français du Sang, Nantes, France) and informed consent was obtained from all individuals. CMV serological status was determined using the chemiluminescent immunoassay LIASON CMV IgG II (DiaSorin). EBV-B cell lines were obtained by EBV transformation of peripheral B cells using EBV supernatant harvested from the cell line B95-8 (American Type Culture Collection). B cells were isolated from PBMCs obtained from healthy individuals. The HLA class I-deficient acute myeloid leukemia K562 cell line was used to determine the degranulation potential of NK cells. K562 cells and EBV-B lymphocytes were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.), containing glutamine (Life Technologies) and penicillin-streptomycin (Life Technologies) and supplemented with 10% FBS (Life Technologies). Mycoplasma tests performed by PCR were negative for all cell lines.

HLA and KIR genotyping

Genomic DNA was extracted from PBMCs using a classical salting-out method (20). HLA-A, -B, and -Cw genes were typed via molecular techniques using PCR-sequence-specific primer (SSP) kit from GenoVision (Olerup SSP HLA-A, -B, -Cw, BioNoBiis, Montfort l’Amaury, France) under the conditions recommended by the manufacturer. KIR genes were typed via molecular techniques using PCR-SSP methods under the conditions recommended by the manufacturers, as described previously (21, 22).

Isolation of monocyte and generation of iDCs

Monocytes were separated from lymphocytes by using a high-density hyperosmotic Percoll density gradient (Sigma-Aldrich) (23) and cultured for 6 d in RPMI 1640 medium (Life Technologies) containing glutamine (Life Technologies), penicillin-streptomycin (Life Technologies), and supplemented with 10% human AB serum (Life Technologies), IL-5 (200 U/ml; CellGenix), and GM-CSF (1000 U/ml; CellGenix).

NK cell isolation and amplification

PBMCs were isolated as previously described (24, 25). Thereafter, NK cells were amplified after in vitro stimulation with irradiated C1+ and C2+ allogeneic PBMCs and EBV-B cells used as feeders following the PBMC/EBV-B cell ratio at 10:1. All amplified KIR2D NK cells were NKG2C negative.

Isolation and amplification of CMV-specific T cell lymphocytes

CMV-specific T lymphocytes were sorted from HLA-A2+ CMV seropositive blood donors using anti–CD3-PerCP (SK7; BD Biosciences) and HLA-A*02:01-pp65 (NLVPMVATV) (tetramer–allophycocyanin, obtained after tetramerization of HLA-A2-pp65 monomer [platform Structure Fédérative de Recherche François Bonamy, Nantes, France] with allophycocyanin-streptavidin [AnaSpec]). Sorted cells were stimulated with irradiated feeder cells (allogeneic PBMCs and a mix of two irradiated EBV-B cell lines) and 1 μg/ml PHA-P (L-7132; Sigma-Aldrich). After 2 wk, specific stimulation using pp65 (1 ng/ml)-loaded autologous EBV-B cells (E:T ratio of 10:1) was performed. pp65-Specific lymphocyte amplification was monitored by flow cytometry using HLA-A2-pp65-PE pentamer (Proimmune, Oxford, U.K.).

CMV preparation and infection of iDCs

Stock suspensions of CMV strain VHL/E (provided by the Prof. James Waldman, Columbus, OH) were produced as previously described (26). Of note, the full genomic sequence of VHL/E is not available and thus its genetic content has not been established. Infectivity titration of the virus was performed on iDCs. After overnight coculture of iDCs alone or with VHL/E, cells were washed and resuspended in RPMI 1640, 10% FBS.

