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Impact of KIR/HLA Incompatibilities on NK Cell Reconstitution and Clinical Outcome after T Cell–Replete Haploidentical Hematopoietic Stem Cell Transplantation with Posttransplant Cyclophosphamide

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Little is known regarding the effect of KIR/HLA incompatibilities (inc.) in the setting of T-replete haploidentical allogeneic hematopoietic stem cell transplantation using posttransplant cyclophosphamide (PTCy). In this retrospective study, the impact of KIR/HLA inc. on clinical outcomes and NK cell reconstitution was studied in a cohort of 51 consecutive patients receiving a T cell–replete haploidentical allogeneic hematopoietic stem cell transplantation after a reduced-intensity conditioning using peripheral blood stem cells as the source of the graft and PTCy as graft-versus-host disease (GvHD) prophylaxis. The NK cell repertoire reconstitution was examined by multiparameter flow cytometry in 34 of these 51 patients from day 0 to day 100 posttransplant. Genetic KIR2DL/HLA inc. were found to be significantly associated with more GvHD (81.2 versus 45.7%, $p = 0.01$) and less relapse (6.2 versus 42.8%, $p = 0.008$) in this context. GvHD is associated with increased levels of differentiated and activated NK cells. A significant loss of KIR2DL2/3$^+$ NK cells was observed at day 30 in patients with inhibitory KIR/HLA inc., suggesting that responsive KIR NK cells are particularly targeted by the immunosuppressive PTCy treatment. Further investigations are needed from a larger cohort with an identical clinical approach to consolidate these results and to identify the NK cell subsets that may be beneficial for the graft-versus-leukemia effect observed. Because many haploidentical donors can be identified in a family, the prediction of KIR NK cell alloreactivity could be of crucial importance for donor selection and patient outcome. ***The Journal of Immunology*****, 2019, 202: 2141–2152.**

Allogeneic hematopoietic stem cell transplantation (HSCT) constitutes a potential curative treatment for many hematologic malignancies. In the absence of a matched sibling donor, one of the most important difficulties linked to this therapy is to find a suitable 10/10, unrelated, HLA-identical donor, because of the broad polymorphism of histocompatibility genes. In this context, an HLA-haploidentical family donor is thus the easiest candidate to identify and can be promptly available among parents, offspring, and nonidentical siblings. However, the major limitation of this transplant modality has traditionally been the high risk of graft failure and of severe acute graft-versus-host disease (GvHD) due to alloreactive donor T cells recognizing the mismatched recipient's HLA haplotype (1). In the 1990s, complete T cell depletion by in vitro selection of CD34$^+$ cells from the graft was proved to be effective to prevent GvHD (2). However, this came at the price of an unacceptably high incidence of relapse. Moreover, T cell depletion led to a profound and prolonged posttransplant immunodeficiency and high mortality due to infections (3). It was also in this context of T cell–depleted haploidentical HSCT that Ruggeri et al. (4) showed for the first time the benefit, in terms of less relapse, of NK cell alloreactivity. The killer cell Ig-like receptors (KIR) are mainly expressed on NK cells. These receptors are specific for given HLA allotypes, and this recognition regulates the cytotoxic activity of NK cells. Failure to establish the KIR/HLA link provides NK cells with the ability to destroy damaged

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Abbreviations used in this article: 221, HLA class I–deficient 721.221; CD56^{int}, CD56 intermediate; CI, confidence interval; DFS, disease-free survival; D/R, donor/recipient; GvHD, graft-versus-host disease; GvL, graft-versus-leukemia; HSCT, hematopoietic stem cell transplantation; inc., incompatibility; inh., inhibitory; KIR, killer cell Ig-like receptor; MAC, myeloablative conditioning; MFC, multiparameter flow cytometry; OS, overall survival; PBSC, peripheral blood stem cell; PTCy, posttransplant cyclophosphamide; RIC, reduced-intensity conditioning.

or allogeneic nucleated cells (5). Thus, KIR/HLA incompatibilities (inc.) can contribute to the so-called graft-versus-leukemia (GvL) effect, reducing the risk of relapse after HSCT (6).

For the last few years, transplant physicians have been performing successful HSCT from haploidentical family donors using high doses of posttransplant cyclophosphamide (PTCy) to eliminate in vivo donor effector T cells, which are liable to attack host tissues (7). This approach is very promising, as it provides the opportunity to perform HSCT, when needed, for patients without a 10/10 HLA-identical donor (8).

However, many issues remain to be clarified, among which is the impact of KIR/HLA inc. in this particular setting. Only a few studies have reported such data, especially in the context of a myeloablative conditioning (MAC) regimen and/or using bone marrow as source of graft (9–11). Moreover, reconstitution of the NK repertoire remains largely unknown after haploidentical HSCT.

In this study, the impact of genetic KIR/HLA inc. on clinical outcomes was retrospectively analyzed in a homogeneous cohort of 51 donor/recipient (D/R) pairs. HSCT was performed for all patients after reduced-intensity conditioning (RIC) with T cell–replete haploidentical peripheral blood stem cells (PBSC). PTCy was administered as GvHD prophylaxis. For 34 of these D/R pairs, the reconstitution kinetics of the NK cell repertoire was thoroughly investigated posttransplant.

Materials and Methods

Study population

This prospective study, conducted between November 2013 and May 2017, enrolled 51 consecutive patients (males: $n = 31$, median age = 57 y [range: 29–70 y]) allografted in the Hematology Department of Nantes University Hospital (Table I). There was a majority of patients with myeloid diseases ($n = 31$) and in complete remission at transplant ($n = 31$). The disease risk index (12) was low, intermediate, and high in 8, 30, and 13 cases, respectively. Nine patients had been previously allografted. A Baltimore conditioning regimen (7) was applied for 25 patients (19 cases with lymphoid diseases, 1 myelofibrosis, 3 acute myeloid leukemias, 1 myelodysplastic syndrome, and 1 blastic plasmacytoid dendritic cells neoplasm). For the remaining 26 patients, conditioning was performed with a Clo-Baltimore regimen (13), in which fludarabine, part of the Baltimore regimen, is replaced by clofarabine. All these patients but one had a myeloid disease, the exception being one case of T-acute lymphoblastic leukemia. The source of graft was PBSC for all cases. As GvHD prophylaxis, and according to the Baltimore regimen, all patients received PTCy (50 mg/kg/d) on days +3 and +4, then cyclosporine A and mycophenolate mofetyl from day +5. Acute and chronic GvHD were classified according to standard criteria (14, 15). The study's involvement with human subjects complies with the Declaration of Helsinki. Healthy volunteers were recruited at the Blood Transfusion Center (Etablissement Français du Sang, Nantes, France), and they gave informed written consent in accordance with the Declaration of Helsinki.

All patients provided informed consent for collecting their own data from the PROMISE database of the EBMT. In addition, declaration of the preparation and conservation of a biocollection (DC-2014-2340) had been submitted to the French Ministry of Research and had received agreement from the Institutional Review Board (2015- DC-1) in 2015 to study NK cell reconstitution posttransplant. This biologic study was approved by the Ethics Review Board of the Nantes University Hospital, and all patients and donors included provided informed consent. The outcomes of some patients have been already reported previously (16).

