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Favorable impact of natural killer cell reconstitution on chronic graft-versus-host disease and cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation

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Key words: Natural Killer cells, CMV reactivation, allo-HSCT, NKG2C, GvHD
ABSTRACT

Natural killer cells are the first lymphocyte subset to reconstitute, and play a major role in early immunity after allogeneic hematopoietic stem cell transplantation. Cells expressing the activating receptor NKG2C seem crucial in the resolution of cytomegalovirus episodes, even in the absence of T-cells. We prospectively investigated Natural Killer cells reconstitution in a cohort of 439 adult recipients who underwent non T-cell depleted allogeneic hematopoietic stem cell transplantation between 2005 and 2012. Freshly collected blood samples were analyzed 3, 6, 12 and 24 months after transplantation. Data were studied with respect to conditioning regimen, source of stem cells, underlying disease, occurrence of Graft-versus-Host Disease, and profiles of cytomegalovirus reactivation. In multivariate analysis, we show that the absolute numbers of CD56<sup>bright</sup>Natural killer cells at month 3 were significantly higher after myeloablative than after reduced intensity conditioning. Acute Graft-versus-Host Disease impaired reconstitution of total and CD56<sup>dim</sup>Natural killer cells at month 3. In contrast, high Natural killer cell count at month 3 was associated with a lower incidence of chronic Graft-versus-Host Disease, independently from a previous episode of acute Graft-versus-Host Disease and stem cell source. NKG2C+CD56<sup>dim</sup> and total Natural killer cell count at M3 was lower in patients reactivating CMV between month 0 and month 3, but expanded greatly afterwards. These cells were also less numerous in patients who experienced subsequent reactivation between month 3 and month 6. Our results advocate a direct role of NKG2C-expressing Natural killer cells in the early control of cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation.
INTRODUCTION

Natural Killer (NK) cells were first described as cytotoxic lymphocytes that do not require prior sensitization to kill tumor cells. Their dual role, both as a main component of the innate immunity and as a modulator of the adaptive immunity, is now well documented\(^1\). NK cells are of major importance in allogeneic hematopoietic stem cell transplantation (allo-HSCT) because of their ability to recognize and destroy abnormal leukocytes, such as leukemic cells\(^2\). They are the first subset of lymphocytes that reconstitute after allo-HSCT, with an early expansion of the cytokine-producing CD56\(^{bright}\) NK cell subset. We found that this is followed by the sequential expansion of an intermediate CD56\(^{bright}\)CD16\(^{low}\) subset, before the development of the dominant CD56\(^{dim}\) subset, characterized by its higher cytotoxic activity\(^3\).

Transplant and disease-related factors can directly influence the upcoming immune reconstitution. Pre-transplant factors such as the source of stem cells, the conditioning regimen (myeloablative conditioning (MAC) versus reduced intensity conditioning (RIC)), and the underlying disease (malignant versus non malignant) greatly influence the speed and the strength of the immune reconstitution after allo-HSCT. This is of utmost importance, as a fast and robust immune reconstitution may affect the clinical outcome in terms of graft versus host disease (GvHD), graft versus leukemia (GvL) and occurrence of opportunistic infections or viral reactivations. In addition, through their alloreactivity against recipient dendritic cells, donor-derived NK cells may well forestall GvHD\(^4\).

Despite great progresses made in the diagnosis and management of GvHD, the outcome of allo-HSCT remains hindered by a high morbidity with organ dysfunctions and poor quality of life. Kim and colleagues have associated the onset of acute GvHD (aGvHD) with an impaired immune reconstitution and a delayed recovery of total NK cells at 1 month post-transplantation, in a cohort of 59 children\(^5\). However, to the best of our knowledge, no study has addressed the potential impact of aGvHD on NK cell subsets recovery and the potential consequences of NK cell reconstitution on chronic GvHD (cGvHD) and clinical outcome in a large cohort of adult patients.

