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Analysis of memory-like Natural Killer cells in Human Cytomegalovirus-infected children undergoing αβ+T- and B-cell depleted Hematopoietic Stem Cell Transplantation for hematological malignancies

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Abstract
We analyzed the impact of human cytomegalovirus infection on the development of Natural Killer cells in 27 pediatric patients, affected by hematological malignancies, who had received a HLA-haploidentical hematopoietic stem cell transplantation, depleted of both $\alpha/\beta^+$ T cells and B cells. In line with previous studies in adult recipients of umbilical cord blood transplantation, we found that human cytomegalovirus reactivation accelerated the emergence of mature Natural Killer cells. Thus, most children displayed a progressive expansion of a memory-like Natural Killer cell subset expressing NKG2C, a putative receptor for human cytomegalovirus, and CD57, a marker of terminal Natural Killer differentiation. NKG2C$^+$CD57$^+$ Natural Killer cells were detectable by month 3 after hematopoietic stem cell transplantation and expanded at least until month 12. These cells were characterized by high Killer Ig-like Receptors (KIRs) and Leukocyte Inhibitory Receptor 1 (LIR-1) and low Siglec-7, NKG2A and Interleukin-18R$\alpha$ expression, killed tumor targets and responded to cells expressing HLA-E (a NKG2C ligand). In addition, they were poor Interferon-$\gamma$ producers in response to Interleukin-12 and Interleukin-18. The impaired response to these cytokines, together with their highly differentiated profile, may reflect their skewing toward an adaptive condition specialized in controlling human cytomegalovirus. In conclusion, also in pediatric patients receiving a type of allograft different from umbilical cord blood transplantation, human cytomegalovirus induced memory-like Natural Killer cells, possibly contributing to control infections and reinforcing anti-leukemia effects.
INTRODUCTION
Natural killer (NK) cells are innate lymphocytes that play an important role in anti-viral and anti-tumor responses. Their function is finely regulated by an array of both activating and inhibitory surface receptors and can be strongly influenced by several other factors, such as exposure to cytokines and/or PAMPs, developmental stage, and licensing. A fundamental role is played by HLA-class I specific inhibitory receptors including: Killer Ig-like Receptors (KIRs) distinguishing among allotypic determinants of the HLA-A, -B and -C; the HLA-E-specific CD94/NKG2A heterodimer and the Leukocyte Inhibitory Receptor 1 (LIR-1 or ILT2) broadly recognizing HLA-class I alleles. Activating KIRs, as well as CD94/NKG2C, represent the activating counterpart of HLA-I specific inhibitory receptors, although the ligand specificity is known only for selected receptors (i.e. KIR2DS1, KIR2DS4 and CD94/NKG2C).

Since NK cells are the first lymphocyte population emerging after hematopoietic stem cell transplantation (HSCT), their role in early recovery of immunity after the allograft is considered crucial, contributing to protect from both tumor recurrence and viral infections before full restoration of T-cell immunity. In KIR/KIR-L mismatched haplo-HSCT recipients, alloreactive NK cells, generated 6-8 weeks after HSCT, are capable of killing residual tumor cells, thus critically improving patients outcome. The first wave of NK cells after HSCT is represented by immature CD56 bright CD94/NKG2A bright NK cells, while more differentiated CD56 dim KIR+ NKG2A- NK cells, containing alloreactive NK cells, emerge only later. To reduce the time window required for fully competent NK cell generation, a new method of graft manipulation has been developed and applied; this approach is based on the elimination of αβ T cells (to prevent graft-versus-host disease, GvHD) and of B cells (to avoid EBV-related post-transplant lymphoproliferative disorders). Notably, together with high numbers of CD34+ HSC, this graft contains donor-derived, mature NK cells and γδ T cells which may confer prompt protection against both leukemia recurrence and infections.

As recently shown, NK cell reconstitution after HSCT can be highly accelerated by human cytomegalovirus (HCMV) infection/reactivation. Indeed, in patients receiving umbilical cord blood transplantation (UCBT), HCMV infection induced a rapid development of mature NK cells characterized by the KIR+NKG2A- phenotype. Importantly, these cells expressed NKG2C. Although the exact mechanism(s) involved in HCMV-induced NKG2C+ NK cell expansion has not been clarified, it is likely that NKG2C may play a role in HCMV recognition and in promoting the expansion and/or maturation of NKG2C+ cells, as well
as in the control of HCMV infection as suggested in the case of a T-cell deficient patient\textsuperscript{25}. In addition, a correlation between early HCMV reactivation and reduced incidence of leukemia relapse has been reported in adult patients with acute myeloid leukemia (AML) receiving allo-HSCT\textsuperscript{26}.

In the present study, we analyzed the impact of HCMV reactivation on the development of NK cells in a cohort of pediatric patients affected by hematological malignancies who received $\alpha/\beta^+$ T cell- and B cell-depleted HSCT. We observed a great expansion of memory-like NK cells in HCMV-reactivating/infected patients that express NKG2C, a putative receptor for HCMV and CD57, a marker of terminal differentiation\textsuperscript{27, 28}.

**METHODS**

**Patients and samples**

A cohort of 27 pediatric patients affected by hematological malignancies (mainly acute lymphoblastic leukemia, ALL) was enrolled in a phase I/II trial (Clinical Trials.gov Identifier NCT01810120) and received HLA-haploidentical HSCT after removal of both $\alpha\beta^+$ T cells and CD19$^+$ B cells\textsuperscript{20}. Patients were transplanted between November 2010 and May 2012. Among them, 13 experienced HCMV infection after transplantation. Of these 13 patients, 12 and 1 had a positive and negative HCMV serology, respectively. However, for the sake of brevity, we considered together all 13 patients as the HCMV-reactivating group throughout the article. Patients’ clinical characteristics and details on graft composition are summarized in Table s1. Assessment of HCMV serology, episodes of HCMV infection/reactivation and therapy are detailed in supplemental methods and summarized in Table s2.

Peripheral blood samples were collected at 1, 3, 6 and 12 months after transplantation. Peripheral blood mononuclear cells (PBMC) were separated from blood samples by Ficoll-Hypaque gradients (Sigma-Aldrich, St. Louis, MO) and used directly for flow-cytometry analyses and functional assays, or frozen and subsequently thawed for further investigations, whenever indicated. NK cell reconstitution was analyzed at the above time points, from month 1 to month 12 for 21 patients, from month 1 to month 6 for 6 patients. PBMC collected from adult healthy donors (hd) were used as controls. Frozen samples from HSC donors peripheral blood (PB) or leukapheresis were also analyzed.
To compare NK cell subsets differentiation, PBMC from 10 pediatric UCBT recipients and 5 pediatric recipients of positively selected CD34+HSC from an HLA-haploidentical parent were also analyzed.

Patients were transplanted at the Bambino Gesù Children’s Hospital, Rome, Italy. Patients’ parents gave their informed consent to participation in this study, which was approved by the Azienda Ospedaliera Universitaria San Martino (Genoa, Italy), by the University of Genoa and by the Bambino Gesù Children’s Hospital (Rome, Italy) ethics committees and was conducted in accordance with the tenants of the Declaration of Helsinki.

Monoclonal antibodies and flow cytometry, functional assays, KIR-ligands and KIR genes profile analyses and statistical analysis
See supplemental methods for details.