HLA and KIR genotyping

High-resolution typing for HLA-A, -B, and -C loci was carried out for all donor and recipient samples by next-generation sequencing using Omixon Holotype HLA (Omixon, Budapest, Hungary) (17). Briefly, DNA samples were amplified for the three HLA class I loci (*HLA-A*, *HLA-B*, and *HLA-C*) by long-range PCR. Amplicons were then cleaned with Exo-SAP (Affymetrix, Santa Clara, CA), quantified with the QuantiFluor kit (Promega, Madison, WI), and normalized. Sequencing libraries were generated for each sample using the Omixon Holotype HLA Genotyping Kit. Libraries from individual HLA amplicons were prepared by enzymatic fragmentation, end-repaired, adenylated, and ligated with indexed adaptors. The indexed libraries were pooled and concentrated with Ampure XP beads (Beckman Coulter, La Brea, CA) before fragment size selection using PippinPrep (Sage Science, Beverly, MA). The size-selected library pool was quantified by quantitative PCR (Kapa Biosystems, Basel, Switzerland), adjusted to 2 nM, denatured, and diluted for optimal cluster density before loading into a MiSeq reagent cartridge (Illumina, San Diego, CA). The reagent cartridge and corresponding flow cell (500 cycles) were placed on an Illumina MiSeq. Samples were demultiplexed, and the resulting FASTQ files were analyzed using Twin Omixon software (Omixon). Besides HLA class I typing, KIR generic typing was performed on all donor samples using a KIR multiplex PCR–Sequence Specific Primer method as previously described (18), using primers provided by Prof. D. Senitzer (City of Hope, Duarte, CA). The presence or absence of KIR2DL1, 2DL2, 2DL3, 2DL5, 3DL1, 2DS1, 2DS2, 2DS3, 2DS4/1D, 2DS5, and 3DS1 genes was assigned. Donor KIR genotypes were then determined based on the presence or the absence of activating KIR. Thus, a KIR AA genotype was defined by the presence of only KIR2DS4 as an activating KIR gene and a KIR B+ genotype by the presence of several activating KIR genes (19). KIR ligands such as C1, C2, and Bw4 were defined based on allelic HLA class I typing.

Immunophenotypic analysis by flow cytometry

The immunophenotype of NK cells was determined by eight-color multiparameter flow cytometry (MFC) on graft aliquots and patient samples using the following mAbs: anti-CD56-allophycocyanin-Cy7 (HCD56), anti-CD3-BV510 (SK7; Sony Biotechnology, San Jose, CA), anti-CD57-FITC (HNK-1), anti-2B4-FITC (2-69), anti-NKp46 (9E2; BD Biosciences, San Jose, CA), anti-NKG2C-PE (134591; R&D Systems, Minneapolis, MN), anti-NKG2D-PerCP-Cy5.5 (1D11), anti-NKp44-PC7 (P44-8; Sony Biotechnology), anti-NKG2A-PC7 (Z199), anti-NKp30-PE (Z25), anti-KIR2DL2/3/2DS2 (GL183), anti-KIR2DL1/2DS1 (EB6), anti-KIR2DS4 (FES172), anti-KIR3DL1/3DS1 (Z27; Beckman Coulter Immunotech, Marseilles, France), and anti-DNAM-1-PerCP-Cy5.5 (11A8; BioLegend, San Diego, CA). MFC data were collected on a FACSCanto II instrument (BD Biosciences) and analyzed with FlowJo 10.2 software (Ashland, OR).

CD107a mobilization assay

The cytolytic potential of NK cells was evaluated at the median time of 25 d (range: 20–33 d) posttransplant for patients developing GvHD ($n = 9$), on day 30 for patients without GvHD ($n = 18$), and on day 100 for 18 patients. This was achieved by a CD107a mobilization assay after stimulation with the HLA class I–deficient 721.221 (221), acute leukemia H9, and acute myeloid KG1 cell lines, kindly provided by Dr. N. Dulphy (INSERM U1160, Paris, France). Briefly, NK cells were preincubated with CD107-BV421 Ab (H4A3; BD Biosciences) and then incubated with 221 target cells for 5 h at an E:T ratio of 1:1, together with brefeldin A (Sigma-Aldrich, Saint Louis, MO) at 10 μg/ml for the last 4 h to block secretion and allow anti-CD107 binding. The cells were surface-stained with the following mouse anti-human mAbs: anti-KIR2DL1/S1-FITC (EB6), anti-KIR2DL2/3/2DS2-PE (GL183), anti-DNAM-1-PerCP-Cy5.5 (11A8; BioLegend), anti-NKG2A-PC7 (Z199), anti-KIR3DL1/3DS1 (Z27; Beckman Coulter Immunotech), CD56-allophycocyanin-Cy7 (HCD56), and anti-CD3-BV510 (SK7; Sony Biotechnology). Degranulation of NK cells, defined by $CD107a^+$ expression, was studied by MFC, using CD3 absence and CD56 expression to target NK cells ($CD3^-$ $CD56^+$). All functional MFC data were collected on a FACSCanto II instrument (BD Biosciences) and analyzed with FlowJo 10.2 software.

Statistical analyses

Overall survival (OS) was defined as the time from day 0 of allogeneic HSCT to death or last follow-up for survivors. Disease-free survival (DFS) was defined as the time from day 0 of allogeneic HSCT to time without evidence of relapse or disease progression, censored at the date of death or last follow-up. Relapse was defined as any event related to reoccurrence of the disease. Categorical data were analyzed by χ^2 test, and univariate comparisons were performed by Student t test. Comparisons of multiple groups were performed by one-way ANOVA using GraphPad Prism v6.0 software (San Diego, CA). The p values <0.05 were considered statistically significant.

Results

Clinical outcomes

All patients were engrafted (Table I). Twenty-nine (57%) and eight (16%) patients developed grade 2–4 and 3–4 acute GvHD, respectively, at a median time of 29 d (range: 17–121 d) posttransplant (Table II). Ten patients (20%) developed moderate or severe chronic

Table I. Characteristics of patients

	All Patients (n = 51)	inh. KIR2D/HLA inc. (n = 22)	No inh. KIR/HLA inc. (n = 29)	p Value
Gender: male	31 (61%)	16 (72.7%)	15 (51.7%)	0.12
Median age, y (range)	57 (29–70)	57.5 (33–70)	55 (29–70)	0.45
Disease				
AML/MDS	18/9	9/1	9/8	
ALL	4	2	2	
HD	3	3	0	
NHL	10	4	6	
CLL	2		2	
Myelofibrosis	2		2	
pDCs neoplasm	1		1	
CML	1		1	
Mycosis fungoides	1			
Myeloid/lymphoid	31 (61%)/20 (39%)	12 (54.5%)/10 (45.5%)	19 (65.5%)/10 (34.5%)	0.56
Status at treatment				0.71
CR1/CR2/CR3	19/10/2 (61%)	8/4/1 (58%)	13/6/1 (66%)	
PR1/PR2/PR3	4/2/4 (19.5%)	0/2/3 (22.7%)	4/0/1 (17.2%)	
Active	10 (19.5%)	4 (6%)	6 (26%)	
Disease risk index				0.23
Low	8 (16%)	4 (18.1%)	4 (13.8%)	
Intermediate	30 (59%)	15 (68.2%)	15 (51.7%)	
High	13 (25%)	3 (13.6%)	10 (34.5%)	
Previous allograft	9 (18%)	5 (22.7%)	4 (13.8%)	0.47
Conditioning				0.4
Baltimore	25 (49%)	9 (44%)	16 (51%)	
Clo-Baltimore	26 (51%)	13 (56%)	13 (49%)	
Haplodonors				
Median age, y	42 (19–71)	42.5 (19–71)	41 (22–71)	0.95
Sister/brother	9/13 (43%)	5/4 (40.9%)	4/9 (44.8%)	0.84
Father/mother	4/2 (12%)	2/1 (13.6%)	2/1 (10.3%)	
Son/daughter	14/5 (37%)	7/2 (40.9%)	7/3 (34.5%)	
Nephew	4 (8%)	1 (4.52%)	3 (10.3%)	
D/R CMV status				0.2
−/−	32 (63%)	12 (54.5%)	14 (48.27%)	
−/+	8 (15%)	4 (18.2%)	4 (13.8%)	
+/−	6 (12%)	5 (22.7%)	1 (3.4%)	
+/+	5 (10%)	1 (4.52%)	4 (13.8%)	
ABO compatibility				
Compatibility	34 (66%)	13 (59.1%)	21 (72.4%)	
Minor inc.	11 (22%)	6 (27.3%)	5 (17.2%)	
Major inc.	6 (12%)	3 (13.6%)	3 (10.3%)	
Donor KIR genotype				0.01
AA	17 (33%)	10 (45.4%)	7 (24.1%)	
AB	31 (61%)	9 (40.9%)	22 (75.8%)	
BB	3 (6%)	3 (13.6%)	0	
Graft composition				
Median $CD34^+$ cells: 10^6/kg	7.88 (2.88–14.16)	7.79 (3.91–14.16)	7.89 (2.88–12.11)	0.86
Median $CD3^+$ T cells: 10^7/kg	24.27 (7.71–66.75)	24.27 (7.71–31.8)	22.87 (13.14–66.75)	0.13
Median $CD45^+$ cells: 10^8/kg	8.76 (4.64–25.36)	8.15 (5.04–16.06)	9.07 (4.64–25.36)	0.33

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CR, complete remission; HD, Hodgkin disease; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; pDC, plasmacytoid dendritic cell; PR, partial remission.