Cytomegalovirus (CMV) is another major cause of morbidity in CMV-seropositive allo-HSCT recipients. We and others have shown the key role of both CD8\(^+\) and CD4\(^+\) CMV-specific T-cells in the resolution of CMV reactivations after allo-HSCT\(^6\)\(^-\)\(^9\).
The high susceptibility to herpesvirus in cases of NK cell deficiencies supports evidence of the critical role of NK cells in host response against viral infections\textsuperscript{10, 11}. NK cell function is regulated by a diverse array of inhibitory and activating receptors, including killer cell Ig-like receptors (KIRs), NKG2A, NKG2C, NKG2D, and natural cytotoxic receptors, defining functionally distinct subsets of NK cells within the total population\textsuperscript{12}. Guma and colleagues reported increased proportions of CD94/NKG2C+ NK cells from healthy CMV seropositive donors, suggesting that viral infection may shape the NK receptor repertoire \textsuperscript{13}. They also showed that direct in vitro stimulation using virus-infected fibroblasts elicits a preferential expansion of CD94/NKG2C+ NK cells from CMV seropositive healthy donors \textsuperscript{14}. In a case report of a SCID child infected with CMV, the control of CMV infection was observed after the expansion of potent NKG2C+ NK cells, advocating the direct role of this subset in anti-CMV immunity \textsuperscript{15}.

In this study, we prospectively evaluated the reconstitution of NK cell subsets in a cohort of 439 consecutive non-T-cell depleted allo-HSCT, excluding cord blood transplantations and children under the age of 15. First, we aimed at evaluating the influences of stem cell sources, conditioning regimen and primary disease on NK subset reconstitution. Then we assessed the impact of NK cell subset reconstitution on clinical outcome, especially the occurrence of cGvHD and transplant-related mortality (TRM). Finally, we evaluated the interplay between the kinetics of NK cell subsets reconstitution and CMV reactivation.
METHODS

Patient characteristics and blood samples
We prospectively studied 439 adult patients over the age of 15, who underwent allo-HSCT from an HLA identical sibling ($n = 237$), an HLA matched ($n = 151$) or mismatched ($n = 51$) unrelated donor. All recipients received non-T-cell-depleted allo-HSCT at the Hematology and Bone Marrow Transplantation Unit, Saint-Louis Hospital (Paris, France), between 2005 and 2012. The source of stem cells was unmanipulated bone marrow (BM) or peripheral blood stem cells (PBSC). Patient and transplant characteristics are presented in Supplementary Table S1.
All patients had given their informed consent for this study, which was approved by the Institutional Review Board of Saint-Louis Hospital, in accordance with the Declaration of Helsinki.

Flow cytometry and monoclonal antibodies
Lymphocyte eight-color immunophenotyping was performed on freshly collected whole blood samples, using a FACS Canto II flow cytometer and FACS DIVA software (BD Biosciences, le Pont de la Claix, France). Lymphocytes absolute count was determined using the TruCount system (BD Biosciences) with anti-CD3-FITC, -CD8-PE, -CD45-PerCP, -CD4-APC, -CCR7-PECy7 and -C45RA-APC mAbs (BD Multitest, BD Biosciences).
Total NK cells, CD56$^{bright}$ and CD56$^{dim}$ NK cell populations were defined using the gating strategy shown in Supplementary Figure 1.

Virological monitoring
Patients were monitored for CMV infection once weekly in EDTA whole blood by quantitative real-time PCR, as previously described$^{16,17}$. CMV reactivations were defined as CMV viral load values over 1000 copies/mL. Pre-emptive antiviral therapy was given when viral load reached this threshold on two successive determinations.

Definition of the main clinical outcomes
aGvHD and cGvHD were diagnosed and graded according to published criteria\textsuperscript{18}. Incidence of aGVHD grades 0-1 and grades 2-4 was 51.8\% and 48.2\%, respectively. TRM was defined as cumulative incidence of death after complete remission. Relapses and deaths resulting from progressive disease were competing events. Patients lost to follow-up were censored at the last known point of study.

**Statistical analyses**

Recipient and transplant-related characteristics such as source of stem cells, conditioning regimen, underlying disease, recipient age, recipient/donor CMV serologic status, and aGvHD were defined as risk factors that could potentially influence the post-HSCT outcomes of interest in this study, i.e. NK reconstitution, TRM, cGvHD, and CMV reactivation. Univariate and multivariate methods developed by Fine and Gray for competing risks studies\textsuperscript{19} were used to estimate the impact of NK cell counts at M3 and other risk factors on these outcomes. All tests were two-sided, with type I error rate fixed at 0.05. All analyses were performed using SPSS 20 and R 2.6.2 statistical software (R packages “cmprsk” for competing risks, The R Foundation for Statistical Computing, Vienna, Austria).