RESULTS

HCMV reactivation/infection accelerates NK cell maturation in αβ\(^+\)T/B-cell depleted HSCT pediatric patients

We analyzed NK cell reconstitution in 27 pediatric patients undergoing αβ\(^+\)T/B cell-depleted HSCT and compared, at different time intervals post-HSCT, data in children who did experience HCMV reactivation (or primary infection in 1 case) (n=13) with those of children who did not (n=14). In all cases, reactivation/infection occurred within month 2 after HSCT and virus was cleared within month 6.

The cells infused with this type of transplantation contain not only CD34+ HSC, but also donor-derived NK and γδ T cells (see also Table s1 for details). Thus, at early time points after transplantation, peripheral blood NK cells contain mature NK cells together with HSC-derived NK cells. Although, for technical limitation, the mature NK cells could not be distinguished from de-novo generated NK cells, a remarkable difference could be detected between patients who either did or did not reactivate HCMV.

HCMV reactivation/infection accelerated the differentiation of mature NK cells, as shown by the higher frequency of KIR\(^+\)NKG2A\(^-\) NK cells by month 3 after HSCT in HCMV-reactivating patients (fig. 1A). Major differences emerged at 6 months after HSCT between HCMV-reactivating and non-reactivating patients (two representative patients are shown in fig. 1B). In line with previous studies\(^{22,23,29}\), HCMV reactivation induced a strong imprinting in NK cell development not only by accelerating KIR\(^+\)NKG2A\(^-\) NK cell differentiation, but also by inducing a remarkable increase of CD56\(^{dim}\) NKG2C\(^+\) NK cells (fig. 1C-D).
Notably, two patients in the HCMV-reactivating group did not expand NKG2C⁺ NK cells while other two in the HCMV-non-reactivating group developed high proportions of NKG2C⁺ NK cells and were also characterized by high percentages of NKG2A⁺KIR⁺ NK cells (see dots in individual plots fig.1A, C).

In line with our previous study showing the emergence of high proportions of hypofunctional and phenotypically aberrant CD56⁻CD16⁺ NK cells in HCMV-reactivating adult recipients of UCBT, we could find also in our pediatric cohort the presence of this peculiar NK cell subset in significantly higher frequencies in HCMV-reactivating patients than in non-reactivating ones (fig. 2A). However, the proportion of these cells at month 6 was significantly lower in the αβ⁺T/B cell-depleted cohort of HCMV-reactivating patients (fig. 2B and s1) than in other two cohorts analyzed in parallel (namely, one undergoing UCBT and the other receiving only megadoses of positively selected CD34⁺ cells from haploidentical donors).

**High proportions of “memory-like” NKG2C⁺ CD57⁺ CD56dim NK cells develop in patients experiencing HCMV reactivation**

In both solid-organ transplanted patients and HSCT recipients, HCMV infection can induce the expansion of NKG2C⁺ CD57⁺ NK cells. This subset is present at variable proportions, also in HCMV-seropositive (HCMV⁺) healthy individuals and may be possibly endowed with “memory” properties. Thus, we analyzed in our cohort of patients the development of such NKG2C± CD57± NK cell subsets.

In fig. 3A, the distribution of different NKG2C/CD57 NK cell subsets (gated on CD56dim NK cells) is reported at different time points after HSCT for patients who did or did not reactivate HCMV (the gating strategy is shown in Fig. s2A). By month 3, a substantial increase of NKG2C⁺CD57⁺ NK cells was detectable in HCMV-reactivating patients. More marked differences were found at month 6, when much higher proportions of NKG2C⁺CD57⁺ cells, paralleled by a sharp decrease of NKG2C⁻ CD57 NK cells, were detectable in HCMV-reactivating patients.

Fig. 3B shows data from two representative patients, one reactivating and the other not reactivating HCMV. A progressive expansion of the NKG2C⁺CD57⁺ NK cell subset (gating on the CD56dim subset) can be observed in the patient reactivating HCMV. Further analysis of PB-NK cells from HCMV⁺ and HCMV⁻ healthy donors (hd) (fig.3C and s2B), showed
that, the NKG2C⁺CD57⁺ NK cell subset was present in significantly lower frequencies in HCMV⁺ hd than in HCMV-reactivating HSCT patients, both at month 6 and 12 (fig. s2C). When PB-NK cells from the various HSC donors of HCMV-reactivating patients were analyzed, we found that NKG2C⁺CD57⁺ NK cells were present at a low median frequency (11% fig. s2D) although all the HSC donors analysed were HCMV⁺ (Table s1). Thus, 3-6 months after HSCT, in most HCMV-reactivating patients, the frequency of NKG2C⁺CD57⁺ NK cells exceeded that measured in their respective donors (fig. 3B, fig. s2D and not shown). In view of this observation, we can suggest that NKG2C⁺CD57⁺ NK cells emerging in HCMV-reactivating patients are mostly de-novo generated.

**Memory-like NK cells from recipients of αβ⁺T/B-cell-depleted HSCT are characterized by the KIR⁺ NKG2A⁻ Siglec7⁻ LIR1⁺/⁻ IL-18Rα low NCR low surface phenotype**

We analyzed in more detail the phenotype of the HCMV-induced NKG2C⁺CD57⁺ CD56dim NK cell subset focusing on HCMV-reactivating patients at month 6 after HSCT, i.e. when these cells were maximally expanded. HCMV⁺ hd were analyzed for comparison. Representative dot plots indicating the gating strategy are shown in fig.4A.

Both NKG2C⁺CD57⁻ and NKG2C⁺CD57⁺ NK cells were mostly KIR⁺NKG2A⁻ in both transplanted patients and HCMV⁺ hd (fig. 4B). Notably, expanded NKG2C⁺ KIR⁺ NK cells preferentially expressed KIRs specific for the respective donor KIR ligands (figure s3 and not shown). As previously reported in UCBT patients, Siglec-7 was significantly downregulated in NKG2C⁺ NK cells isolated from patients, but only marginally in NK cells from HCMV⁺ hd (fig. 4B)²². Importantly, Siglec-7 downregulation was correlated with HCMV recurrence. Indeed, non-reactivating patients did not significantly downregulated this marker at any time points after HSCT (fig. s4), with the exception of the two outliers mentioned before.

Analysis of LIR-1 in our cohort of patients revealed a more frequent expression in NKG2C⁺CD57⁺ than in NKG2C⁺CD57⁻ NK cells. This different expression was more evident in HCMV⁺ hd, where most NKG2C⁺CD57⁺ NK cells were LIR-1⁺ (fig. 4B), in line with previous studies³¹.

The NKG2C⁺CD57⁻ and NKG2C⁺CD57⁺ NK cells subsets of both HSCT recipients and hd displayed remarkable differences in the expression levels of IL-18Rα (fig. 4C). This is in line with previous reports that terminally differentiated CD57⁺ NK cells express low levels of different cytokine receptors²⁷, ²⁸. Interestingly, both the proportion of IL-18Rα (not
shown) and the median surface intensity (fig. 4C) were lower in patients than in hd in all CD56dim NK cell subsets. Fig. 4C also shows that NKG2C+CD57+ (both in patients and hd) display lower expression of NKp46 and NKp30 as compared to NKG2C+ CD57- NK cells. No significant differences were detected for other triggering receptors including CD16, DNAM1, NKG2D and 2B4 (fig. 4C and s5).