GvHD. At last updating (April 2018), and with a median follow-up of 29.1 mo (range: 12.4–50 mo; one patient alive at 7 mo was lost to follow-up afterward) for alive patients, 16 (31%) have relapsed and 19 (37%) died. The causes of death were relapse in 11 cases, infection in 4, GvHD in 3, and dermatomyositis in 1. Two-year OS and DFS for the whole cohort are 61.6 (95% confidence interval [CI]: 49–76%) and 50.3% (95% CI: 38–66%), respectively.

Comparing Baltimore (fludarabine, n = 25) versus Clo-Baltimore (clofarabine, n = 26) subgroups, no differences were observed in terms of grade 2–4 acute (64 versus 50%, p = 0.46) or moderate/severe chronic (24 versus 15%, p = 0.67) GvHD incidence, relapses (32 versus 31%, p = 1), or deaths (36 versus 38%, p = 1). Similarly, there was no difference in 2-y DFS (68.1 [95% CI: 48–95%] versus 42.6% [95% CI: 29–62%], p = 0.09) or 2-y OS (74.4 [95% CI: 55–99%] versus 56% [95% CI: 47–75%], p = 0.16). Patients with lymphoid or myeloid malignancies also had similar grade 2–4 acute (65 versus 52%, p = 0.51) or moderate/severe chronic (25 versus 16%, p = 0.67) GvHD incidences, relapses (35 versus 29%, p = 0.88), deaths (30 versus 42%, p = 0.57), and 2-y DFS (54.5 [95% CI: 36–81%] versus 45.4% [95% CI: 27–74%], p = 0.93) and OS (68.7 [95% CI: 50–93%] versus 46.5% [95% CI: 28–75%], p = 0.31). However, patients developing grade 2–4 acute GvHD (n = 29) had a lower incidence of relapse compared with other patients (21 versus 45%, p = 0.059). As Baltimore versus Clo-Baltimore patients had similar outcomes (Fig. 1A), they were pooled to study the impact of NK cell alloreactivity posttransplant.

Inhibitory KIR_D/HLA-C_R inc. favors GvHD and protects against relapse in haploidentical RIC PBSC HSCT with PTCy

To investigate the impact of KIR/HLA inc. on clinical outcome after T cell–replete haploidentical RIC PBSC HSCT with PTCy, we determined the HLA and KIR genetic profiles of the 51 D/R pairs included in the study. The C1, C2, and Bw4 KIR ligands, defined from donor and recipient HLA class I typings, are shown in Table III. Inhibitory (inh.) KIR/HLA inc. were defined by the presence of inh. KIR genes (KIR2DL1, KIR2DL2/3, and KIR3DL1)

Table II. Patient outcomes

	All Patients (n = 51)	inh. KIR/HLA inc. (n = 22)	No inh. KIR/HLA inc. (n = 29)	p Value
Follow-up, mo (range)	29.1 (12.4–50)	29.1 (12.4–50)	28.1 (16.7–49)	0.81
Median neutrophils recovery >0.5 Giga/l, d (range)	18 (13–30)	18 (13–30)	20 (13–27)	0.25
Median platelets recovery >50 Giga/l, d (range)	27 (9–104)	26 (9–104)	27.5 (16–99)	0.29
Acute GvHD	29 (57%)	13 (59%)	13 (44.8%)	0.04
Grade 2	21 (41%)	1 (4.5%)	8 (25.6%)	0.02
Grade 3	4 (8%)	2 (9%)	3 (10.3%)	
Grade 4	4 (8%)	4 (8%)	2 (6.9%)	
Median time of occurrence, d (range)	29 (5.7–121)	30 (5.7–121)	28 (17–59)	0.88
Chronic GvHD				1
Extensive	8 (15.7%)	4 (18.18%)	4 (13.8%)	
Relapse	16 (31%)	3 (13.3%)	13 (44.8%)	0.01
2-y DFS	50.3% (95% CI: 38–66%)	67.8% (95% CI: 50–90%)	37.6% (95% CI: 23–60%)	0.03
2-y OS	61.6% (95% CI: 49–76%)	76.2% (95% CI: 60–96%)	51.2% (95% CI: 35–73%)	0.04
Deaths	19 (37%)	5 (22.7%)	14 (48.3%)	0.12
Causes of death				0.35
Relapse	11 (58%)	2 (40%)	9 (64.3%)	
Infection	4 (21%)	1 (20%)[a]	3 (21.4%)[b]	
GvHD	3 (16%)	2 (40%)	1 (7%)	
Dermatomyositis	1 (5%)		1 (7%)	

[a]Respiratory syncytial virus.
[b]Mucomycosis, n = 1; *Toxoplasma gondii*, n = 1; adenovirus plus *Escherischia coli*, n = 1.

in the donor concomitant with the absence of the respective cognate HLA ligands in the recipient. KIR2DL1$_D$/C2^-_R inc. was found in 10 D/R pairs, KIR2DL2/3$_D$/C1^-_R inc. in 6, and KIR3DL1$_D$/Bw4^-_R inc. in another 6 (Table III). Similarly, activating KIR/HLA inc. were defined by the presence of activating KIR genes (KIR2DS1 and KIR2DS2) in the donor concomitant with the presence of the respective cognate HLA ligands in the recipient, absent in the donor. From the 51 D/R pairs, only two cases showed a KIR2DS1$_D$/C2^+_R inc., and another two a KIR2DS2$_D$/C1^+_R inc. According to clofarabine versus fludarabine treatment, both subgroups of patients presented a similar distribution of inh. KIR/HLA inc. (Fig. 1A). Characteristics of patients, donors, and outcomes are displayed in Tables I and II according to the D/R pair distribution of inh. KIR/HLA inc.

There was a higher incidence of acute GvHD for patients with inh. KIR/HLA inc. (n = 22) compared with those without (n = 29) at 72.7 versus 45% (p = 0.04), whereas significantly fewer relapses were observed in the former group (13.6 versus 45%, p = 0.01) (Fig. 1B). Moreover, these patients have better 2-y DFS and OS at 67.8 (95% CI: 50–90%) versus 37.6% (95% CI: 23–60%, p = 0.03) and 76.2 (95% CI: 60–96%) versus 51.2% (95% CI: 35–73%, p = 0.04), respectively (Table II).