Phenotypic analysis by flow cytometry

The NK cell surface phenotype was determined by three- or four-color flow cytometry using the following mouse anti-human mAbs: anti–KIR2DL1-PerCP (143211; R&D Systems), anti–KIR2DL2/3/2DS2-PE (GL183; Beckman Coulter), anti–KIR2DL1/2/3/2DS2-FTC (IA6) (27), anti–NKp46-allophycocyanin (9E2; Becton Dickinson), anti–NKG2C-PE (134591; R&D Systems), anti–NKG2A (Z199; Beckman Coulter), anti–CD3-PerCP (SK7; BD Biosciences), anti–CD56-allophycocyanin (B159; BD Biosciences), anti–NKG2D (DI1); BD Biosciences), anti–NKp30 (Z25; Beckman Coulter), and anti–NKp44 (Z231, Beckman Coulter). iDCs were stained with the following mouse anti-human mAbs: anti–HLA-A, -B, -C (F41-IE3H1D2; EFS), anti–HLA-C (L3.1; MediaPharma), anti–HLA-E-PE (3D12; Miltenyi Biotec), anti–CD40-PE (5C3; BD Biosciences), anti–CD80-PE (L307.4; BD Biosciences), anti–CD83-allophycocyanin (HB15e; BD Biosciences), anti–CD86-PE (2331; BD Biosciences), anti–HLA-A, -B, -C (F41-IE3H1D2; EFS), anti–MICA/B-PE (6D4; BD Biosciences), anti–CD4–AF647 (F41-IE3H1D2; EFS), anti–CD56-allophycocyanin (9E2; Becton Dickinson), anti–NKG2A (Z199; Beckman Coulter), anti–KIR2DL1/2/3/2DS2-PE (GL183; Beckman Coulter), and anti–NKG2C-PE (134591; R&D Systems).

Preferential expression of KIR2D on amplified NKG2C+ NK cells

Results

Statistical analyses

Comparisons of NK cell frequencies between two different series of individuals were performed using ANOVA test. A p value of <0.05 was considered to be statistically significant.

Results

Preferential expression of KIR2DL on amplified NKG2C+ NK cells in CMV seropositive individuals

Recently, some studies have shown that a variable expansion of NK cell subsets coexpressing NKG2C and KIR are associated with CMV infection (8–11). We therefore assessed the expression of KIR and NKG2C to determine what NK cell subsets are mainly

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observed in CMV seropositive individuals \((n = 31)\) compared with seronegative individuals \((n = 29)\) (Fig. 1). By four-color flow cytometry, three different NK cell subsets were defined on the basis of NKG2C and KIR2D expression (NKG2C+KIR2D−, NKG2C−KIR2D+, and NKG2C−KIR2D+) using a KIR2DL1/L2/L3/2DS1/2S-specific mAb (1A6) (27) and NKG2C-specific mAb in CMV− versus CMV+ individuals (Fig. 1A). In agreement with previous reports, the frequencies of NKG2C+ and NKG2C−KIR2D+ NK cells are significantly higher in CMV seropositive than seronegative individuals \((p = 0.004\) and \(p = 0.005\), respectively) (Fig. 1B). NKG2C+ NK cells did not express NKG2A and preferentially expressed CD57 (data not shown), as previously described in HSCT recipients and immunocompetent CMV seropositive individuals (9). Interestingly, expansion of the NKG2C+KIR2D+ NK cell population in CMV seropositive individuals is associated to the AA KIR genotype (Fig. 1C), although the probability is not significant \((p = 0.08)\), which is confirmed by preferential expression of inhibitory KIR on the NKG2C+ NK cell subset (Fig. 1D). Indeed, of the 12 individuals with an NKG2C+KIR2D+ NK cell frequency superior to the mean value (6.8%), 11 are KIR2DL3 genotyped and coexpressed KIR2DL3 and NKG2C+ whatever the HLA background (2 C2C2, 4 C1C1, and 5 C1C2 individuals) (Fig. 1D). However, only one individual (C1C2) coexpressed KIR2DL1 with KIR2DL3 and NKG2C (Fig. 1D). In B+ KIR genotyped individuals, activating KIR2DS2 was coexpressed on NKG2C+ cells in one KIR2DL3−KIR2DL2/S2+ C2C2 genotyped individual (data not shown). KIR2DS1 expression was evaluated using the combination of KIR2DL1/2DL2/2DL3/2DS2-specific 8C11 and KIR2DL1/S1-specific EB6 mAbs in 2 KIR2DS1 genotyped individuals, and no KIR2DS1 expression was observed on expanded NKG2C+ NK cells. Of note, all three KIR3DS1 genotyped individuals expressed KIR3DS1 on the expanded KIR2DL3−NKG2C+ NK cell subset. KIR3DL1 was coexpressed with NKG2C+ and KIR2DL3+ in 5 of 10 individuals expressing KIR3DL1 (data not shown).