**Memory-like NKG2C+CD57+ NK cells respond efficiently to tumor cells and HLA-E+ targets, but show impaired IFN-γ production in response to rhIL-12 plus rhIL-18 stimulation.**

We next evaluated the functional capabilities of the expanded NKG2C+CD57+ memory-like NK cell subset (in comparison with the other subsets identified by the expression of NKG2C/CD57) by assessing degranulation and IFN-γ production upon K562 stimulation and/or exposure to cytokines. Fig. 5A (left panel) shows that the different NK subsets (analyzed by gating on CD56dim NK cells) displayed comparable levels of CD107a degranulation, further increased by overnight exposure to rhIL-15. Fig. 5A (right panel) shows that, in the absence of exogenous rhIL-15, PB-NK cells from HCMV+ hd displayed slightly higher degranulation as compared to patients.

Regarding the production of IFN-γ, upon stimulation with K562, both the NKG2C+CD57- and NKG2C+CD57+ NK cell subsets (both in patients and hd) were slightly better producers than NKG2C+ NK cell subsets. Also, IFN-γ production was increased upon overnight exposure to rhIL-15 (fig. 5B).

In parallel experiments, the ability of the various NKG2C/CD57 subsets to produce IFN-γ was assessed after overnight incubation in the presence of rhIL-12 plus rhIL-18. Remarkably, under these conditions, NKG2C+CD57+ memory-like NK cells from patients resulted poor IFN-γ producers (fig. 5B), while the less differentiated NKG2C+CD57+ NK cells were the best producers. Intermediate levels of IFN-γ were produced by the NKG2C+CD57- and NKG2C+CD57+ NK subsets. These differences in response to rhIL-12 plus rhIL-18 might reflect, at least in part, the levels of expression of IL-18Rα (fig. 4C) in the different NKG2C/CD57 subsets. It is of note, in this context, that all the NK subsets analyzed expressed IL-12R at similar levels (not shown). A lower production of IFN-γ, in response to rhIL-12 plus rhIL-18, was detected also in NKG2C+CD57+ NK cells from hd. However, downregulation of IL-18Rα expression was less marked and IFN-γ production was only partially compromised. At variance with IFN-γ production, exposure to rhIL-12
plus rhIL-18 induced comparable levels of degranulation in the different NKG2C/CD57 NK cell subsets after stimulation with K562 (fig. s6).

In another set of experiments, NKG2C⁺ NK cells from HCMV-reactivating patients were assessed for degranulation in reverse ADCC assays, either in the presence or in the absence of specific anti-NKG2C mAb. Antibody-mediated triggering of NKG2C efficiently induced degranulation in both NKG2C⁺CD57⁻ and NKG2C⁺CD57⁺ NK cell subsets at levels comparable to those induced by anti-CD16 mAbs (fig. 6A). The simultaneous mAb-mediated triggering of NKG2C and LIR-1 did not affect degranulation induced by anti-NKG2C mAb alone (not shown). On the contrary, the simultaneous mAb-mediated triggering of NKG2C and KIR almost completely abolished NKG2C-triggered degranulation (fig. 6A). Similar results were obtained in the analysis of HCMV⁺ hd NK cells (fig. s7).

Next, we examined the ability of NKG2C⁺ NK cell subsets (either CD57⁺ or CD57⁻) to recognize HLA-E, a specific ligand of NKG2C¹⁰, by assessing their degranulation, in CD107a assays, in the presence of the HLA-E expressing (transfected) 721.221 cell line (221.AEH)³². As shown in fig. 6B both NKG2C⁺CD57⁻ and NKG2C⁺CD57⁺ NK cell subsets displayed enhanced degranulation as compared to 221wt (i.e. lacking HLA-E surface expression, fig. s8A), indicating that NKG2C recognizes HLA-E on target cells and induces activation/degranulation of NKG2C⁺ cells. This finding was further substantiated by masking experiments performed using a specific anti-NKG2C mAb. In the presence of this mAb, degranulation of NKG2C⁺ NK cells in response to 221.AEH was inhibited (not shown). On the contrary, NKG2C⁻ CD57⁻ and NKG2C⁻CD57⁺ NK cells that express high levels of NKG2A (fig. 4B), displayed decreased degranulation against 221.AEH than to 221wt, in line with the inhibitory effect mediated by NKG2A upon interaction with HLA-E.

Similar results were obtained in parallel experiments with HCMV⁺ hd. However, in this case, NKG2C⁺ NK cells displayed less increases in degranulation when comparing 221wt to 221.AEH (fig. s8B). This is likely due to the higher levels of NKG2A expressed on the surface of NKG2C⁺ NK cells from hd (fig. 4B). In line with this possibility, when degranulation was evaluated after gating on NK cells lacking NKG2A (fig. s8C), the increment became significant also in hd (fig. s8D).

**DISCUSSION**

NK cells play a crucial role in the early immunity following HSCT in leukemic patients³³; therefore the possibility to improve and to control NK cell maturation and function in HSCT recipients may result in important clinical benefits.
We have recently documented that in UCBT recipients HCMV infection/reactivation early after HSCT results in a remarkable acceleration of NK cell reconstitution and maturation. In agreement with this study, we found that, also in pediatric patients receiving a different cell composition in the HLA-haploidentical HSCT setting, depleted of TCR-αβ+T and CD19+ B cells, HCMV reactivation could accelerate the development of mature NK cells characterized by the KIR+NKG2A+NKG2C+Siglec7+ phenotypic signature. Noteworthy, as compared to pediatric patients receiving either UCBT or positively-selected CD34+ HSC from an HLA-haploidentical relative, this cohort of patients had reduced proportions of hypofunctional CD56-CD16+ NK cells. Since these cells usually develop in subjects infected by HCMV when T cell immunity is impaired, its reduced presence suggests that NK and γδ T cells contained in the graft may exert a protective role against severe infections.

It is well documented that HCMV infection influences NK cell maturation and induces a long-term reconfiguration of the NK cell receptor repertoire. This imprinting induced by HCMV infection also suggested that NK cells might keep memory of past infections, thus sharing features with cells of the adaptive immunity. Indeed, in mice, the expansion and persistence of memory Ly49H+ NK cells, endowed with specific anti-MCMV properties, has been clearly documented. It is possible that NKG2C+ NK cells that expand in humans after HCMV infection and preferentially acquire CD57 may represent the human counterpart of murine memory Ly49H+ NK cells.

In the present study, we show that most pediatric patients reactivating HCMV display a progressive expansion of this putative memory NK cell population expressing both NKG2C and CD57. The frequency of NKG2C+ NK cells (both CD57+ and CD57-) at 6 and 12 months after HSCT was higher than in both adult HCMV-seropositive hd and in the respective HSC donors (fig.s2, 3B). This may reflect a recent or ongoing HCMV infection occurring in patients, but could also depend on the status of immunosuppression allowing a better imprinting of NK cells. Notably, we found a correlation between the duration of the anti-viral treatment (Table s2) and the percent of Siglec-7 negative NK cells, which was only a tendency at month 3 (Spearman r=0.47 p=0.14) and became significant at month 6 (Spearman r=0.6 p=0.04), possibly indicating that the loss of Siglec-7 might be a marker of an efficient anti-HCMV response.