**RESULTS**

*Early reconstitution of NK cell subsets is dependent on the conditioning regimen and the occurrence of acute GvHD*

NK cell counts and subsets were determined at months M3, M6, M12 and M24 after allo-HSCT. Our results confirmed previous data showing that early NK cell reconstitution is due to a substantial expansion of the CD56\textsuperscript{bright} population at M3, which remains increased over a year following transplantation. HLA compatibility was not associated with any difference in NK cell subset count at any time point (data not shown). We analyzed the reconstitution of CD56\textsuperscript{bright}, CD56\textsuperscript{dim} and total NK cells in different transplantation settings, with regard to the underlying primary disease, the conditioning regimen, the source of stem cells and the occurrence of aGvHD. Absolute CD56\textsuperscript{bright} NK cell counts were significantly increased at M3 and M6 in patients treated for a non-malignant disease. Similar findings were observed in those transplanted with BM and in patients who received MAC as conditioning regimen
A multivariate analysis by ANOVA showed that the conditioning regimen was the decisive factor influencing CD56^{bright} subset counts at M3 ($P = 0.008$, Table 1).

CD56^{dim} and total NK cell counts at M3 and M6 were not statistically different between patients with either underlying malignant or non-malignant disease, transplanted with either BM or PBSC, and conditioned with either MAC or RIC (Figures 1B and C, three left panels). However, they were significantly lower in patients who had aGvHD grades 2-4 ($P = 0.0001$ at M3 and $P = 0.043$ at M6, Figures 1B and 1C, right panel). Although MAC was a factor of higher risk of aGvHD than RIC (MAC = 58% versus RIC = 41%, $P = 0.00005$), aGvHD was shown to be the decisive factor influencing total NK and CD56^{dim} subset counts at M3 in a multivariate analysis by ANOVA ($P = 0.0001$ and $P = 0.001$, respectively, Table 1).

Finally, NK subsets absolute counts reached similar values at M24, regardless of the pre-transplantation settings.

**Absolute NK cell count is an independent risk factor for cGvHD**

As expected\textsuperscript{20-23}, the cumulative incidence of cGvHD was significantly higher in the group of patients who had aGvHD (grades 2-4 versus grades 0-1, $P = 0.0009$, Figure 2A). The risk of cGvHD was also increased in patients who received PBSC transplantation as compared with BM transplantation ($P = 0.004$, Figure 2A). We did not find any association between the CD34+ cell dose or the conditioning regimen and the incidence of cGvHD (data not shown).

To assess the influence of NK reconstitution on subsequent occurrence of cGvHD, we separated the patients in two groups according to their NK cell subset counts at M3 below or above the median of the entire cohort. Figure 2B shows a side-by-side comparison of these groups in terms of cumulative incidence of cGvHD. The occurrence of cGvHD was less frequent among patients with total NK, CD56^{dim} and, to a lesser extend, CD56^{bright} NK cell counts above the median at M3 ($P = 0.0005$, $P = 0.0002$ and $P = 0.047$, respectively). A multivariate analysis using the Fine-Gray regression model confirmed that the occurrence of aGvHD grades 2-4, the source of stem cells and the total NK cell count at M3 were all independent risk factors for cGvHD ($P = 0.018$, $P = 0.013$ and $P = 0.012$, respectively). The relative risk of cGvHD associated with NK cell count below the median (<111.7/µL) at M3 was 1.8,
while the relative risk of cGvHD associated with previous occurrence of aGvHD was 1.7.

In a combinatorial analysis presented in Figure 2C, we showed that these three factors had a cumulative impact on the incidence of cGvHD. The group of patients with the lowest risk of cGvHD, far below any other group of patients, combined an above-the-median NK cell count at M3, a BM transplantation and absence or low grade aGvHD.