**Downregulation of HLA class I molecule expression on CMV-infected iDCs**

It has been previously reported that monocyte-derived DCs are susceptible to in vitro CMV infection by the endothelial cell–adapted strain VHL/E (29). Thus, after 48 h of CMV infection, iDCs were stained with an mAb specific for the CMV major immediate-early IE-1 protein (Fig. 2A). Based on the percentage of IE-1+ cells, 50% of values from 20 independent experiments are comprised between first quartile \((Q1 = 39.5\%)\) and the third quartile \((Q3 = 56\%)\) around a median of 48% (Fig. 2B). Interestingly, HLA ligands of NK receptors as HLA class I and especially HLA-C molecules (ligands of KIR2DL) were downregulated on CMV-infected iDCs. In the same line, the nonclassical HLA-E molecule (ligand of CD94/NKG2A or CD94/NKG2C), weakly expressed on iDCs, was downregulated on CMV-infected iDCs (Fig. 2C). As previously described, CMV infection does not induce maturation of iDCs (30).

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**FIGURE 1.** Preferential expression of KIR2DL on amplified NKG2C+ NK cells in human CMV seropositive individuals. (A) Representative density plots illustrating NK cell phenotype from CMV− and CMV+ individuals. NK cells were stained with anti-CD56, anti-NKG2C, and anti-KIR2DL1/L2/L3/2DS1/2S (1A6) mAbs. (B) Scatter plots displaying the NKG2C−KIR2D−, NKG2C−KIR2D+, and NKG2C+ NK cell frequencies from CMV− \((n = 29)\) and CMV+ \((n = 31)\) individuals. (C) Summary box and whisker plot showing the frequency of NKG2C−KIR2D−, NKG2C−KIR2D+, and NKG2C+ NK cells from CMV− AA KIR genotyped \((n = 10)\) and AB and BB KIR genotyped \((n = 21)\) individuals. (D) Representative density plots of four C1C1 and one C1C2 CMV+ individuals. KIR2DL3−NKG2C+ and KIR2DL1−NKG2C− NK cells are targeted with anti-NKG2C and GL183 and with anti-NKG2C and EB6, respectively. Statistical significance \((**p < 0.01)\) between both groups was determined using an ANOVA test.
However, the expression of CD86 decreased on CMV-infected iDCs compared with uninfected iDCs (Fig. 2C, 2D). Moreover, the uninfected iDC subset in CMV-treated cultures present two levels of expression of HLA class I, that is, CD86 and CD83 molecules. Similar results were obtained at 96 h after infection (Fig. 2D). This could be explained by binding of viral products or soluble factors. Experiments were performed with UV-inactivated virus in the same conditions to confirm that the productive CMV infection of iDCs is necessary to modulate the biology of CMV-treated iDCs (data not shown).

CMV-infected iDCs escape to HLA-A2-pp65–specific CD8+ T cell recognition but activate NK cell degranulation

Consistent with previous reports indicating that several viral proteins inhibit HLA class I expression on CMV-infected cells to escape to T lymphocyte response, we further evaluate our in vitro model in assessing the CD107a mobilization of HLA-A2-pp65–specific T lymphocytes against CMV-infected iDCs versus uninfected iDCs. HLA-A2-pp65–specific T lymphocytes, targeted using HLA-A2-pp65 pentamer (Fig. 3A), reacted against pp65-loaded HLA-A2+ iDCs, but not against uninfected HLA-A2+ iDCs and CMV-infected HLA-A2+ iDCs (Fig. 3B). This result obtained in our cellular model is consistent with the ability of CMV to escape to T lymphocyte response by inhibiting HLA class I expression on iDCs. We therefore investigated NK cell ability to recognize decreased expression of HLA class I molecules on CMV-infected iDCs by focusing on Nkp46+ NK cells (Fig. 3C). Interestingly, NK cells reacted against CMV-infected iDCs almost as well as against an HLA class I–deficient K562 cell line, used as a positive control of degranulation, as illustrated for one individual (Fig. 3D) and confirmed for six individuals with a significant probability ($p = 0.01$) (Fig. 3E).