The phenotypic characterization of NK cells developing in our patients reactivating HCMV revealed that NKG2C+CD57+ NK cells represent a highly differentiated subset, displaying lower levels of expression of Siglec-7, IL-18Rα, NKG2A, NKp46 and NKp30 and higher
levels of KIRs and LIR-1 than NKG2C+CD57- NK cells. It is likely that NKG2C+CD57- NK cells emerge first in response to HCMV infection and rapidly shift to a more differentiated CD57-LIR-1^ phenotype. It cannot be ruled out that LIR-1 expression may be progressively acquired by NKG2C^+ NK cells following HCMV infection, and represent a viral evasion strategy. Indeed, the HCMV-derived viral glycoprotein UL-18 is a high-affinity ligand for LIR-1^39. Although in reverse ADCC experiments LIR-1 did not substantially inhibit NKG2C activation, it is possible that, in vivo, UL-18 expressed by infected cells may efficiently engage LIR-1 and weaken NKG2C-mediated signaling.

Whether the NKG2C+CD57^+ NK cell subset can persist for a long time after resolution of infection, or is continuously replenished by differentiating NKG2C+CD57^- NK cells^36, 40 is still unknown. In support of the first hypothesis is the finding that HCMV infection can induce resistance to cell death in NK cells developing after UCBT^41. In some patients, we could observe an increase in NK cell numbers after HCMV infection (Table s3) that was followed by the acquisition of a memory-like phenotype, suggesting that both proliferation and differentiation are likely contributing to the generation of this subset.

In our cohort of patients, the expanded memory-like NKG2C+CD57^+ NK cells were functionally competent in terms of cytokine production and cytotoxicity/degranulation in response to tumor targets. On the other hand, they displayed poor capabilities of producing IFN-\( \gamma \) in response to rhIL-12 plus rhIL-18. This may be consequent, at least in part, to the reduced expression of IL-18R. However, we cannot exclude the involvement of other mechanisms affecting the signaling pathway downstream the receptors. The diminished responsiveness to cytokines of NKG2C+CD57^+ NK cells may reflect their specialization in controlling HCMV infection and their memory-like signature, in agreement with recent findings in mice^42. Indeed, in mice, MCMV-induced, memory Ly49H^+ NK cells show an impaired response to cytokines alone (IL-12 and IL-18). Whether this poor response was determined by the reduced expression of cytokine receptors, or by an altered signaling downstream the receptors, has not been established. On the other hand, these MCMV-induced memory Ly49H^+ NK cells were characterized by a higher responsiveness to m157 antigen (the specific viral ligand for Ly49H) in the presence of cytokines, as compared to naive Ly49H^+ NK cells^42. In this context, we also show that cytokine-treated NKG2C^+ CD57^+ NK cells can efficiently degranulate in response to HLA-E^+ targets (HLA-E is a ligand of NKG2C) (fig.6)^10. It is of note that differently from mice, the putative viral ligands recognized by NKG2C, expressed by HCMV-infected cells, have not
been identified. They may be HLA-E molecules bound to viral peptides (e.g. UL40-derived peptides)\textsuperscript{37,43}, as well as other undefined molecules.

Interestingly, two patients receiving grafts from HCMV seropositive donors containing donor-derived NKG2C\(^+\) NK cells did not experience HCMV reactivation after transplantation, but displayed a significant expansion of highly differentiated NKG2C\(^+\) NK cells. These data suggest that donor-derived, transplanted NK cells may persist in the recipient and favor anti-viral responses. Since these patients received grafts from seropositive donors, it is likely that transplanted, NKG2C\(^+\) NK cells, primed by a previous encounter with HCMV in the donor, had undergone expansion in response to viral antigens present in low levels in infected peripheral tissues of the recipient (subclinical HCMV reactivation). This would be in line with previous data in recipients receiving T-cell replete HSCT\textsuperscript{44,45}. Interestingly, one of these two patients (#26) expanding NKG2C\(^+\) NK cells, experienced infection with viruses other than HCMV, namely Adenovirus and BK, early after HSCT (Table s1). Thus, it cannot be ruled out that these viral infections could have favored the expansion of HCMV-primed, donor-derived NKG2C\(^+\) NK cells, in agreement with a previous study showing that Hantavirus infection could induce expansion of NKG2C\(^+\) NK cell in HCMV\(^+\) individuals\textsuperscript{46}.

The response to HCMV may be influenced also by the number of donor-derived mature NK cells contained in the graft, highly variable among patients receiving this type of HSCT (Table s1). It is possible that protection from HCMV reactivation is achieved only with suitable numbers of NKG2C\(^+\)KIR\(^+\) NK cells in the graft. Notably, the two patients displaying high frequencies of NKG2C\(^+\) NK cells and absence of HCMV reactivation received grafts containing high numbers of donor-derived NK cells (pts #26 and #27, Table s1).

Thus, in HCMV-reactivating patients, as well as in the few non-reactivating ones who were transplanted with seropositive donors, the proportions of mature NKG2C\(^+\)CD57\(^+\) NK cells were significantly higher than those of non-reactivating patients. The capability of killing patient leukemia blasts and of protecting from acute HCMV infection and even from other viral infections should be investigated in assays against autologous leukemia blasts and autologous infected targets. However, it is conceivable that these cells, which efficiently respond against tumors and HLA-E\(^+\) targets, may indeed play a beneficial role. Indeed AML blasts (and to a lesser extent ALL blasts) express HLA-E at significant levels\textsuperscript{47,48} and could be directly targeted by NKG2C\(^+\)NK cells (especially when a KIR/KIR-L mismatch occurs in the graft versus host direction). Along this line, a protective role for
NKG2C+CD57+ CD56dim NK cells emerging after HSCT in HCMV-reactivating recipients has been recently suggested, although in a different transplantation setting. Interestingly, it has recently been shown that NKG2C+CD57+ NK cells, isolated from HCMV+ individuals, are characterized by an epigenetic remodeling at the IFN-γ locus which is similar to the one found in memory CD8+ T cell or Th1 cells. This epigenetic imprinting could be responsible of the enhanced IFN-γ production observed in NKG2C+ NK cells and may be involved in the regulation of NK cell adaptive immune mechanisms. Moreover, very recently, an altered pattern of expression of signalling proteins has been described in HCMV-induced memory-like NK cells. In particular, HCMV infection could promote the generation of adaptive NK cells that lack the expression of FcεRγ, EAT-2 and SyK. These molecular characteristics depend on given DNA methylation patterns that memory NK cells share in part with CTLs. Such epigenetic alterations could be responsible for the functional skewing shown by HCMV-induced NK cells that appear to be specialized in target cell recognition, especially via ADCC mechanisms, and impaired in cytokine-induced responses (at least IL-12 and IL-18). Indeed, previous studies showed that memory-like NKG2C+CD57+NK cells, isolated from HCMV+ hd, can efficiently kill HCMV-infected targets in the presence of anti-HCMV antibodies, i.e. through CD16 cross-linking. Notably, in HSCT recipients, memory-like NKG2C+CD57+ NK cells displayed efficient mAb-mediated CD16 triggering (fig.6A) and could kill infected cells via ADCC. The precise definition of the signals capable of inducing this selective imprinting confined to NKG2C+ NK cells would be important for providing a molecular basis for the regulation and, possibly, the manipulation of adaptive features in innate cells.

A larger cohort of patients should be investigated in future studies to definitively establish whether HCMV reactivation actually confer beneficial effects against infections and leukemia relapses, as suggested by other studies. In conclusion, we show that HCMV reactivation in pediatric patients receiving a novel type of haplo-HSCT (αβ+T/B-cell depleted) deeply influence NK cell maturation and induce the emergence of memory-like NK cells. Learning to harness the recently unraveled adaptive features of NK cells may reveal useful to achieve better recovery of immunity in HSCT recipients.