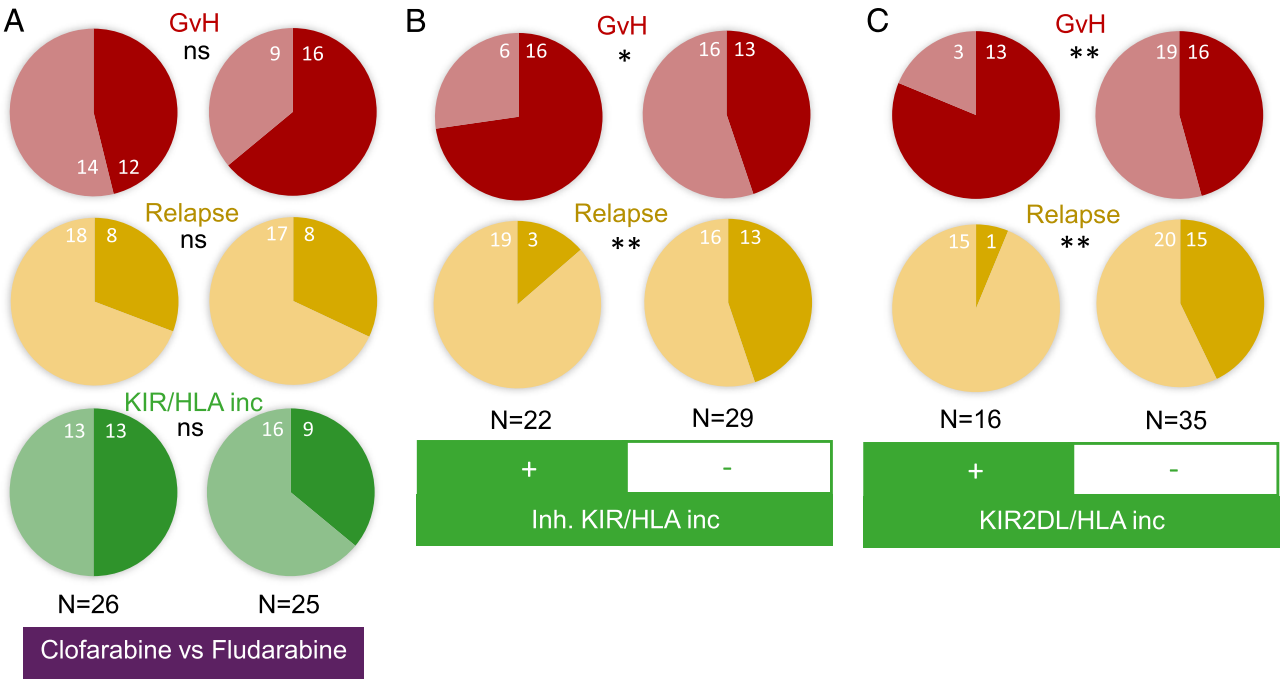

FIGURE 1. Factors influencing graft outcome. (**A**) Comparison of groups of patients treated with clofarabine (n = 26) or fludarabine (n = 25) according to GvHD (no GvHD in light red versus GvHD in dark red), relapse occurrence (no relapse in light yellow versus relapse in dark yellow), and KIR/HLA inc. (no inc. in light green versus inc. in dark green). (**B**) Comparison of groups of patients with (n = 22) or without inh. KIR (KIR2DL and KIR3DL1)/HLA inc. (n = 29) and (**C**) with (n = 16) or without KIR2DL/HLA inc. (n = 35) according to GvHD and relapse occurrence. *$p < 0.05$, **$p < 0.01$, χ^2 test.