KIRs are required for a robust response against allogeneic CMV-infected iDCs

It has been reported that KIRs are required on NKG2C+ NK cells for a strong production of IFN-γ (9). Nevertheless, the role of KIRs in triggering NK cell degranulation is undocumented. To investigate the functional reactivity of NKG2C*KIR2D−, NKG2C−KIR2D+, and NKG2C*KIR2D+ NK cell subsets (Fig. 4A) against allogeneic CMV-infected iDCs, degranulation assays were carried out with PBMCs from CMV+ individuals ($n = 6$) stimulated overnight with IL-2 (Fig. 4B). As seen previously, PBMCs were incubated alone or in the presence of K562 cells, mock-infected iDCs, or CMV-infected iDCs (E:T ratio = 10:1). All of these NK cell populations reacted significantly against CMV-infected iDCs ($p = 0.02$ for NKG2C*KIR2D− NK cells, $p = 0.0006$ for NKG2C−KIR2D+ NK cells, and $p = 3.5 \times 10^{-5}$ for NKG2C*KIR2D+ NK cells).
cells). However, NK cells bearing KIR2D receptors (NKG2C−KIR2D+ and NKG2C+KIR2D+ NK cell subsets) reacted more robustly than did NKG2C+KIR2D− NK cells. Indeed, when KIR2D receptors were neutralized with anti-KIR2DL1/2/3/2DS2 mAb, NKG2C+KIR2D+ NK cell degranulation against CMV-infected iDCs was decreased by half (Fig. 4C). Otherwise, because NKG2C+ NK cells are mostly NKG2A+ (Fig. 4C), we neutralized HLA-E molecules with anti–HLA-E mAb to assess the impact of NKG2C on NK cell degranulation against CMV-infected iDCs. The NKG2C+KIR2D+ NK cell degranulation was slightly decreased in this blocking condition (Fig. 4C). These results suggest a predominant impact of KIR2DL in triggering NK cell response against CMV-infected iDCs. Based on our results showing a decreased expression of HLA class I molecules on CMV-infected iDCs and the expansion of NKG2C+KIR2D+ NK cell subsets in CMV infection, we hypothesized that the absence of inhibitory KIR engagement with the cognate ligand and the activating NKG2C engagement with HLA-E on human CMV-infected iDCs might both contribute to drive the expansion process.

C1C1 but not C2C2 allogeneic iDCs activate KIR2DL1+ NK cell degranulation following the self-missing model

To determine the nature of KIR2DL receptors involved in the NK cell response to CMV-infected iDCs, we first focused on NK cell subsets expressing well-characterized inhibitory KIR2DL1, which recognizes HLA-C molecules of C2 group. KIR2DL1+ NK cells were sorted from KIR2DS1+ genotyped C2+ individuals and amplified following polyclonal stimulation. The phenotype (KIR2DL, NKG2A, NKp44, NKp30, and NKG2D) of the studied NK cell lines was stable following culture alone or coculture with iDCs or CMV-infected iDCs for 5 h. All selected and amplified NK cells did not express the inhibitory receptor ILT2 (data not shown). However, most amplified NK cells expressed the NKG2A marker (Fig. 5A). Thus, NKG2A expression on KIR2D NK cells may inhibit NK cell response and override the signal mediated by KIR2D receptors. We therefore assessed the function of KIR2DL1+ KIR2DL2/3/2S2+ NK cells. We showed that the degranulation of C2+ educated KIR2DL1+ NK cells, controlled with K562, is strongly induced in contact to C1C1 allogeneic iDCs (p = 0.002) but not in contact to C2C2 allogeneic iDCs (Fig. 5B). These results