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Authorship Contributions
L.M. designed research and performed experiments, A.B. designed research, recruited study subjects and contributed to data analysis and paper writing, M.F. performed experiments and contributed to data analysis and paper writing, D.P. interpreted data and critically revised the paper, M.L-B. provided fundamental reagents and critically revised the paper; R.M. performed experiments and analyzed data, F.L. designed research, interpreted data and critically revised the paper; L.M. provided economic support and revised the paper, A.M. interpreted data, provided economic support and wrote the paper, M.D.C. designed research, performed experiments, interpreted data and wrote the paper.

Conflict of interest disclosure: A.M. is a founder and shareholder of Innate-Pharma (Marseille, France). The remaining authors declare no conflicts of interest.

References


29. Della Chiesa M, Falco M, Bertaina A, et al. Human cytomegalovirus infection promotes rapid maturation of NK cells expressing activating killer Ig-like receptor in


49. Cichocki F, Cooley S, Davis Z, et al. CD56CD57NKG2C NK cell expansion is associated with reduced leukemia relapse after reduced intensity HCT. Leukemia. 2015; doi: 10.1038/leu.2015.260


FIGURE LEGENDS

**Figure 1.** HCMV induces rapid differentiation of NKG2A*KIR+ NKG2C+ NK cells in patients receiving αβ*T/B-depleted haplo-HSCT.

Freshly collected PB NK cells from the various patients were analyzed by multicolor immunofluorescence and FACS analysis at different time intervals after HSCT. NK cells were gated from PBMC samples as CD3-CD19- lymphocytes. In **A** the expression of NKG2A in combination with KIRs was analyzed and the percentages of NKG2A*KIR+ NK cells in patients who did (empty circles, n=13) or did not (filled black squares, n=14) experience HCMV after transplantation are reported at 1, 3, 6 and 12 months after HSCT. 95%CI for the mean and statistical significance are indicated (*p<0.05; ** p<0.01; *** p<0.001). In **B** reciprocal expression of NKG2A and KIR are shown for two representative patients, one reactivating (left panel) and the other non-reactivating HCMV (right panel), at 6 months after transplantation. The percentage of NKG2A*KIR+ NK cells is indicated in the lower right quadrant. In **C**), after gating on CD56<sup>dim</sup> NK cells, the percentages of CD56<sup>dim</sup> NK cells expressing NKG2C are reported, at the different time points, in patients experiencing (empty circles, n=13) or not (filled black squares, n=14) HCMV reactivation after transplantation. 95% CI for the mean and statistical significance are indicated. In **D** NK cells from two representative patients are shown at 6 months after HSCT. The percentages of NKG2C+ NK cells are depicted in the upper right quadrants. Numbers in brackets represent the percentages of NKG2C+ NK cells by gating on the CD56<sup>dim</sup> NK cell subset.

**Figure 2.** Modest accumulation of aberrant CD56<sup>-</sup>CD16<sup>+</sup> NK cells in patients undergoing HCMV reactivation after αβ*T/B-depleted transplant as compared to patients receiving UCBT or purified CD34<sup>+</sup> cells.

PB NK cells from the various patients were analyzed for the expression of CD56 and CD16 at 1, 3, 6 and 12 months after HSCT. In **A**), the percentages of CD56<sup>-</sup>CD16<sup>+</sup> NK cells in patients experiencing HCMV (empty circles, n=13) or not (filled black squares, n=14) after transplantation are reported at the different time points. 95% CI for the mean and statistical significance are indicated (*p<0.05; ** p<0.01; *** p<0.001). In **B**), the percentages of CD56<sup>-</sup>CD16<sup>+</sup> NK cells measured in αβ*T/B-depleted haplo-HSCT (n=13) patients are compared to those measured in two different groups of pediatric patients reactivating HCMV after transplantation who received either cord blood transplantation...
(UCBT) (n=5) or positively selected CD34+ HSC (CD34+ haplo-HSCT) (n=5). The values reported correspond to 6 months after HSCT for all patients. 95%CI for the mean and statistical significance are indicated.

**Figure 3. Different NK cell subsets identified by NKG2C/CD57 expression: progressive expansion of memory-like NKG2C+CD57+ NK cells in patients reactivating HCMV after HSCT**

PB NK cells from the various patients were analyzed for the expression of NKG2C and CD57 at 1, 3, 6 and 12 months after HSCT. After gating on CD56+CD3-CD19- lymphocytes, CD56dim NK cells were evaluated.

In **A**) the size of the different subsets identified by NKG2C and CD57 on CD56dim NK cells (i.e. NKG2C+CD57-, NKG2C+CD57+, NKG2C-CD57- and NKG2C-CD57+, indicated for brevity as 2C and 57) in patients experiencing (empty circles, n=13) or not (filled black squares, n=14) HCMV reactivation after transplantation are shown from month 1 to month 12 after HSCT. 95% CI for the mean and statistical significance are indicated (*p<0.05; ** p<0.01; *** p<0.001). In **B)**, reciprocal expression of NKG2C and CD57 on CD56dim NK cells from two representative patients, one reactivating (upper panels), and the other non-reactivating HCMV (lower panels), is shown at different time intervals after transplantation in comparison to NK cells isolated from their respective HSC donors (left panels). The percentages of positive cells are indicated in each quadrant. In **C**) reciprocal expression of NKG2C and CD57 on CD56dim NK cells from three hd (one HCMV-, left panel; two HCMV+, middle and right panels) are shown. The percentages of positive cells are indicated in each quadrant.

**Figure 4. NKG2C+ CD57+ NK cells expanded in patients receiving αβ+T/B-depleted HSCT and experiencing HCMV are characterized by a KIR1 NKG2A- Siglec7- LIR1+/− IL18Rα− NCR− low surface phenotype**

PB NK cells collected at 6 months after HSCT from HCMV reactivating patients and from HCMV+ hd were analyzed for the expression of the indicated surface markers, after gating on the different CD56dim NK cell subsets identified by NKG2C and CD57 (i.e. NKG2C+CD57−, NKG2C+CD57+, NKG2C−CD57− and NKG2C−CD57+, indicated for brevity as 2C and 57). In **A**) the gating strategy is shown for a representative patient and a donor, in **B**) 95% CI for the mean percentage of positive CD56dim NK cells is shown for HCMV reactivating patients (black bars, n=8) and for HCMV+ hd (grey bars, n=7). In **C**) 95% CI for
the median fluorescence intensity (mfi) is similarly shown. Statistical significance was calculated for NKG2C⁺CD57⁻ vs NKG2C⁺CD57⁺ NK cell subsets for each surface marker (*p<0.05; ** p<0.01; *** p<0.001).

Figure 5. Memory-like NKG2C⁺CD57⁺ NK cells from patients are capable of both degranulating and producing IFN-γ in response to tumor targets but show impaired IFN-γ production in response to rhIL-12 plus rhIL-18 stimulation.