Table III. KIR and HLA genotyping of D/R pairs

Donor	Conditioning Regimen	KIR Genes[a] 2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	1D	2DS5	3DS1	KIR Genotype	HLA Typing HLA-A*		HLA-B*		HLA-C*		KIR Ligands	Recipient	HLA Typing HLA-A*		HLA-B*		HLA-C*		KIR Ligands	KIR/HLA inc.
D1	Clofarabine	1	0	1	1	0	1	1	1	0	0	0	0	1	0	0	AA	02:01	24:02	55:01	35:02	01:02	04:01	Bw4 C1C2	R1	02:01	24:02	55:01	08:01	01:02	07:01	Bw4 C1C1	$KIR2DL1_D/C2-_R$
D2	Clofarabine	1	0	1	1	0	1	1	1	0	0	0	0	1	0	0	AA	01:01	68:01	51:01	57:01	01:02	06:02	Bw4 C1C2	R2	23:01	68:01	49:01	51:01	01:02	07:02	Bw4 C1C1	$KIR2DL1_D/C2-_R$
D3	Clofarabine	1	0	1	1	0	1	1	1	0	0	0	0	1	0	0	AA	11:01	31:01	14:01	35:01	04:01	08:02	C1C2	R3	03:01	31:01	14:01	14:02	08:02	08:02	C1C1	$KIR2DL1_D/C2-_R$
D4	Clofarabine	1	1	0	1	1	1	1	1	1	1	1	0	1	0	1	AB	03:02	29:02	51:01	58:01	07:01	15:02	Bw4 C1C2	R4	02:01	29:02	35:03	58:01	07:01	12:03	Bw4 C1C1	$KIR2DL1_D/C2-_R$
D5	Clofarabine	1	0	1	1	0	1	1	1	0	0	0	1	1	0	0	AA	29:02	33:03	49:01	50:01	06:02	07:01	Bw4 C1C2	R5	29:02	01:15N	49:01	08:01	07:01	07:01	Bw4 C1C1	$KIR2DL1_D/C2-_R$
D6	Clofarabine	1	0	1	1	1	0	1	1	1	0	0	0	0	1	1	BB	03:02	23:01	44:03	49:01	04:01	07:01	Bw4 C1C2	R6	03:02	01:01	08:01	49:01	07:01	07:01	Bw4 C1C1	$KIR2DL1_D/C2-_R$
D7	Fludarabine	1	0	1	1	0	1	1	1	0	0	0	0	1	0	0	AA	02:01	29:02	27:05	35:03	01:02	04:01	Bw4 C1C2	R7	02:01	25:01	18:01	27:05	01:02	12:03	Bw4 C1C1	$KIR2DL1_D/C2-_R$
D8	Fludarabine	1	0	1	1	0	1	1	1	0	0	0	0	1	0	0	AA	29:02	29:02	08:01	50:01	06:02	07:01	C1C2	R8	02:01	29:02	08:01	08:01	07:01	07:01	C1C1	$KIR2DL1_D/C2-_R$
D9	Fludarabine	1	1	1	1	0	1	1	1	0	1	0	0	1	0	0	AB	02:01	32:01	14:02	13:02	06:02	08:02	Bw4 C1C2	R9	02:01	32:01	14:02	49:01	07:01	08:02	Bw4 C1C1	$KIR2DL1_D/C2-_R$
D10	Fludarabine	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	AB	03:01	11:01	50:01	14:02	06:02	08:02	C1C2	R10	03:01	68:01	07:02	14:02	07:02	08:02	C1C1	$KIR2DL1_D/C2-_R$
D11	Clofarabine	1	0	1	1	0	1	1	1	0	0	0	0	1	0	0	AA	02:01	11:01	07:02	57:01	06:02	07:02	Bw4 C1C2	R11	02:01	31:01	51:01	57:01	06:02	15:02	Bw4 C2C2	$KIR2DL3_D/C1-_R$
D12	Clofarabine	1	0	1	1	0	1	1	1	0	0	0	1	1	0	0	AA	02:01	24:02	44:02	51:01	05:01	14:02	Bw4 C1C2	R12	11:01	24:02	27:02	44:02	02:02	05:01	Bw4 C2C2	$KIR2DL3_D/C1-_R$
D13	Clofarabine	1	0	1	1	1	0	1	1	1	0	0	0	0	1	1	BB	03:01	29:02	07:02	35:01	04:01	07:02	C1C2	R13	02:01	29:02	35:01	44:02	04:01	05:01	Bw4 C2C2	$KIR2DL3_D/C1-_R$
D14	Fludarabine	1	0	1	1	1	0	1	1	1	0	0	0	0	1	1	BB	02:01	32:01	44:02	51:01	01:02	05:01	Bw4 C1C2	R14	26:01	32:01	35:03	44:02	04:01	05:01	Bw4 C2C2	$KIR2DL3_D/C1-_R$
D15	Fludarabine	1	1	1	1	0	1	1	1	0	1	0	0	1	0	0	AB	11:01	33:01	40:02	14:02	02:02	08:02	C1C2	R15	11:01	29:02	40:02	57:01	02:02	06:02	Bw4 C2C2	$KIR2DL2/3_D/C1-_R$
D16	Fludarabine	1	0	1	1	0	1	1	1	0	0	0	1	1	0	0	AA	23:01	11:01	45:01	52:01	06:02	12:02	Bw4 C1C2	R16	23:01	24:02	45:01	50:02	06:02	06:02	Bw4 C2C2	$KIR2DL3_D/C1-_R$
D17	Clofarabine	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	AB	02:01	68:02	14:02	44:02	05:01	08:02	Bw4 C1C2	R17	30:02	68:02	14:02	15:03	02:10	08:02	C1C2	$KIR3DL1_D/Bw4-_R$
D18	Fludarabine	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	AB	01:01	02:01	08:01	51:01	07:01	14:02	Bw4 C1C1	R18	01:01	11:01	08:01	18:01	07:01	07:01	C1C1	$KIR3DL1_D/Bw4-_R$
D19	Fludarabine	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	AB	31:01	02:01	44:02	40:01	03:04	05:01	Bw4 C1C2	R19	31:01	68:01	35:02	40:01	03:04	04:01	C1C2	$KIR3DL1_D/Bw4-_R$
D20	Clofarabine	1	0	1	1	0	1	1	1	0	0	0	1	1	0	0	AA	03:01	24:02	07:02	41:01	07:02	17:01	Bw4 C1C2	R20	03:01	26:01	07:02	45:01	06:02	07:02	C1C2	$KIR3DL1_D/Bw4-_R$
D21	Clofarabine	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	AB	02:01	24:02	14:02	55:01	03:03	08:02	Bw4 C1C1	R21	02:01	26:08	14:02	35:01	03:03	08:02	C1C1	$KIR3DL1_D/Bw4-_R$
D22	Clofarabine	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	AB	01:01	24:02	08:01	39:06	06:02	07:02	Bw4 C1C2	R22	01:01	01:01	08:01	08:01	06:02	07:01	C1C2	$KIR3DL1_D/Bw4-_R$
D23	Clofarabine	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	AB	01:01	11:01	08:01	51:01	07:01	15:02	Bw4 C1C1	R23	11:01	11:01	35:01	51:01	04:01	15:02	Bw4 C1C2	$KIR2DS1_D/C2+_R$
D24	Clofarabine	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1	AB	31:01	03:01	44:02	51:01	05:01	15:02	Bw4 C2C2	R24	02:01	03:01	44:02	51:01	05:01	07:01	Bw4 C1C2	$KIR2DS2_D/C1+_R$
D25	Fludarabine	1	0	1	1	1	1	1	1	1	0	1	0	1	0	1	AB	68:01	32:01	15:03	44:02	07:04	16:01	Bw4 C1C1	R25	02:01	32:01	15:03	27:02	02:02	16:01	Bw4 C1C2	$KIR2DS1_D/C2+_R$
D26	Fludarabine	1	1	1	1	1	1	1	1	0	1	1	0	1	0	1	AB	68:01	68:01	27:05	44:02	02:02	05:01	Bw4 C2C2	R26	02:01	68:01	27:05	39:01	02:02	12:03	Bw4 C1C2	$KIR2DS2_D/C1+_R$
D27	Clofarabine	1	0	1	1	0	1	1	1	0	0	0	0	1	0	0	AA	03:01	33:01	14:02	44:03	16:01	08:02	Bw4 C1C1	R27	24:02	33:01	14:02	27:05	02:02	08:02	Bw4 C1C2	No
D28	Clofarabine	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	AB	32:01	32:01	56:01	08:01	01:02	07:02	Bw4 C1C1	R28	32:01	29:02	56:01	45:01	01:02	06:02	Bw4 C1C2	No
D29	Clofarabine	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	AB	02:01	24:02	40:01	44:03	03:04	16:01	Bw4 C1C1	R29	03:01	24:02	14:02	44:03	08:02	16:01	Bw4 C1C1	No
D30	Clofarabine	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	AB	02:01	30:02	08:01	45:01	07:02	16:01	C1C1	R30	30:02	30:02	08:01	35:01	07:02	04:01	C1C2	No
D31	Clofarabine	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	AB	26:01	01:01	08:01	35:03	07:01	04:01	C1C2	R31	26:01	66:01	15:01	35:03	03:04	04:01	C1C2	No
D32	Clofarabine	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	AB	03:01	24:02	07:02	51:01	07:02	14:02	Bw4 C1C1	R32	02:05	03:01	07:02	47:01	07:02	07:18	Bw4 C1C1	No
D33	Clofarabine	1	0	1	1	0	1	1	1	0	0	0	0	1	0	0	AA	03:01	66:01	15:18	41:02	07:04	17:03	C1C2	R33	03:01	03:01	15:18	27:02	07:04	02:02	Bw4 C1C2	No
D34	Clofarabine	1	0	1	1	0	1	1	1	0	0	0	0	1	0	0	AA	23:01	24:02	07:02	07:02	07:02	12:03	C1C1	R34	23:01	32:01	07:02	49:01	07:01	12:03	Bw4 C1C1	No
D35	Clofarabine	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	AB	02:01	23:01	27:05	15:01	01:02	01:02	Bw4 C1C1	R35	11:01	23:01	27:05	52:01	01:02	12:02	Bw4 C1C1	No
D36	Clofarabine	1	1	1	1	0	1	1	1	0	1	0	1	1	0	0	AB	01:01	31:01	08:01	39:01	07:01	12:03	C1C1	R36	25	31	39	39	12:03	12:03	C1C1	No
D37	Clofarabine	1	1	1	1	0	1	1	1	0	1	0	0	1	0	0	AB	02:01	23:01	27:02	44:03	02:02	04:01	Bw4 C2C2	R37	01:01	23:01	44:03	51:01	04:01	15:02	Bw4 C2C2	No
D38	Fludarabine	1	1	1	1	0	1	1	1	0	1	0	1	1	0	0	AB	26:01	26:01	38:01	38:01	12:03	12:03	Bw4 C1C1	R38	26:01	32:01	38:01	55:01	01:02	12:03	Bw4 C1C1	No
D39	Fludarabine	1	0	1	1	0	1	1	1	0	0	0	1	1	0	0	AA	24:02	32:01	44:03	27:05	04:01	01:02	Bw4 C1C2	R39	01:01	32:01	18:01	27:05	02:02	12:03	Bw4 C1C2	No
D40	Fludarabine	1	0	1	1	1	1	1	1	1	0	0	1	0	1	1	AB	01:01	03:01	07:02	08:01	07:01	07:02	C1C1	R40	01:01	29:02	08:01	44:04	07:01	16:01	C1C1	No
D41	Fludarabine	1	1	1	1	0	1	1	1	0	1	0	0	1	0	0	AB	02:01	02:05	18:01	51:01	07:01	15:02	Bw4 C1C2	R41	02:05	29:02	51:01	58:01	07:18	15:02	Bw4 C1C2	No
D42	Fludarabine	1	0	1	1	0	1	1	1	0	0	0	0	1	0	0	AA	02:01	11:01	07:02	18:03	07:01	07:02	C1C1	R42	02:01	26:01	08:01	18:03	07:01	07:01	C1C1	No
D43	Fludarabine	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	AB	02:01	02:01	08:01	35:08	04:01	07:01	C1C2	R43	02:01	29:02	44:03	35:08	04:01	16:01	Bw4 C1C2	No
D44	Fludarabine	1	1	1	1	0	1	1	1	0	1	0	0	1	0	0	AB	03:01	29:02	55:01	38:01	03:02	12:03	Bw4 C1C1	R44	03:01	33:01	55:01	58:01	03:02	03:03	Bw4 C1C1	No
D45	Fludarabine	1	0	1	1	0	1	1	1	0	0	0	1	1	0	0	AA	03:01	01:01	40:01	13:02	05:01	06:02	Bw4 C2C2	R45	03:01	68:01	40:01	49:01	05:01	07:01	Bw4 C1C2	No
D46	Fludarabine	1	0	1	1	0	1	1	1	0	0	0	0	1	0	0	AA	03:01	32:01	40:01	52;01	03:04	12:02	Bw4 C1C1	R46	25:01	32:01	18:01	52:01	12:02	12:03	Bw4 C1C1	No
D47	Fludarabine	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	AB	01:01	03:01	15:17	07:02	07:01	07:02	C1C1	R47	01:01	32:01	15:17	38:01	07:01	12:03	Bw4 C1C1	No
D48	Fludarabine	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	AB	24:02	02:01	14:01	14:02	02:02	08:02	Bw4 C1C2	R48	24:02	30:01	08:01	14:02	02:02	07:02	Bw4 C1C2	No
D49	Fludarabine	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	AB	02:01	02:01	14:01	40:01	03:04	08:02	C1C1	R49	02:01	26:01	14:01	15:01	03:03	08:02	C1C1	No
D50	Fludarabine	1	0	1	1	1	1	1	1	1	0	0	1	0	1	1	AB	01:01	24:02	57:01	44	02:02	06:02	Bw4 C2C2	R50	24:02	24:02	35	44	02:02	04:01	Bw4 C2C2	No
D51	Fludarabine	1	1	0	1	1	1	1	1	0	1	1	0	1	0	0	AB	02:01	26:01	18:01	50:01	05:01	06:02	C2C2	R51	02:01	02:01	18:01	35:01	04:01	05:01	C2C2	No