FIGURE 3. CMV-infected iDCs escape to HLA-A2-pp65-specific CD8+ T cell recognition but activate NK cell degranulation. (A) HLA-A2-pp65-specific T cells were cell sorted and amplified in vitro from CMV seropositive individuals and targeted as CD3+HLA-A2-pp65-pentamer+. (B) After 2 wk of stimulation, these cells were phenotyped by flow cytometry and were incubated for 5 h alone or in the presence of mock-infected, pp65-loaded, or human CMV-infected allogeneic iDC HLA-A2+ at an E:T ratio of 10:1. Results of CD107a mobilization obtained for a representative experiment of three performed are shown. Percentages of CD107a+ HLA-A2-pp65-specific T cells observed by flow cytometry are indicated on the density plots for all conditions of stimulation. (C) NK cells are targeted as NKp46+ cells. (D) PBMCs stimulated overnight with IL-2 were incubated for 5 h alone or in the presence of CMV-infected allogeneic iDCs at an E:T ratio of 10:1. Surface CD107a expression on NKp46+ NK cells was analyzed by flow cytometry. Representative density plots from six experiments are shown. The percentage of CD107a+ NK cells is indicated on each density plot. (E) Dot representation displaying the percentage of CD107a+ NK cells from all six experiments. Statistical significance (**p < 0.01) between both groups was determined using an ANOVA test.
validated our in vitro model to explore the impact of CMV infection of iDCs on KIR + NK cell degranulation.

**CMV infection of C2C2 iDCs triggers alloreactivity of C2+ KIR2DL1+ NK cells**

CMV infection of C2C2 iDCs activated significantly KIR2DL1+ NK cell degranulation ($p = 0.0003$) (Fig. 5C). This result suggests that HLA class I downregulation in CMV-infected C2C2 iDCs was sufficient to trigger KIR2DL1+ NK cell cytotoxicity. To confirm this point, we evaluated CMV infection of C1C1 iDCs on KIR2DL1+ NK cell degranulation. Indeed, when KIR2DL1+ NK cells were incubated in the presence of CMV-treated C1C1 iDCs, the frequency of CD107a+KIR2DL1+ NK cells was high and similar to that observed in the presence of mock-infected C1C1 iDCs (Fig. 5C). The activation of KIR2DL1+ NK cell degranulation with only CMV-infected C2C2 iDCs but not CMV-infected C1C1 iDCs supports the hypothesis that the protective KIR2DL1/C2 interaction is directly targeted.

**KIR2D receptors were blocked with 8C11 mAb and isotype control. HLA-E molecules expressed on iDCs were blocked with 3D12 mAb and isotype control. Statistical significance ($p < 0.05$, **$p < 0.01$) between both groups was determined using an ANOVA test.**

**KIR2DL3+ NK cells respond to CMV-infected allogeneic C2C2+ iDCs but not to CMV-infected allogeneic C1C1 iDCs**

Additionally, we investigated the role of KIR2DL3+ NK cells in the response to CMV-infected allogeneic iDCs. KIR2DL3+ NK cells were sorted and in vitro amplified from KIR2DS1/L2/S2 genotyped C1C1 individuals (Fig. 5D). Fifteen days after stimulation, we foremost assessed the degranulation of KIR2DL3+KIR2DL1/NKG2A+ NK cells against allogeneic iDCs. Both C1C1+ and C2C2+ iDCs inhibited KIR2DL3+ NK cell degranulation (Fig. 5E). Indeed, when HLA-C molecules were neutralized with anti–HLA class I mAb, KIR2DL3+ NK cell degranulation was partially restored, showing the functional interaction of KIR2DL3 with C2 ligand (Fig. 5G). These results are in accordance with recently reported data showing that KIR2DL3 could recognize not only the expected group of HLA-C molecules belonging to the C1 group, but also those belonging to C2 group (32–34). To determine whether the decreased HLA class I expression on CMV-infected iDCs impacts the KIR2DL3+ NK cell function, degranulation of the KIR2DL3+ NK cell subset against CMV-infected iDCs has been evaluated. CMV infection of C2C2+ iDCs activated significantly KIR2DL3+ NK cell degranulation ($p = 0.01$) (Fig. 5F). This result may imply that HLA class I
downregulation on CMV-infected C2C2+ iDCs is sufficient to trigger KIR2DL3+ NK cell reactivity. However, CMV infection of C1C1+ iDCs did not activate KIR2DL3+ NK cell degranulation (Fig. 5F). These findings support the functional contribution of KIR2DL3+ NK cells in response to CMV infection of C2C2+ iDCs, but they suggest a potential evasion of CMV to KIR2DL3+ NK cell control of C1C1+ target cells.