Freshly drawn PBMC from HCMV-reactivating patients (n=8) at 6 months after HSCT, and from HCMV⁺ hd (n=6) were cultured overnight in the presence or in the absence of rhIL-15 or rhIL12 plus rhIL18. Then PB cells were incubated with either medium alone (not shown) or K562 for 3 hours. In A), after incubation with K562, CD107a expression was evaluated in the different NKG2C/CD57 CD56dim NK cell subsets shown in fig.4. Black bars represent cells cultured overnight in medium alone, while white bars cells cultured overnight in rhIL-15. In B), parallel cultures were assessed for intracellular IFN-γ production upon overnight culture with medium alone (black bars) or rhIL15 (white bars) followed by stimulation for 3 hours in the presence of K562 or upon overnight exposure to rhIL12 plus rhIL18 (grey bars). 95%CI for the mean of CD107a/IFN-γ positive NK cells is shown for each subset. Left panels show data relative to patients and right panels data relative to hd.

Figure 6. Both anti-NKG2C mAbs and HLA-E⁺ target cells efficiently trigger degranulation of the memory-like NKG2C⁺CD57⁺ NK cell subset of HCMV-reactivating transplanted patients

Thawed PBMC from HCMV-reactivating patients, collected at 6 months after HSCT, were cultured in the presence of rhIL-15. In A), after overnight culture, cells were incubated for 1h with the FcγR⁺ murine cell line p815 either in the presence or in the absence of anti-CD16, anti-NKG2C and anti-KIR specific mAbs alone or in combination (for each patient an anti-KIR mAb recognizing the KIR that was most expanded on NKG2C⁺CD57⁺ cells was used, i.e the KIR specific for the corresponding donor KIR ligand). CD107a expression is shown for each NK cell subset as 95%CI for the mean summarizing data for n=7 HCMV-reactivating HSCT patients. (dark bars: NKG2C⁺CD57⁺, light grey: NKG2C⁺CD57⁺, dark grey: NKG2C⁺CD57⁺, white bars: NKG2C⁺CD57⁺). CTR indicates NK cells cultured in the presence of p815 and in the absence of mAbs. In B), after 3 days of culture with rhIL-15, cells were incubated in medium alone (black bars) or with 221wt (light grey bars) or with 221 expressing HLA-E (221 AEH, dark grey bars). CD107a expression
is shown for each NK cell subset as 95%CI for the mean summarizing data for n=5 HCMV-reactivating HSCT patients. Statistical significance is indicated (*p<0.05).
Figure 1

A

% NKG2A-KIR+

0 20 40 60 80 100

1m 3m 6m 12m

** ** **

B

HCMV-reactivating pt Non reactivating pt

HCMV-reactivating pt

NKG2A

58

KIR

14

CD56

HCMV-reactivating pt

67 (76)

NKG2C

20 (21)

% CD56dim NKG2C+

0 20 40 60 80 100

1m 3m 6m 12m

* **

HCMV-reactivating patients

HCMV-non reactivating patients
Figure 5

A

HCMV-reactivating patients

%CD107α+ NK cells

HCMV+ hd

%CD107α+ NK cells

NKG2C+ CD57- NKG2C+ CD57+ NKG2C- CD57- NKG2C- CD57+

NKG2C+ CD57- NKG2C+ CD57+ NKG2C- CD57- NKG2C- CD57+

B

HCMV-reactivating patients

%IFN-γ + NK cells

HCMV+ hd

%IFN-γ + NK cells

NKG2C+ CD57- NKG2C+ CD57+ NKG2C- CD57- NKG2C- CD57+

NKG2C+ CD57- NKG2C+ CD57+ NKG2C- CD57- NKG2C- CD57+
Supplemental material

Material and methods

HCMV serology and therapy

HCMV serology was assessed prior to transplantation using enzyme-linked immunoassay for virus-specific immunoglobulin IgM and IgG. Patients were monitored for HCMV infection in blood by determination of DNAemia twice a week from day 0 until discharge from the hospital, and then once a week for the first three months after transplantation. Subsequently, patients were monitored for HCMV at time of control medical visits or in the presence of clinical symptoms suggestive of HCMV infection. Pre-emptive therapy was administered when the viral load was greater than 5,000 DNA copies/mL and was based on administration of i.v. ganciclovir (5 mg/kg twice a day), replaced by foscarnet (90 mg/kg twice a day) in case of ganciclovir-induced neutropenia (less than 0.5x10^9 neutrophils/l) or sustained increase of HCMV levels in blood during therapy with ganciclovir. Anti-viral treatment was discontinued after virus clearance from blood, defined as two consecutive negative results. Episodes of HCMV relapse were treated similarly.

Monoclonal antibodies and flow cytometry

The following mAbs, all produced in our lab, were used in this study: c127, (IgG1, anti-CD16), AZ20 (IgG1, anti-NKp30), BAB281 (IgG1, anti-NKp46), z231 (IgG1, anti-NKp44), BAT221 (IgG1, anti-NKG2D), KRA236 (IgG1 anti-CD226), PP35 (IgG1, anti-CD244), c218 and FS280 (IgG1 and IgG2a, respectively, anti-CD56), QA79 and Z176 (IgG1 and IgG2b, respectively, anti-siglec-7 or p75/AIRM1), 11PB6 (IgG1, anti-KIR2DL1 and KIR2DS1), GL183 (IgG1, anti-KIR2DL2/L3/S2), FES172 (IgG2a, anti-KIR2DS4), Z27 (IgG1, anti-KIR3DL1/S1), DF200 (IgG1 anti-KIR2DL1/S1/L2/L3/S2/S5), AZZ158 (IgG2a, anti-KIR3DL1/S1/L2), ECM41 (IgM, anti-KIR2DL3), Z199 and Z270 (IgG2b and IgG1 respectively, anti-NKG2A), F278 (IgG1, anti-LIR-1).

Anti-KIR2DL1-PE, -APC or non-conjugated mAb (clone 143211) and anti-NKG2C (mouse IgG2b) were purchased from R&D Systems Inc (Abingdon, United Kingdom). Anti-CD56-PC7 (C218 clone), anti-CD3-Pacific Blue (UCHT1 clone), anti-CD19-Pacific Blue (J3-119 clone), anti-NKG2A-APC (Z199 clone) were purchased from Beckman Coulter, Immunotech (Marseille, France).

Anti-CD16-PerCPCy5.5, anti-CD16-APC-Cy7 (clone 3G8), anti-KIR2DL2/L3-S2-FITC (CHL clone), anti-CD107a-PE (anti-LAMP1), anti-IFN-γ-PE and anti-IL12R-PE were obtained...
from BD Bioscience Pharmingen (San Diego, CA). Anti-CD3-VioGreen, anti-CD20-VioGreen, anti-CD14-VioGreen, anti-CD57-VioBlue or purified, biotin-conjugated anti-NKG2A, anti-KIR3DL1-FITC (DX9 clone), anti-KIR2DL2/L3-S2-APC (DX27 clone), biotin-conjugated anti-KIR2DL1-S1 (11PB6 clone), anti-biotin VioBlue and anti-biotin-PerCPVio700 mAbs were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-IL18Rα (mouse IgG1, clone B-E43) was purchased from ABCAM (Cambridge, UK). Goat anti-mouse isotype-specific secondary reagent was purchased from Southern Biotech (Birmingham, AL) and Jackson ImmunoResearch Laboratories (Suffolk, UK).

NK cell phenotype and effector functions were analyzed on either freshly derived PBMC or thawed PBMC where indicated, gating NK cells by physical parameters and by the combined use of anti-CD56, anti-CD3 and anti-CD19 mAbs. The CD56dim NK cell subset was evaluated whenever indicated.