**NK cell count is a reliable risk stratification tool of treatment-related mortality (TRM)**

In our cohort of 439 patients, the cumulative incidence of TRM was significantly higher in patients with grades 2-4 aGvHD versus grades 0,1 \( (P = 0.0088) \). To assess the influence of NK cell counts at M3 on TRM, we distinguished patients with total NK cell count below and above the median. We observed no significant difference between the two groups (data not shown). Nonetheless, as reported in Figure 3A, the cumulative incidence of TRM was much higher in the group of patients with total NK cell counts in the lower quartile \( (P = 0.0005) \), and this was observed regardless of the aGvHD grade (Figure 3B, C). No significant difference was observed between all three other quartiles. This result was supported by a multivariate analysis taking into account total NK cell count at M3, the source of stem cells, the conditioning regimen and the occurrence of aGvHD. Patients with NK cell counts at M3 in the lower quartile had a three times greater risk of TRM than all the others \( (P = 0.0003, \text{Table } 2) \). The same result was observed concerning CD56\text{dim} \( (P = 0.00053) \) but not CD56\text{bright} NK cells \( (NS) \).

**CMV reactivations and NK cell reconstitution**

248 patients were CMV-seropositive before transplantation and 61.5% of them developed CMV reactivation before M3. We assessed whether NK cell count at M3 was associated with previous CMV reactivation. Our data showed that total NK cells and CD56\text{dim} NK cell counts were decreased in patients with previous CMV reactivation \( (P = 0.01 \text{ and } P = 0.036, \text{respectively}) \), whereas CD56\text{bright} NK cell count was not significantly different. The difference was even more significant when considering NKG2C+ subsets whereas the counts of NK cells expressing NKG2A were similar regardless of CMV reactivation status (Figure 4A). CMV-seropositive
recipients (R+) who did not develop CMV reactivation before M3 had higher counts of NKG2C+ NK cells ($P = 0.004$), whereas reactivating patients displayed similar low levels than seronegative recipients (R-) transplanted with seronegative donors (D-) at M3 (Figure 4B). In case of CMV reactivation, NKG2C+ NK cell count increased rapidly and reached higher values at M12 than without CMV reactivation. The kinetics of NKG2C+ CD56$^{dim}$ NK cells was then evaluated according to CMV reactivation and to the serological status of donor/recipient pairs. In the absence of CMV reactivation (i.e. viral load below 1000 copies/mL), CMV-seropositive patients who received an allo-HSCT from seropositive donors (D+/R+) had higher counts of NKG2C+ CD56$^{dim}$ NK cells at M3 than reactivating patients ($P = 0.003$). Strikingly, NKG2C+ CD56$^{dim}$ NK cell count was significantly higher at M3 in D+/R+ than in D-/R+ patients who did not reactivate CMV ($P = 0.03$) (Figure 4C). NKG2C+ NK cell count remained low and stable in the absence of CMV reactivation before M3 in D-/R+ patients. Similarly to total NKG2C+ NK cells, the NKG2C+ CD56$^{dim}$ NK subset markedly increased after CMV reactivation, and during 12 months of follow up.

Finally, being aware of the known increased risk of CMV reactivation after a previous one, and to get insight into whether NK cell count at M3 might be predictive of subsequent CMV reactivations, we separated patients in two other groups depending on the occurrence of CMV reactivations between M3 and M6. Our results showed that NKG2C+ total and CD56$^{dim}$ NK cells were significantly increased at M3 in patients who did not experience any subsequent reactivation ($P = 0.011$ and 0.007, respectively, Supplementary Figure 2A to C).

T-cell-mediated immunity represents an essential host factor in the control of CMV reactivation. We found a strong correlation between CD56$^{dim}$ NK cell and total CD8+ T-cell counts, at M3 and afterwards, only in case of reactivation. This correlation reached a higher significance with activated CD25+CD8+ T-cells, ($P < 0.0001$) (Supplementary Figure 3), and to a lesser extend with terminally differentiated memory CD8+ T-cells ($P = 0.01$, data not shown). These data suggest that CMV may drive the magnitude and the shape of both innate and adaptive immunity.