[a]0 = absence and 1 = presence.

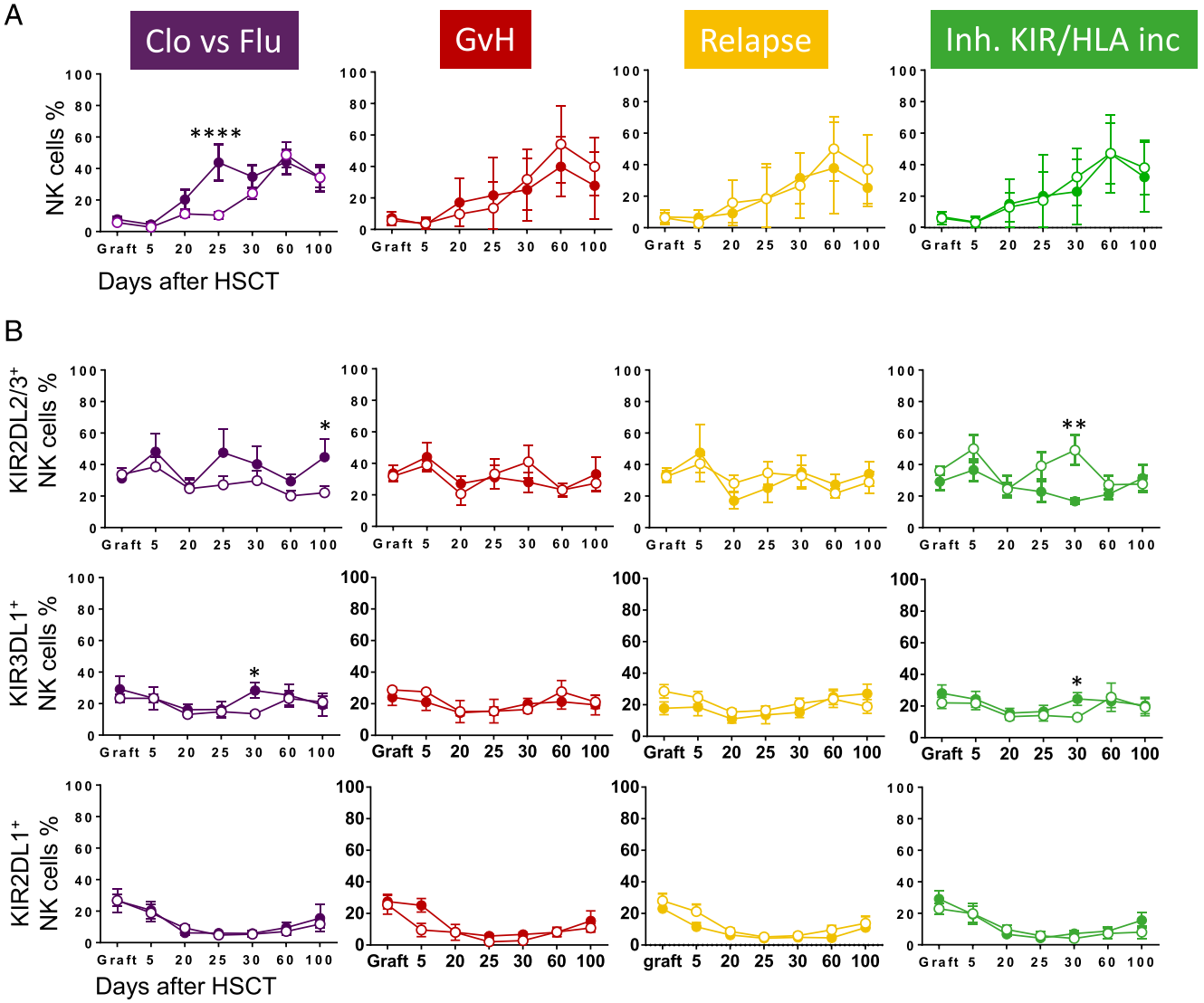

FIGURE 2. Reconstitution of the KIR NK cell repertoire. (**A**) NK cell ($n = 34$) and (**B**) KIR2DL2/3$^+$ ($n = 34$), KIR3DL1$^+$ ($n = 27$), and KIR2DL1$^+$ ($n = 27$) NK cell frequencies determined by flow cytometry from graft and recipients following the posttransplantation kinetics (days 5, 20, 25, 30, 60, and 100) according to clofarabine (open purple circle) versus fludarabine (filled purple circle) treatment, GvHD (open red circle for no GvHD and filled red circle for GvHD), relapse occurrence (open yellow circle for no relapse and filled yellow circle for relapse), and inh. KIR/HLA inc. (open green circle for no inc. and full green circle for inc.). *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, Student t test.

Focusing on specific KIR2DL (KIR2DL1 and KIR2DL2/3)/HLA-C inc. ($n = 16$), it was observed that patients with those inc. presented a significantly higher incidence of acute GvHD (81.2 versus 45.7%, $p = 0.01$) and again a significantly lower incidence of relapse (6.2 versus 42.8%, $p = 0.008$) (Fig. 1C), a better 2-y DFS (68.1 [95% CI: 48–95%] versus 42.6% [95% CI: 29–62%], $p = 0.09$) and a better 2-y OS (74.4 [95% CI: 55–99%] versus 56% [95% CI: 41–75%], $p = 0.16$). Chronic GvHD was not associated with inh. KIR/HLA inc., relapse, DFS, or OS in this series. Of note, donor AA and B+ genotypes were not associated with GvHD or relapse (data not shown). Thus, our results showed that inh. KIR/HLA inc. impact GvHD and relapse after RIC PBSC HSCT with PTCy and confer better survivals.

KIR2DL2/3$^+$ and KIR3DL1$^+$ NK cell recoveries at day 30 postgraft are inversely impacted by KIR/HLA inc.