Discussion

Recent studies have reported the expansion of NKG2C+ NK cells expressing self-specific inhibitory KIR (8–10) in CMV infection. Our data provide evidence that inhibitory KIR2DL3 is preferentially coexpressed on NKG2C+ NK cells in CMV seropositive individuals whatever the HLA-C background, and even KIR2DL3 seems to recognize the HLA-C ligand from the C1 and C2 groups with a lower affinity to C2 ligands (32). Our results are in agreement with a previous report revealing the expansion of NKG2C+KIR2DL2/DS2/DS3+ NK cells in a T+ B+NK SCID patient with a CMV infection representing 80% of NK cells (35). In contrast to Charoudeh et al. (12) who reported a specific expansion of NK cells expressing the inhibitory receptors KIR2DL1 and KIR2DL3 in response to in vitro exposure to CMV, we observed preferential KIR2DL3 coexpression on NKG2C+ NK cells but seldom KIR2DL1 coexpression. Similarly, we observed mainly inhibitory KIRs coexpressed on NKG2C+ NK cells. Additionally, our phenotypic study showed a frequent coexpression of KIR3DL1
or KIR3DS1 on NKG2C+ KIR2DL3+ NK cells in CMV seropositive individuals, whatever the Bw4 environment. Thus, the predominant expression of KIR2DL3 suggests a driven clonal-like expansion of KIR2DL3+NKG2C+ NK cells during CMV infection that can coexpress another KIR as KIR3DL1 or KIR3DS1, as previously described for Ly49H+ NK cells in mice challenged with murine CMV (36). Moreover, numerous hypotheses regarding HIV-1 infection (37) and recent data (12) suggest a potential impact of KIR3DL1/S1 in controlling CMV infection. The role of KIR3DL1/S1 engagement in CMV infection should be investigated to answer this point.

We showed that NK cells bearing KIR2D receptors react more robustly than do KIR2D*NKG2C+ NK cells. Indeed, KIR2D+ NK cell subsets degranulated and secreted INF-g (data not shown) more than did the KIR2D*NKG2C+ NK cell subset in response to CMV-infected iDCs. Our results are in accordance with previous results showing that KIRs are required for robust IFN-g production (9). As previously described (38), degranulation of C2+ educated KIR2DL1+ NK cells is strongly induced in contact to allogeneic C1C1+ iDCs but not in contact to C2C2+ iDCs. We demonstrated that infection of C2C2+ iDCs with CMV significantly enhances KIR2DL1+ NK cell degranulation. Thus, HLA class I downregulation observed on CMV-infected iDCs seems sufficient to activate KIR2DL1+ NK cells. Interestingly, KIR2DL1+ NK cell alloreactivity against C1C1+ iDCs is maintained with CMV infection. Additionally, KIR2DL1+ and KIR2DL3+ NK cell subsets reacted against CMV-infected C2C2+ iDCs. This point could explain recent work showing that early CMV infection is associated with a reduced risk of relapse in acute myeloid leukemia patients undergoing allogeneic HCT (19). Thus, it is possible that CMV infection activates and mobilizes the NK cell subset able to recognize “missing-self” and that expanded NKG2C*KIR2D+ NK cells constitute boosted alloreactive NK cells that are more efficient against leukemia cells.