**DISCUSSION**

The first few months following allo-HSCT remain hampered by the risk of aGvHD and infections. In particular, the possibility of reactivation of latent viruses such as EBV$^{24}$
and CMV\textsuperscript{25} has been associated with the long-lasting immunodeficiency that follows transplantation. In this context, NK cells are the first lymphoid subset to replenish and are quantitatively normalized in 1-2 months after HSCT\textsuperscript{26}, so their role is likely to be predominant in early outcome of HSCT. We have previously reported that NK cell frequency in lymphocytes – but not their absolute number – is increased in HSCT recipients during the first 3 months after transplant, compared with HSCT donors\textsuperscript{3}. In this monocentric longitudinal study, we analyzed the association between NK subsets reconstitution and allo-HSCT outcome in the largest cohort of non-T-cell depleted hematopoietic transplantation studied so far to our best knowledge.

These data are consistent with our previous report on 43 HLA-matched non-T-cell-depleted HSCT, showing that early post-transplant period is dominated by CD56\textsuperscript{bright} NK cells\textsuperscript{3}. Here, we clearly demonstrate that CD56\textsuperscript{bright} NK cell count depended on the conditioning regimen and was higher among patients who received MAC. This result may be explained by a higher availability of the bone marrow, allowing their primary expansion within a poor cellular environment. We also show that persistently low CD56\textsuperscript{dim} NK cell counts were associated with the occurrence of grade 2-4 aGvHD. As the CD56\textsuperscript{bright} subset was not affected, we observed therefore a profound defect in CD56\textsuperscript{dim} NK cells during aGvHD, which mechanism remains to be elucidated. In addition, low total NK cell count at M3 was associated with a higher risk of cGvHD and constituted another independent risk factor, together with the source of stem cells and aGvHD\textsuperscript{27-29}. The favorable impact of NK cells on GvHD has been extensively reported both in mice and humans receiving mismatched hematopoietic transplant or allo-reactive infused NK cells \textsuperscript{2, 30}. NK cells are assumed to be protective from GvHD by inhibiting alloreactive donor T-cells and to support GvL effect in mouse models \textsuperscript{31, 32}. In a contingent of 43 patients, Chang and colleagues have suggested that NK cell count can be used to assert the clinical outcomes after unmanipulated haploidentical transplantation \textsuperscript{33}. Only few studies have investigated the role of NK cells in HLA matched allo-HSCT \textsuperscript{34}. The dose of infused donor-derived NK cells was found associated with a lower incidence of cGvHD\textsuperscript{35}. Low total NK cell count was detected at M3, M6 and M12 after graft in patients with extensive cGvHD, but due to a limited cohort of patients, multivariate analysis especially taking into account previous aGvHD could not be performed\textsuperscript{36}.

Furthermore, we show here that low NK cell count at M3 was strongly associated with an increased risk of TRM, which is mainly due to cGvHD and infections. This
relationship was not linear as only patients with NK cell count in the bottom quartile (< 62.8/mL) displayed a three-fold increased risk of TRM.

NK cells provide a first line of defense against several types of viruses including CMV. Although these cells have traditionally been classified as cells of the innate immune system, compelling evidences indicate that they share many similarities with cytotoxic T lymphocytes. Consistent studies have pointed that CMV stimulates the expansion of a specific CD56^{dim} NKG2C+ NK cell subset in different settings. Levels of NK cells expressing NKG2C were increased in patients experiencing acute CMV infection after solid organ transplantation\textsuperscript{37,38} and expansion of potent NKG2C+ NK cells was evidenced in CMV-reactivating recipients from allo-HSCT or unrelated CMV naïve cord blood grafts\textsuperscript{39-41}. In addition, the recovery of NK cell function has been correlated with protection against CMV\textsuperscript{42}. Although these studies were conducted on relatively small cohorts, the results indicate that the reconstitution of NK cells post allo-HSCT may be decisive in the early control of CMV reactivation. NK cells from mice challenged with murine CMV undergo all four phases of an immune response against a pathogen: clonal-like expansion, involving the Ly49H\textsuperscript{+} NK cell subset, contraction and then memory-like phase, with heightened responses to rechallenge by the same antigen\textsuperscript{43}. Similarly, human CMV has been reported to induce a persistent reconfiguration of the NK-cell compartment, with the expansion NK cells bearing the NKG2C receptor, in healthy individuals as well as in patients with different pathologies\textsuperscript{13,44,45}.

