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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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[1]. Then, quantification of CMV was carried out with two successive fully automated real time PCR assays[2] on the same *m*2000 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.

1. Gouarin S, Vabret A, Scieux C, Agbalika F, Cherot J, Mengelle C, et al. Multicentric evaluation of a new commercial cytomegalovirus real-time PCR quantitation assay. Journal of virological methods. 2007 Dec;146(1-2):147-54.

2. Schnepf N, Scieux C, Resche-Riggon M, Feghoul L, Xhaard A, Gallien S, et al. Fully automated quantification of cytomegalovirus (CMV) in whole blood with the new sensitive Abbott RealTime CMV assay in the era of the CMV international standard. Journal of clinical microbiology. 2013 Jul;51(7):2096-102.

SUPPLEMENTARY DISPLAY ITEMS

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+ $CD56^{bright}$ NK cells. (B) NKG2C+ $CD56^{dim}$ NK cells. (C) NKG2C+ total NK cells.

Figure S3. Correlation between $CD56^{dim}$ NK and CD25+ activated CD8+ T cells at M3 in case of CMV reactivation

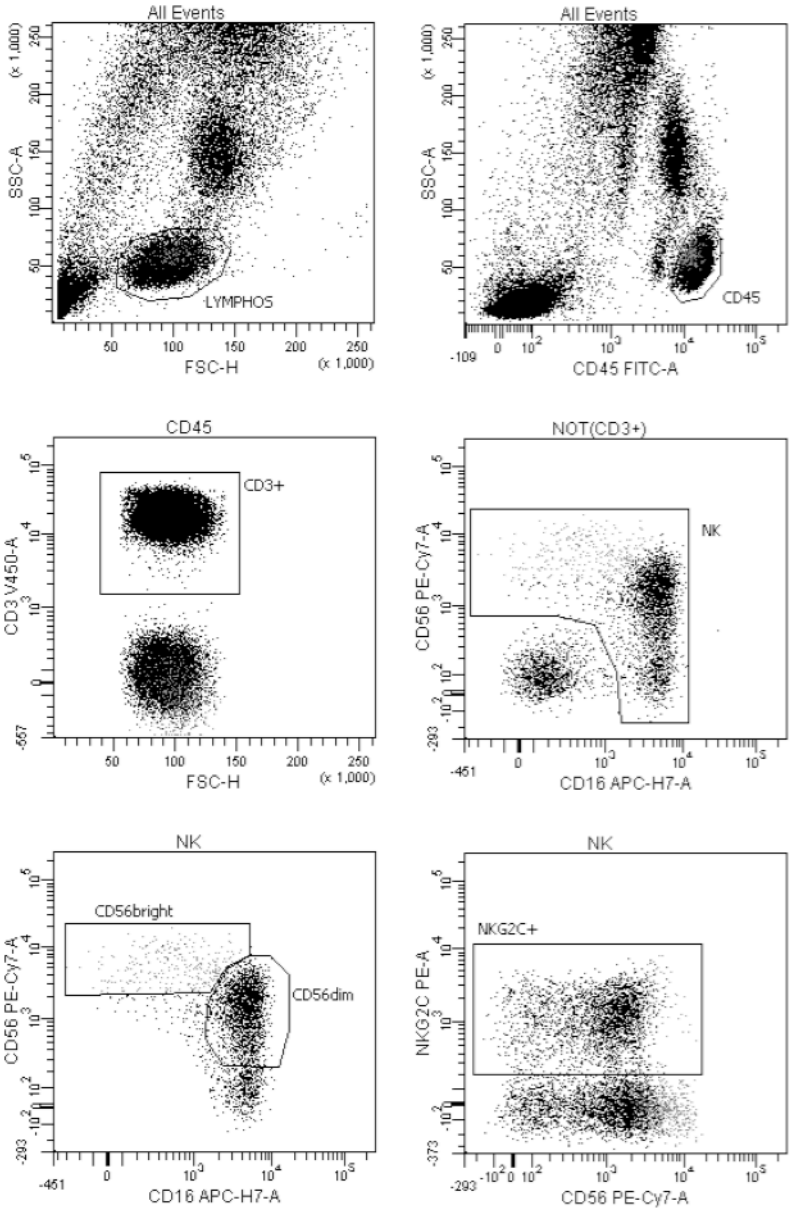
Supplementary Table S1. Patient characteristics