Recent studies performed on a large cohort has revealed the expansion of activating KIR* (KIR2DS2, KIR3DS1, KIR2DS4) NK cell subsets independently of NKG2C, suggesting a contribution of both activating KIR* and NKG2C+ NK cell subsets in CMV infection (11). In a murine model, activating Ly49H recognition of the viral protein m157 drove expansion of the cells that control murine CMV infection (15). Thus, we evaluated amplified and sorted C2+ KIR2DS1+ NK cell degranulation against C1C1+ and C2C2+ iDCs infected or not by CMV. In our model, we did not observe a potential activation of KIR2DS1+ NK cells stimulated with CMV-infected iDCs (data not shown). However, the CMV seronegative status of the studied individuals may explain the absence of the KIR2DS1+ NK cell response. Indeed, it is possible that “memory” status of NK cells expanded during CMV infection is necessary to involve KIR2DS1 engagement with viral ligands.

Interestingly, we show that KIR2DL3 recognizes not only C1 but also C2 ligands, as demonstrated in our cellular model using C1C1 or C2C2 iDCs. In contrast, KIR2DL1+ NK cells recognize only C2C2+ iDCs. These results are in accordance with previous works indicating that KIR2DL3 interacts with HLA-C molecules belonging to C1 and C2 groups (32). Additionally, KIR2DL3+ NK cell degranulation is significantly increased in contact to CMV-infected C2C2+ iDCs, probably due to the decreased expression of HLA class I molecules on CMV-infected C2C2+ iDCs. Unexpectedly, KIR2DL3+ NK cell degranulation is not increased in contact to CMV-infected C1C1 iDCs. This last result suggests a potential evasion of CMV to KIR2DL3+ NK cell control in HLA-C1 environment as observed in our model at 48 h postinfection and confirmed at 96 h postinfection (data not shown). However, we do not exclude that KIR2DL3+ NK cell degranulation is activated by CMV-infected target cells earlier or later during the viral cycle.

The existence of multiple CMV strategies to escape immune system control opens a range of hypotheses. Indeed, the high frequency of KIR2DL3+ NK cells observed in CMV infection may lead to an antiviral immune pressure mediated in vivo by these KIR2DL3+ NK cells. The modulation of KIR/HLA interactions by viruses has been reported for some inhibitory KIR (39–43). This suggests that viral CMV peptides may increase the affinity of C1 ligands for inhibitory KIR2DL3 and prevent the activation of specific KIR2DL3+ NK cells.

Different groups have recently reported a specific NK cell phenotype marked by the acquisition of CD57, NKG2C, and KIR on NK cells in different viral infections including in nonexclusive fashion CMV (44), hantavirus (45), chikungunya virus (46), and HIV-1 (47). Interestingly, all of these viruses developed evasion strategies based on HLA class I downregulation to escape T cell recognition. Although the ligand of NKG2C is still elusive in CMV infection, it is conceivable that NKG2C*KIR2DL3+ NK cell expansion is mutually driven by the recognition of HLA-E by CD94/NKG2C and the absence of engagement of KIR2DL with specific HLA KIR ligands on CMV-infected cells. Additionally, the large specificity of KIR2DL3 may explain its frequent expression on NKG2C+ NK cells associated to CMV infection.

In conclusion, our results provide evidence that inhibitory KIR2DL3 is preferentially coexpressed with NKG2C on amplified NK cells in CMV infection. Our findings support the functional contribution of KIR2DL1+ and KIR2DL3+ NK cell subsets in controlling CMV infection in C2+ recipients of allogeneic HSCT, suggesting a beneficial impact of CMV on GVL effect in this clinical context. In C2- recipients, the beneficial KIR2DL1+ NK cell alloreactivity is maintained despite infection, whereas CMV seems to have developed a strategy to escape to KIR2DL3+ NK cell control. This study opens new perspectives of investigation to determine the molecular mechanisms involved in KIR2DL3 NK cell escape employed by CMV.

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Disclosures

The authors have no financial conflicts of interest.