Cytofluorimetric analyses were performed on FACSVerse (Becton Dickinson & Co, Mountain View, CA) and data were analyzed by FacsSuite software version 1.0.5. Single fluorescence cytofluorimetric analyses were performed on FACSCalibur (Becton Dickinson) and analyzed by CellQuestpro.

**CD107a degranulation and IFN-γ production**

The medium used throughout the experiments was RPMI-1640 medium supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin-neomycin mixture and 10% heat-inactivated FCS(1).

Whenever indicated, PBMC were cultured either in the presence or in the absence of rhIL-15 or rhIL-12 plus rhIL-18 (Peprotech, London, UK) at the final concentration of 10 ng/ml, 2 ng/ml and 20 ng/ml respectively.

Freshly drawn PBMC from 8 HCMV-reactivating patients at 6 months after HSCT and from 6 HCMV seropositive hd were cultured overnight (18 hours) either in the presence or in the absence of rhIL-15; then PBMC were washed and incubated with the target cells K562 at an effector-to-target cell (E/T) ratio of 1:1 (where effector cells are PB-NK cells) for 3 hours in culture medium supplemented with anti-CD107a-PE mAb. Thereafter, cells were stained with anti-CD56-PC7, anti-CD3 and anti-CD20-VioGreen, anti-CD16-PercpCy5.5, anti-CD57-VioBlue, anti-NKG2C followed by appropriate secondary reagents (anti-mouse IgG2b APC-conjugated, Jackson ImmunoResearch, Suffolk UK), for 35 min on ice. Cells were then washed and analyzed by flow cytometry (FACSCalibur, Becton Dickinson). To detect NK intracellular production of IFN-γ, overnight cultured PBMC were washed and
incubated with K562 or with medium alone, for 4 hours in the presence of GolgiStop (BD Biosciences Pharmingen). PBMCs overnight cultured in the presence of rhIL-12 plus rhIL-18 were analyzed, as well. Thereafter, cells were washed, stained as described above for CD107a assays and then fixed and permeabilized with the BD Cytofix/Cytoperm kit (BD Bioscience Pharmingen). IFN-γ production was detected by subsequent intracellular staining with anti-IFN-γ-PE and cytofluorimetric analysis. The percent of positive cells was calculated subtracting the baseline CD107a or IFN-γ expression in controls cultures without stimuli from targets.

In reverse Ab-dependent cellular cytotoxicity (ADCC) assays, thawed PBMC from 7 HCMV-reactivating patients and from 6 HCMV seropositive hd were cultured overnight with rhIL-15, washed and then incubated with the FcγRII+ p815 murine mastocytoma cell line, either in the presence or in the absence of mAbs specific for the different surface receptors indicated in the text/figures at an effector-to-target cell (E/T) ratio of 1:1 for 1 hour (this time window was chosen so that NKG2C surface expression was detected when NK cells were triggered by anti-NKG2C mAb).

In another set of degranulation assays, thawed PBMC were cultured for 3 days in the presence of rhIL-15, then washed and incubated either in the presence or in the absence of the lymphoblastoid 721.221wt cell line (221wt) or the 721.221.AEH cell line (221.AEH) that has been transfected with a hybrid HLA-E containing the HLA-A2 signal sequence (2), at an effector-to-target cell (E/T) ratio of 1:1 for 3 hours in culture medium supplemented with anti-CD107a-PE mAb. Cells were then stained with anti-CD56-PC7, anti-CD3- and anti-CD20-Viogreen, anti-CD16-APC-Cy7, anti-NKG2A biotin-conjugated plus anti-BiotinPercpvio700, anti-CD57-VioBlue, anti-NKG2C and then analyzed as described above.

**KIR-ligand and KIR gene profile analyses**

DNA of the tested samples was extracted using the QIAamp DNA Blood Mini kit (QIAGEN) according to the manufacturer’s instruction. KIR ligand and the KIR gene profile analyses were performed using a sequence specific primer-PCR (SSP-PCR) protocol. In particular KIR gene profile, performed using KIR genotype kit (GenoVision, Saltsjoebaden, Sweden), allowed the detection of the presence/absence of all the KIR genes, while, by the use of KIR HLA ligand kit (GenoVision, Saltsjoebaden, Sweden), we typed HLA-C alleles on the basis of the dimorphism present at position 80 (analysis of C1 and C2 epitope), and we
detected the presence of HLA-B and –A alleles characterized by the Bw4 motifs (dividing the HLA-B Bw4⁺ alleles in two groups according to the residue present at position 80).

NKG2C genotype was analyzed as described elsewhere²⁹. All patient samples have been typed as NKG2C⁺/+ or NKG2C⁺/-, i.e. are characterized by the presence of at least one NKG2C ORF allele²⁹.

**Statistical analysis**

Wilcoxon-Mann-Whitney non-parametric tests were employed. The statistical significance (p) is indicated (*p<0.05; ** p<0.01; *** p<0.001). Median fluorescence intensity (MFI) values were normalized before calculating statistical significance. Graphic representations and statistical analysis were performed using GraphPad Prism 6 (GraphPad Software, Inc. La Jolla, CA).

**Supplementary figures**

Figure s1. Analysis of CD56⁻CD16⁺ NK cells in patients undergoing HCMV reactivation after receiving αβ⁻T/B-depleted HSCT or UCBT or purified CD34⁺ HSCT

PB NK cells from the various patients were analyzed for the expression of CD56 and CD16. Three representative patients receiving the different type of transplantation indicated (reactivating HCMV after transplantation) are shown at 6 months after HSCT. The percentages of CD56⁻CD16⁺ NK cells are indicated in the lower right quadrant.

Figure s2. Distribution of NK cell subsets identified by NKG2C/CD57 expression in healthy donors and in HSC donors as compared to reactivating HSCT patients

PB NK cells from healthy donors, from the various patients, and from given HSC donors were analyzed for the expression of NKG2C and CD57. After gating on CD56⁺CD3⁻CD19⁻ lymphocytes, CD56dim NK cells were evaluated. In A) the gating strategy is shown for a representative HCMV-reactivating patient and a HCMV⁺ donor. In B) the percentage of the different subsets identified by NKG2C and CD57 on CD56dim NK cells (i.e. NKG2C⁺CD57⁺, NKG2C⁺CD57⁻, NKG2C⁻CD57⁻ and NKG2C⁻CD57⁺), in HCMV⁺ (black bars, n=10) or HCMV⁻ hd (grey bars, n=7) is reported as 95% CI for the mean. Statistical significance is indicated. In C) the percentage of the different NKG2C/CD57 subsets in HCMV⁺ hd (black bars, n=10) are compared to NK cells from HCMV-reactivating αβ⁻T/B-depleted haplo-HSCT at 6 months (dark grey bars, n=13) and 12 months (light grey bars, n=10). 95% CI for the mean and statistical significance are reported. In D) the percentage of
CD56^{dim}NKG2C^{+}CD57^{+} PB-NK cells isolated from the HSC donors of HCMV-reactivating patients is shown (black bar, n=11) in comparison to PB-NK cells isolated from HCMV-reactivating patients at month 1 (white bar), 3 (light grey bar), 6 (very light grey bar), 12 (dark grey bar) after HSCT. 95% CI for the mean and statistical significance are reported.