The kinetics of NK cell repertoire reconstitution was analyzed afterward for 34 donor/patient pairs by immunophenotyping in MFC from day 0 to day 30, three times per week, and then at day 60 and day 90/100 posttransplant. Nineteen of these patients developed acute GvHD at a median time of 34 d (range: 21–119 d) posttransplant. Proliferative NK cells in the graft were particularly targeted by the early administration of PTCy, as shown by the profound depletion in NK cells from day 5 to day 20, confirming previous data from our group (16). Although GvHD, relapse, or inh. KIR/HLA inc. did not impact NK cell frequency during the first 30 d, the fludarabine-based regimen was associated with higher NK cell recovery compared with the clofarabine-based regimen at day 25 ($p = 0.0001$) (Fig. 2A). Because of the significant impact of genetic KIR/HLA inc. on GvHD and relapse, investigations were pursued focusing on KIR2DL2/3$^+$, KIR3DL1$^+$, and KIR2DL1/S1$^+$ NK cell subsets, based on the positivity for the KIR of interest. Interestingly, only inh. KIR/HLA inc. modulated KIR NK cell reconstitution posttransplant (Fig. 2B). Indeed, inh. KIR/HLA inc. were associated with a significant decrease in KIR2DL2/3$^+$ NK cell recovery ($p = 0.004$) but conversely fostered KIR3DL1$^+$ NK cell recovery at day 30 ($p = 0.01$). Neither inh. KIR/HLA inc. nor type of conditioning nor relapse nor GvHD had any impact on KIR2DL1/S1$^+$ (Fig. 2B) and KIR2DS4$^+$ NK cell recoveries (data not shown). Our results suggest that KIR/HLA inc. impact the KIR NK cell repertoire recovery after RIC PBSC

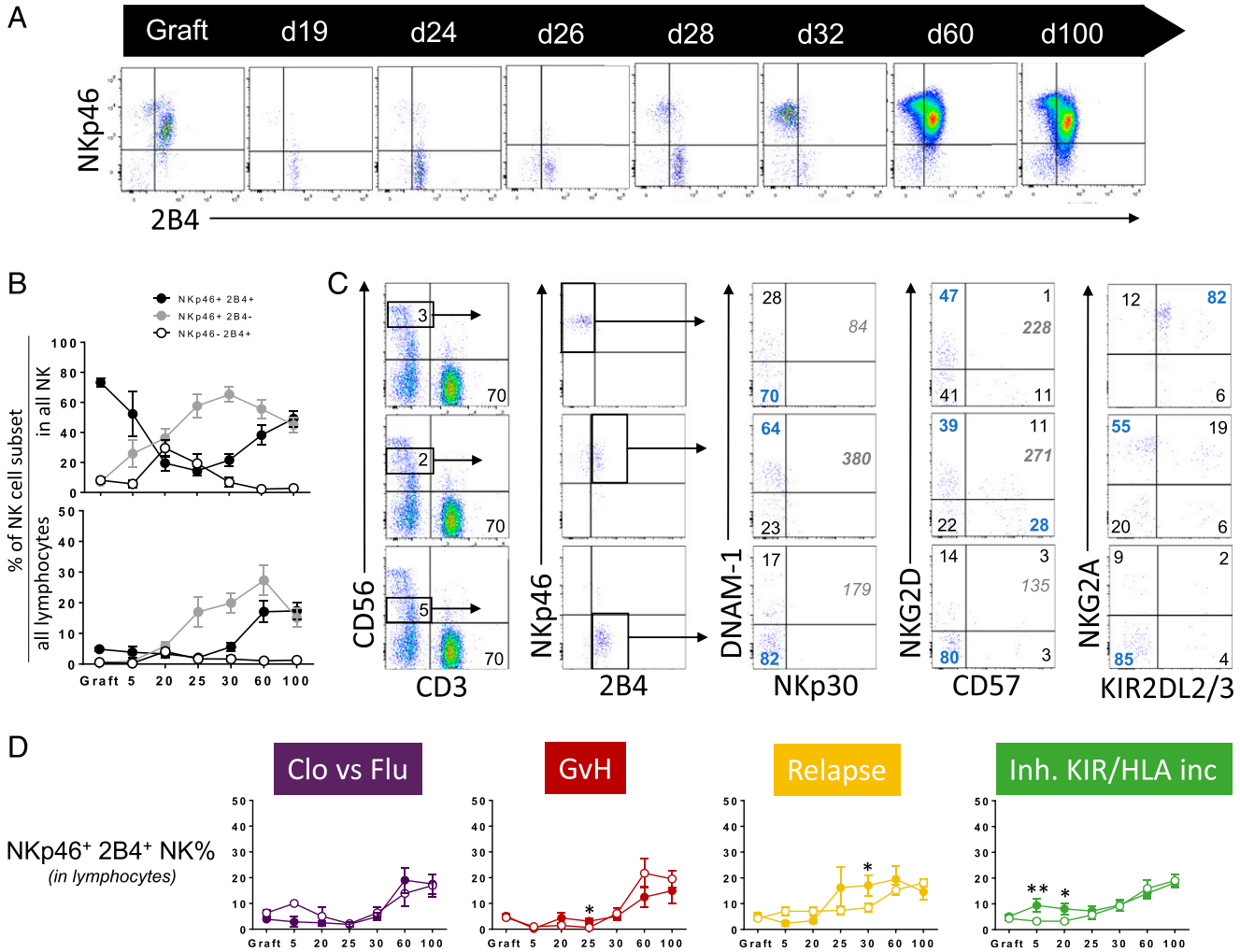

FIGURE 3. Identification of three NK cell subsets according NKp46 and 2B4 expression posttransplantation. (**A**) Both NKp46 and 2B4 NK cell receptors were investigated during NK cell recovery by flow cytometry. Representative density plots illustrating different stages of the NKp46/2B4 NK cell subset from graft to recipient at day 100, moving through days 19, 24, 26, 28, 32, and 60. (**B**) Evolution of NKp46$^+$ 2B4$^+$, NKp46$^+$ 2B4$^-$, and NKp46$^-$ 2B4$^+$ NK cell subsets in the NK cell compartment and in all lymphocytes following the posttransplantation kinetics (days 5, 20, 25, 30, 60, and 100). (**C**) Representative density plots illustrating the phenotype of all NKp46$^+$ 2B4$^+$, NKp46$^+$ 2B4$^-$, and NKp46$^-$ 2B4$^+$ NK cell subsets, taking into account CD3, CD56, DNAM-1, NKp30, NKG2D, CD57, NKG2A, and KIR2DL2/3 expression. The frequency of cells in each gate is indicated. The mean fluorescence intensity for DNAM-1 and NKG2D is indicated in italics. (**D**) NKp46$^+$ 2B4$^+$ NK cell subset frequencies in all lymphocytes were represented following the posttransplantation kinetics (days 5, 20, 25, 30, 60, and 100) according to clofarabine (open purple circle) versus fludarabine (filled purple circle) treatment, GvHD (open red circle for no GvHD and filled red circle for GvHD), relapse occurrence (open yellow circle for no relapse and filled yellow circle for relapse), and inh. KIR/HLA inc. (open green circle for no inc. and filled green circle for inc.). *$p < 0.05$, **$p < 0.01$, Student t test.

HSCT with PTCy with opposite effects on KIR2DL2/3$^+$ and KIR3DL1$^+$ NK cell subsets.

Patients developing GvHD harbor more differentiated and activated NK cells

Different membrane markers inform about the differentiated and activated status of NK cells (20). Thus, NKp46 is a marker of NK cells (21, 22), and 2B4 is a receptor corresponding to an activating marker at the early stage of NK cell differentiation. Conversely, it becomes an inh. receptor on differentiated NK cells (23). Expression of these markers was examined in MFC on graft aliquots and during reconstitution of the NK cell repertoire (Fig. 3A). In the grafts, NK cells displayed a differentiated phenotype and expressed essentially NKp46 and 2B4. In patients, between day 20 and day 30, NK cells were NKp46$^-$ and 2B4$^+$ and then lost progressively 2B4 expression to acquire NKp46 expression. At day 60, NK cells displayed a similar phenotype to that of grafted NK cells. The evolution of all three NK cell subsets (NKp46$^-$ 2B4$^+$, NKp46$^+$ 2B4$^-$, and NKp46$^+$ 2B4$^+$) during immune reconstitution in the NK cell compartment showed that the frequency of NKp46$^+$ 2B4$^+$ decreased until day 20 but increased at day 30 (Fig. 3B). Inversely, the NKp46$^+$ 2B4$^-$ NK cell subset increased until day 30 only to progressively decrease. In the first part of the immune reconstitution, until day 20, donor NK cells were still present, and in the second part, from day 20, new recipient NK cells appeared from donor hematopoietic stem cells. Taking into account all lymphocytes, NKp46$^+$ 2B4$^-$ NK cell frequency increased from day 20 to day 60 (Fig. 3B). However, the NKp46$^+$ 2B4$^+$ NK cell subset appeared later, from day 30, in accordance with the profile shown for a representative patient in Fig. 3A. Interestingly, all three subsets based on NKp46 and 2B4 expression match NK cell subsets identified by the level of CD56 expression (Fig. 3C). Indeed, NKp46$^+$ 2B4$^-$ are CD56^{high}, NKp46$^+$ 2B4$^+$ are CD56 intermediate (CD56^{int}), and NKp46$^-$ 2B4$^+$ are CD56^{low}. Different phenotypes are associated with these three NK subsets. NKp46$^+$ 2B4$^-$ (CD56^{high}) NK cells express high levels of