References

reactivation after allogeneic transplantation promotes a lasting increase in 

Bézat, V., O. Dalgarp, T. Asselah, P. Hafon, P. Bedossa, A. Boudifia, B. Hervier, 
I. Theodorou, M. Martinot, P. Debré, et al. 2012. CMV drives clonal expansion of 
NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. 

Bézat, V., L. L. Liu, J.-A. Malmberg, M. A. Iverson, E. Sohliberg, 
A. T. Bjerklund, C. Retière, E. Sverremark-Ekström, J. Traherne, P. Ljungman, 
et al. 2013. NK cell responses to cytomegalovirus infection lead to stable 
imprints in the human KIR repertoire and involve activating KIRs. Blood 121: 
2678–2688.

Charoudeh, H. N., G. Terszowski, K. Czaja, A. Gonzalez, K. Schmitter, and 
M. Stern. 2013. Modulation of the natural killer cell KIR repertoire by cyto-

Cosman, D., N. Fanger, L. Borges, M. Kubin, W. Chin, L. Peterson, and 
M. L. Hsu. 1997. A novel immunoglobulin superfamily receptor for cellular and 

Kubin, M., L. Cassiano, J. Chalupny, W. Chin, D. Cosman, W. Fanslow, 
J. Müllierr, A. M. Rousseau, D. Uleff, and R. Artiman. 2001. ULPB1, 2, 3, 
new MHC class I-related molecules that bind to human cytomegalovirus gly-

Babić, M., A. Krmpotić, and S. Jonjic. 2011. All is fair in virus-host interactions: 

number of activating KIR genes inversely correlates with the rate of CMV 
infection/reactivation in kidney transplant recipients. Am. J. Transplant. 8: 1312–
1317.

Cook, M., D. Briggs, C. Craddock, P. Mahendra, D. Milligan, C. Fegan, 
has a major impact on the rate of cytomegalovirus reactivation following T-

Chen, C., M. Busson, V. Rocha, M.-L. Appert, V. Lepage, N. Dulphy, P. Haas, 
G. Socie, A. Toubert, D. Charron, and P. Loiseau. 2006. Activating KIR genes are 
associated with CMV reactivation and survival after non-T-cell depleted 
HLA-identical sibling bone marrow transplantation for malignant disorders. 
Bone Marrow Transplant. 38: 437–444.

Della Cnese, M., M. Falco, M. Podestà, F. Locatelli, L. Moretta, F. Frassoni, and 
A. Moretta. 2012. Phenotypic and functional heterogeneity of human NK cells 
developing after umbilical cord blood transplantation: a role for human cyto-

cedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 
16: 2317–2321.

expression in populations from France, Guadeloupe, Finland, 

Koprowski, M., C. Cabrera, I. Erkizia, M. Bofill, B. Clotet, L. Ruiz, and M. Lo 
7. Lopez-Martos, S., J. M. Milush, B. S. Schwartz, M. J. Pando, J. Jarjoura, 
of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. 

Babić, M., A. Krmpotić, and S. Jonjic. 2011. All is fair in virus-host interactions: 

number of activating KIR genes inversely correlates with the rate of CMV 
infection/reactivation in kidney transplant recipients. Am. J. Transplant. 8: 1312–
1317.

Cook, M., D. Briggs, C. Craddock, P. Mahendra, D. Milligan, C. Fegan, 
has a major impact on the rate of cytomegalovirus reactivation following T-

Chen, C., M. Busson, V. Rocha, M.-L. Appert, V. Lepage, N. Dulphy, P. Haas, 
G. Socie, A. Toubert, D. Charron, and P. Loiseau. 2006. Activating KIR genes are 
associated with CMV reactivation and survival after non-T-cell depleted 
HLA-identical sibling bone marrow transplantation for malignant disorders. 
Bone Marrow Transplant. 38: 437–444.

Della Cnese, M., M. Falco, M. Podestà, F. Locatelli, L. Moretta, F. Frassoni, and 
A. Moretta. 2012. Phenotypic and functional heterogeneity of human NK cells 
developing after umbilical cord blood transplantation: a role for human cyto-

cedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 
16: 2317–2321.

expression in populations from France, Guadeloupe, Finland, 