Characteristic	Number (Percentage)			
N	439 (100.0%)			
Donor matching and relation to patient				
HLA identical siblings	237 (54.0%)			
Matched unrelated*	149 (33.9%)			
Mismatched unrelated†	53 (12.1%)			
Source of stem cells				
Peripheral blood	336(76.5%)			
Bone marrow	103 (23.5%)			
Conditioning regimen				
Total Body Irradiation	161 (36.7%)			
Reduced Intensity Conditioning	266 (60.6%)			
Myeloablative conditioning	173 (39.4%)			
Sex matching (D/R)				
Female/male	97 (22.1%)			
Female/female	87 (19.8%)			
Male/female	96 (21.9)			
Male/male	157 (35.8%)			
Age, medians (min-max)				
Recipients	44.0 (15.0-68.0)			
Donors	35.9 (10.4-67.7)			
Diagnosis				
Acute lymphoblastic leukemia	57 (13.0%)			
Non-Hodgkin lymphoma	49 (11.2%)			
Hodgkin disease	19 (4.3%)			
Myeloma	42 (9.6%)			
Acute myeloid leukemia	104 (23.7%)			
Myeloproliferative neoplasia	40 (9.1%)			
Myelodysplastic syndrome	52 (11.8%)			
Other hematological malignancies	37 (8.4%)			
Non hematological malignancies	39 (8.9%)			
Matching CMV status				
D+R+	148 (33.7%)			
D-R+	100 (22.8%)			
D+R-	62 (14.1%)			
D-R-	128 (29.1%)			
Acute GvHD				
Grades 0, 1	221 (51.9%)			
Grades 2, 3, 4	205 (48.1%)			
Chronic GvHD	240 (54.7%)			
Relapse	89 (22.3%)			
Survival status				
Alive	320 (75.1%)			
Dead	106 (24.1%)			
Number of patients during follow up	M3	M6	M12	M24
	439	376	265	249

* 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 S1



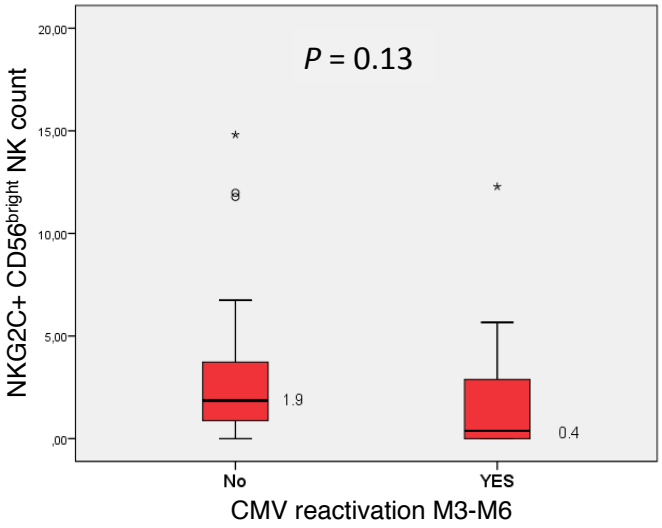
Tube: NK gating

Population

- All Events
  - LYMPHOS
    - CD45
      - CD3+
        - NOT(CD3+)
          - NK
            - CD56dim
            - CD56bright
            - NKG2C+

Supplementary Figure S2

A



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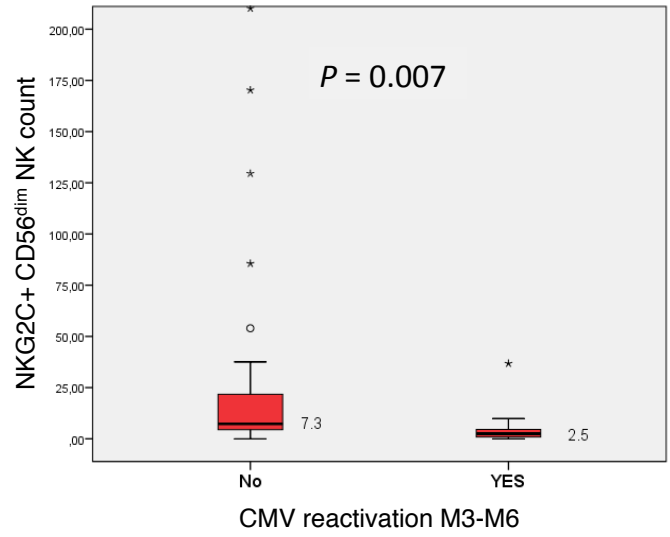
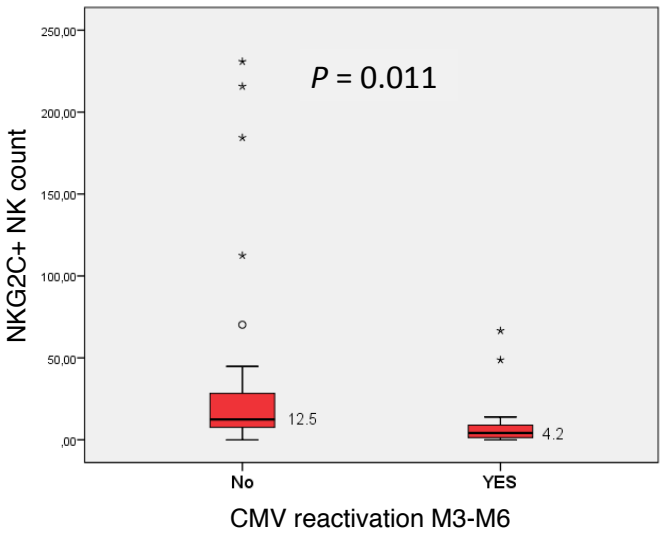


C



Supplementary Figure S3