This expansion of activated and differentiated NKG2C+ NK cells was recently described upon additional encounters with viruses, such as Hantavirus, HIV, hepatitis B virus and hepatitis C virus\textsuperscript{46-48}. However, the mechanism by which CMV drives NKG2C\textsuperscript{+} NK cells is unknown, and the ligand for NKG2C remains elusive. In our study, patients who reactivated CMV before M3 had significantly fewer CD56^{dim} NKG2C\textsuperscript{+} NK cells at M3 but their counts significantly increased afterwards. Strikingly, in the absence of CMV reactivation (or in case of viral load below 1000 copies/mL), this NK subset was at higher levels in seropositive patients who received HSCT from seropositive donors (R+/D+) than in D-/R+ and D-/R- patients. Such observation may be related to the fact that CMV viral loads below the therapeutic threshold are sufficient to provide low levels of chronic stimulation in D+/R+ recipients, allowing the expansion of transplantable NKG2C\textsuperscript{+} NK cells with memory-like properties, as
previously suggested\textsuperscript{40}. Therefore, we hypothesize that a threshold level of NKG2C\textsuperscript{+} NK cells above normal values in the first 3 months after allo-HSCT may protect against CMV reactivation. In turn, in case of CMV reactivation, the proliferation of NKG2C\textsuperscript{+} NK cells is boosted.

To our knowledge, this is the first study analyzing the reconstitution of CD56\textsuperscript{bright}, CD56\textsuperscript{dim}, and total NKG2C\textsuperscript{+} NK cells with regard to acute and chronic GvHD, CMV reactivation and TRM in allo-HSCT settings. Our data highlight the favorable role of an early efficient NK cell reconstitution after allo-HSCT on clinical outcome. Determination of NK cell subset count is a simple flow cytometry analysis feasible in a routine basis to monitor post-transplant immune reconstitution. We suggest its use to help in stratifying the risk of cGvHD, TRM and CMV reactivation.
ACKNOWLEDGEMENTS

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AUTHORSHIP AND DISCLOSURES

VDK processed clinical samples, analyzed the data, and wrote the paper; RPL and GS provided clinical samples and critically reviewed the paper; MB analyzed the data and performed statistical analysis; CS performed and interpreted virological analysis; GM processed clinical samples and critically reviewed the paper; PH processed clinical samples; MC processed clinical samples; AX, MR, and PR provided clinical samples; DC critically reviewed the paper; AT and HMT designed the study, analyzed the data, and wrote the paper.

The authors report no potential conflicts of interest.

REFERENCES


### TABLES

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<th>Parameter</th>
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<th>(P^{\text{CD56}^{\text{dim}}})</th>
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* \(\geq\) or < 25

**Table 1. Multivariate analysis of variance (ANOVA test) of parameters influencing NK cell subset counts at M3.**

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*< 1st quartile versus \(\geq\) 1st quartile

**Table 2. Multivariate analysis (Fine and Gray’s competing risk model) of risk factors of Treatment-Related Mortality.**
FIGURE LEGENDS

**Figure 1. Kinetics of NK cell subset reconstitution after allo-HSCT.** The median absolute counts of NK cell subsets (cells/µL) are shown at M3, M6, M12 and M24. (A) CD56\textsuperscript{bright} NK cells. (B) CD56\textsuperscript{dim} NK cells. (C) Total NK cells. *P* < 0.05; **P* ≤ 0.01; ***P* ≤ 0.001.

**Figure 2. Risk factors for cGvHD.** Cumulative incidence of cGvHD is displayed (A) according to the severity of aGvHD and the source of stem cells, and (B) according to absolute counts of NK cell subsets at M3. (C) Combinatorial analysis of the risk factors for cGvHD in patients transplanted with peripheral blood stem cells (left panel) and with bone marrow (right panel).

**Figure 3. NK cell counts at M3 and Treatment-Related Mortality (TRM).** Cumulative incidence of TRM is displayed according to absolute counts of total NK cells at M3 (lower quartile Q1 = 62.8 /µL) in the entire cohort (A), in patients who underwent acute GVHD grades 0-1 only (B), and in patients who underwent acute GVHD grades 2-4 only (C).