**Figure s3. KIR gene and KIR ligand analyses.**
Donor genotypes were analyzed for the presence (grey boxes) or absence (white boxes) of the indicated KIR genes using SSP-PCR approach. Two different sets of primers were used to discriminate between the KIR2DS4 alleles coding for membrane bound receptors (reported as S4) and the alleles coding for putative soluble receptors (reported as d). KIR3DL1*004 indicates the presence of a KIR3DL1 allele coding for intracellularly retained receptor. *004/? indicates the presence of at least one KIR3DL1 allele coding for intracellularly retained receptor. Dark grey boxes indicate educated KIRs.

A/A indicates that the sample is homozygous for group A KIR haplotypes, B/X indicates genotypes containing either one (A/B heterozygous) or two (B/B homozygous) group B haplotypes.

**Figure s4. Siglec-7 expression in patients receiving $\alpha\beta^{+}$T/B-depleted haplo-HSCT stratified on HCMV recurrence**
Peripheral blood NK cells from the various patients were analyzed for the expression of Siglec-7 at 1, 3, 6 and 12 months after HSCT. After gating on CD56^{+}CD3^{−}CD19− lymphocytes, CD56^{dim} NK cells were evaluated. The percent of positive CD56^{dim} NK cells in patients either experiencing (empty circles) or not experiencing (filled black squares) HCMV after transplantation are reported at the different time points. 95% CI for the mean and statistical significance are indicated.

**Figure s5. Analysis of the expression of activating receptors by NKG2C^{+} CD57^{+} NK cells from HCMV-reactivating patients and HCMV^{+} hd**
PB NK cells collected at 6 months after HSCT from HCMV reactivating patients and from HCMV^{+} hd were analyzed for the expression of the indicated surface markers, after gating on the different CD56^{dim} NK cell subsets identified by NKG2C and CD57 as shown in fig.4 (i.e. NKG2C^{+}CD57^{−}, NKG2C^{−}CD57^{+}, NKG2C^{−}CD57 and NKG2C^{+}CD57^{−}, indicated for brevity as 2C and 57). 95% CI for the median fluorescence intensity (mfi) is shown for
HCMV reactivating patients at 6m after HSCT (black bars, n=8) and for HCMV+ hd (grey bars, n=7).

**Figure s6. Comparable levels of degranulation of the different NKG2C/CD57 NK cell subsets exposed to rhIL-12 plus rhIL-18**

Thawed PBMC from HCMV-reactivating patients (n=6), collected at 6 months after HSCT, were cultured overnight in the presence of rhIL-12 plus rhIL-18 or rhIL-15 for comparison. Then PB cells were incubated either with or without K562 cell line for 3 hours. CD107a expression was evaluated in the different NKG2C/CD57 CD56dim NK cell subsets (dark bars: NKG2C+CD57-, light grey: NKG2C+CD57+, dark grey: NKG2C+CD57-, white bars: NKG2C+CD57+). 95%CI for the mean of CD107a positive NK cells is shown.

**Figure s7. Anti-NKG2C mAbs trigger degranulation of memory-like NKG2C+ CD57+ NK cell subset from HCMV+ hd in reverse ADCC assays**

Thawed PBMC from HCMV+ hd were cultured in the presence of rhIL-15. After overnight culture, cells were incubated for 1h with the FcγR+ murine cell line p815 in the presence or in the absence of anti-CD16, anti-NKG2C, and anti-KIR specific mAbs alone or in combination. CD107a expression is shown for each NK cell subset as 95%CI summarizing data for n=6 HCMV+ hd (dark bars: NKG2C+CD57-, light grey: NKG2C+CD57+, dark grey: NKG2C+CD57-, white bars: NKG2C+CD57+). CTR indicates NK cells cultured in the presence of p815, in the absence of mAbs.

**Figure s8. Recognition of HLA-E+ 221 by NKG2C+CD57+ NK cells from both HCMV+ hd and patients is enhanced by gating on NKG2C+CD57+NKG2A- NK cells**

A) 221 wt and 221 AEH were analysed by flow cytometry for the expression of both non-classic HLA-E molecules (3D12 mAb) and HLA-class I (HLA-I) molecules (W63/2 mAb). Empty histograms represent cells incubated with the secondary reagent only. Mean Fluorescence Intensity values are indicated in each histogram plot. In B) and D) thawed PBMC were cultured in the presence of rhIL-15. As indicated in figure 6B, after three days of culture, cells were incubated in medium alone (black bars) or with 221 wt (light grey bars) or with 221 expressing HLA-E (221.AEH, dark grey bars). In B) CD107a expression is shown for each NK cell subset as 95%CI for the mean summarizing data for n=7 HCMV+ hd. In C), the gating strategy to exclude NKG2A+ NK cells before analysing degranulation in the different NKG2C/CD57 NK cell subsets, is shown for a representative hd. In D) CD107a expression is shown, after gating on NKG2A- NK cells, as
95% CI for the mean summarizing data for n=7 HCMV$^+$ donors (left panel) and n=5 HCMV$^-$ reactivating HSCT patients (right panel). Statistical significance is reported.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>WBC</th>
<th>Diagnosis</th>
<th>Disease stage</th>
<th>Conditioning Scheme</th>
<th>HCMV serostatus</th>
<th>HCMV infection/reactivation</th>
<th>GvHD, grade</th>
<th>other infections</th>
<th>n° TN cells infused (10^6)</th>
<th>n° CD34+ cells infused (10^6)</th>
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<th>cause of death</th>
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**Table s1 legend:** AML, acute myeloid leukemia; BCP-ALL, B-cell precursor acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; CR, complete remission; FLT3-ITD, fms-related tyrosine kinase 3-internal tandem duplications; TBI, Total Body Irradiation; TT, Thiotepa; FLU, Fludarabine; TREO, Treosulfan; BUS, busulfan; L-PAM, melphalan; EDX, endoxan (cyclophosphamide); R/D, recipient/donor; GvHD, Graft-versus-Host Disease; HSCT, hematopoietic stem cell transplantation; HCMV, human cytomegalovirus; AdV, adenovirus; VZV, varicella zoster virus.

**Table s2. HCMV infection/reactivation characteristics**

<table>
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<tr>
<th>HCMV reactivating patient #</th>
<th>Day after HSCT when first HCMV reactivation occurred</th>
<th>Multiple HCMV reactivations (n°)</th>
<th>n°of days with HCMV DNAemia</th>
<th>Duration of anti-viral treatment (days)</th>
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Table s3. NK cells absolute numbers in patient peripheral blood after HSCT.

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Table s3 legend: n.a. = not available
Patients #1-13 (bold) are those reactivating HCMV after HSCT.
The reported values correspond to absolute numbers of NK cells/µl at the different time points after HSCT.
Supplemental figures

Figure s1

Figure s2

(A) HCMV-reactivating patient

(B) HCMV+ Healthy Donors

(C) HCMV-reactivating pts 6m after HSCT

(D) HCMV-reactivating pts 12m after HSCT
### Figure s3

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**Inhibitory KIRs**

**Activating KIRs**

### Figure s4

![Graph showing %CD56dim SIGLEC-7+ over time](image)

- **HCMV-reactivating patients**
- **HCMV-non-reactivating patients**
Figure s7

Figure s8

A

HLA-E

HLA-I

221 WT

221 AEH

B

HD

%CD57+ NK cells

C

CD56
gate NK
gate NK NKG2A-

CD3CD19
NKG2A
CD57

D

HD

%CD57+ NK cells

PATIENTS

%CD57+ NK cells