**Figure 4. Reconstitution kinetics of NKG2C+ NK cells.** (A) and (B) Counts of total NKG2A+ and NKG2C+ NK cells (cells/µL) (respectively) are shown at M3, M6 and M12, according to the occurrence of CMV reactivation between M0 and M3. (C) Counts of NKG2C+ CD56\textsuperscript{dim} NK cells (cells/µL) are shown according to the serological status of donor/recipient pairs and CMV reactivation. “D-/R-” stands for CMV-seronegative recipients, transplanted from CMV-seronegative donors with no evidence of CMV primo-infection. Grey area indicates the interquartile range in a blood donor population (n = 45).
Figure 4

A

B

C

- R+, with M0-M3 CMV reactivation
- R+/D+, without M0-M3 CMV reactivation
- R+/D-, without M0-M3 CMV reactivation
- D-/R-
SUPPLEMENTARY MATERIALS AND METHODS

Patient characteristics and blood samples
Unrelated donors were considered HLA matched if compatible at the allelic level for HLA-A, -B, -C, -DRB1, and -DQB1 loci. Prior to transplantation, serum samples from recipients and donors were analyzed by ELISA for CMV-specific IgG antibodies. EDTA-treated blood samples were collected at months M3, M6, M12 and M24 after transplantation for flow cytometric analysis. NKG2C and NKG2A staining on NK cell subsets were implemented in our immunophenotyping workflow from 2009 and relates to the last 207 patients.

Flow cytometry and monoclonal antibodies
All reagents were obtained from BD Biosciences, Beckman Coulter (Villepinte, France), or Miltenyi Biotec (Paris, France). The following mouse monoclonal antibodies (mAbs) were used: anti-CD45-FITC, -NKG2C-PE, -NKG2A-APC, -CD8-PerCP, -CD56-PE-Cy7, -CD16-APC-H7, -CD3-V450 and -CD4-V500.

Virological monitoring
From 2005 to 2009, the clinical samples were tested with an in-house real-time PCR assay. Then, quantification of CMV was carried out with two successive fully automated real time PCR assays on the same m2000 RealTime platform (Abbott Molecular Inc., Des Plaines, IL, USA), the IVD/CE-labeled CMV ABI Prism SDS assay (Qiagen/Artus GmbH, Hilde, Germany) between 2009 and 2012 and the IVD/CE-labeled Abbott RealTime CMV assay, from may 2012. The lower limit of quantification was 200 copies/mL and 40 copies/mL for the last real-time CMV assay.

Definition of the main clinical outcomes
All patients received cyclosporine as GvHD prophylaxis, with either methotrexate or mycophenolate mofetil. All patients were considered at risk for aGvHD as of day +1 after transplant. Occurrence of cGvHD was evaluated among patients who survived with sustained engraftment from day +100 after transplant.
**Statistical analyses**

Differences in categorical variables between groups were evaluated by Chi-square or Fisher test. Comparisons of absolute median values of total NK and subsets at M3, M6, M12, and M24 among groups of risk factors were performed with non-parametric Kruskal-Wallis test.

Log10 values of total NK and subsets were used to calculate multivariate ANOVA in order to evaluate risk factors influencing total NK and subsets reconstitution at M3.

In order to evaluate their potential impact on TRM, cGvHD and CMV reactivation, four groups based on the 25th, 50th and 75th percentiles of total NK and subsets at M3 were used. Cumulative incidence of TRM and cGvHD were estimated for these 4 groups, considering relapse (for TRM) and death (for cGvHD) as competing events.

Table S1. Patient characteristics.

**Figure S1. NK cells gating strategy.**
Populations and the relationship between them are displayed in the population hierarchy. NK cells were defined as CD45+ CD3- and CD16 or CD56+ lymphocytes.

**Figure S2. NKG2C+ NK subset counts at M3 in accordance with CMV reactivations between M3 and M6.** (A) NKG2C+ CD56$^{\text{bright}}$ NK cells. (B) NKG2C+ CD56$^{\text{dim}}$ NK cells. (C) NKG2C+ total NK cells.

**Figure S3. Correlation between CD56$^{\text{dim}}$ NK and CD25+ activated CD8+ T cells at M3 in case of CMV reactivation**
## Supplementary Table S1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number (Percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>439 (100.0%)</td>
</tr>
<tr>
<td><strong>Donor matching and relation to patient</strong></td>
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</tr>
<tr>
<td>HLA identical siblings</td>
<td>237 (54.0%)</td>
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<tr>
<td>Matched unrelated*</td>
<td>149 (33.9%)</td>
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<tr>
<td>Mismatched unrelated†</td>
<td>53 (12.1%)</td>
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<tr>
<td><strong>Source of stem cells</strong></td>
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</tr>
<tr>
<td>Peripheral blood</td>
<td>336 (76.5%)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>103 (23.5%)</td>
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<tr>
<td><strong>Conditioning regimen</strong></td>
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</tr>
<tr>
<td>Total Body Irradiation</td>
<td>161 (36.7%)</td>
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<tr>
<td>Reduced Intensity Conditioning</td>
<td>266 (60.6%)</td>
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<tr>
<td>Myeloablative conditioning</td>
<td>173 (39.4%)</td>
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<tr>
<td><strong>Sex matching (D/R)</strong></td>
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<tr>
<td>Female/male</td>
<td>97 (22.1%)</td>
</tr>
<tr>
<td>Female/female</td>
<td>87 (19.8%)</td>
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<tr>
<td>Male/female</td>
<td>96 (21.9)</td>
</tr>
<tr>
<td>Male/male</td>
<td>157 (35.8%)</td>
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<tr>
<td><strong>Age, medians (min-max)</strong></td>
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<tr>
<td>Recipients</td>
<td>44.0 (15.0-68.0)</td>
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<tr>
<td>Donors</td>
<td>35.9 (10.4-67.7)</td>
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<td><strong>Diagnosis</strong></td>
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<tr>
<td>Acute lymphoblastic leukemia</td>
<td>57 (13.0%)</td>
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<tr>
<td>Non-Hodgkin lymphoma</td>
<td>49 (11.2%)</td>
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<tr>
<td>Hodgkin disease</td>
<td>19 (4.3%)</td>
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<tr>
<td>Myeloma</td>
<td>42 (9.6%)</td>
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<tr>
<td>Acute myeloid leukemia</td>
<td>104 (23.7%)</td>
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<tr>
<td>Myeloproliferative neoplasia</td>
<td>40 (9.1%)</td>
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<tr>
<td>Myelodysplastic syndrome</td>
<td>52 (11.8%)</td>
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<tr>
<td>Other hematological malignancies</td>
<td>37 (8.4%)</td>
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<tr>
<td>Non hematological malignancies</td>
<td>39 (8.9%)</td>
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<tr>
<td><strong>Matching CMV status</strong></td>
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</tr>
<tr>
<td>D+R+</td>
<td>148 (33.7%)</td>
</tr>
<tr>
<td>D-R+</td>
<td>100 (22.8%)</td>
</tr>
<tr>
<td>D+R-</td>
<td>62 (14.1%)</td>
</tr>
<tr>
<td>D-R-</td>
<td>128 (29.1%)</td>
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<tr>
<td><strong>Acute GvHD</strong></td>
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<tr>
<td>Grades 0, 1</td>
<td>221 (51.9%)</td>
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<tr>
<td>Grades 2, 3, 4</td>
<td>205 (48.1%)</td>
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<tr>
<td><strong>Chronic GvHD</strong></td>
<td>240 (54.7%)</td>
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<tr>
<td><strong>Relapse</strong></td>
<td>89 (22.3%)</td>
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<td><strong>Survival status</strong></td>
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<tr>
<td>Alive</td>
<td>320 (75.1%)</td>
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<tr>
<td>Dead</td>
<td>106 (24.1%)</td>
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<tr>
<td><strong>Number of patients during follow up</strong></td>
<td>M3</td>
</tr>
<tr>
<td></td>
<td>439</td>
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</tbody>
</table>

* 10/10 HLA-A, B, C, DR, DQ allelic match
† 8/10 or 9/10 HLA-A, B, C, DR, DQ allelic match for the 2005-9 and 9/10 for the 2009-10 period
Supplementary Figure S2

A

CMV reactivation M3-M6

\[ P = 0.13 \]

B

CMV reactivation M3-M6

\[ P = 0.007 \]

C

CMV reactivation M3-M6

\[ P = 0.011 \]
Supplementary Figure S3

R = 0.55
P < 0.0001