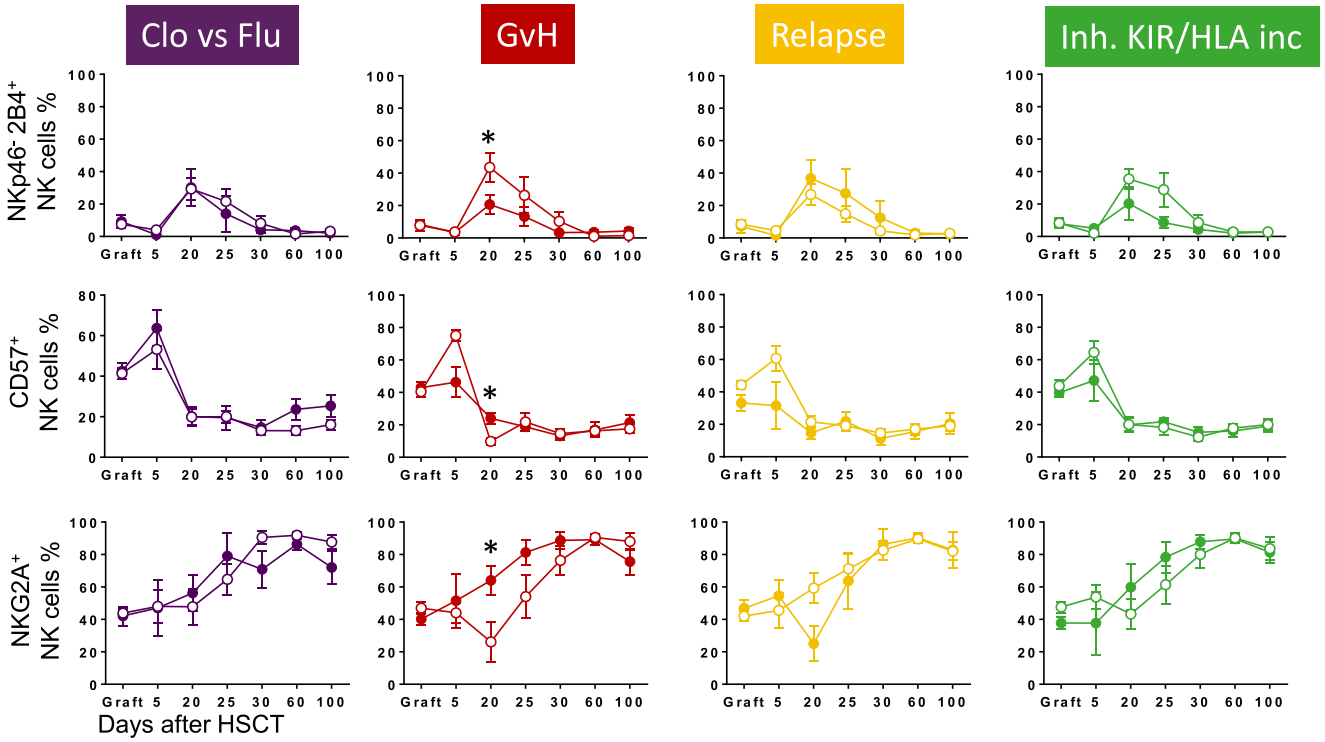

FIGURE 4. Differentiated status of NK cells from patients developing GvHD. Immature NKp46$^-$ 2B4$^+$ NK cell subset frequencies and mature CD57$^+$ and NKG2A$^+$ NK cell subset frequencies were represented following the posttransplantation kinetics (days 5, 20, 25, 30, 60, and 100) according to clofarabine (open purple circle) versus fludarabine (filled purple circle) treatment, GvHD (open red circle for no GvHD and filled red circle for GvHD), relapse occurrence (open yellow circle for no relapse and filled yellow circle for relapse), and inh. KIR/HLA inc. (open green circle for no inc. and filled green circle for inc.). *$p < 0.05$, Student t test.

NKG2A and intermediate levels of NKG2D and KIR. They do not express CD57. NKp46$^-$ 2B4$^+$ (CD56^{low}) NK cells do not express DNAM-1, NKG2D, NKp30, CD57, NKG2A, and KIR. In contrast, NKp46$^+$ 2B4$^+$ (CD56^{int}) NK cells associated with the late stage of differentiation express all these receptors. The NKp46$^+$ 2B4$^+$ NK cell subset was significantly more represented early in patients developing GvHD at day 25 ($p = 0.03$) and in those with KIR/HLA inc. at days 5 ($p = 0.01$) and 20 ($p = 0.04$) before the median time of GvHD occurrence (day 29). In contrast, the NKp46$^+$ 2B4$^+$ NK cell subset was low until day 25 in patients with relapse occurrence but became significantly higher at day 30 ($p = 0.03$) (Fig. 3D).

Patients developing GvHD showed a differentiated profile of NK cells characterized by less frequent undifferentiated 2B4$^+$ NKp46$^-$ NK cells at day 20 ($p = 0.04$) (Fig. 4). Naive NKG2A$^+$ KIR$^-$ CD57$^-$ NK cells will acquire KIR and CD57 expression to become differentiated. NK cell activation will translate in increased numbers of NKG2A$^+$ NK cells (20, 24). A significant increase of CD57$^+$ ($p = 0.02$) and NKG2A$^+$ NK cell frequencies ($p = 0.01$) was observed at day 20 posttransplant in patients who developed GvHD (Fig. 4). NKG2A$^+$ KIR2DL2/3$^-$ NK cells were predominant in patients developing GvHD at day 20 ($p = 0.03$) and also in patients with KIR/HLA inc. at days 25 ($p = 0.004$) and 30 ($p = 0.007$) (Fig. 5A, 5B). In accordance with these results, NKG2A$^+$ KIR2DL2/3$^+$ NK cells were observed mainly in patients with no KIR/HLA inc. at day 25 ($p = 0.05$) and day 30 ($p = 0.01$) (Fig. 5B).

The expression of different activating receptors, such as DNAM-1 (25), NKG2D (26, 27), NKp30 (28), and NKp44 (29, 30), potentially implicated in the GvL effect induced by NK cells (31) was also investigated during immune reconstitution posttransplant. At days 20 and 25, even though the difference was not statistically significant, DNAM-1 and NKG2D were more expressed on NK cells in patients developing GvHD and those with KIR/HLA inc., highlighting an activated status of these NK cells (Fig. 5C, 5D).

Significant differences in expression were also observed for DNAM-1 at day 20 ($p = 0.02$) and for NKG2D at day 25 ($p = 0.02$), considering patients with or without KIR/HLA inc. (Fig. 5C, 5D). Conversely, DNAM-1 expression was significantly lower at day 100 on NK cells from patients developing GvHD compared with controls ($p = 0.01$). As the NKp46$^+$ 2B4$^+$ NK cell subset expresses DNAM-1 and NKG2D, these different profiles are in accordance with the profile of NKp46$^+$ 2B4$^+$ NK cells presented in Fig. 3D during immune reconstitution. Interestingly, NKG2D expression, but not that of DNAM-1, was significantly higher on NK cells at day 30 for patients who relapsed ($p = 0.03$). Of note, we cannot associate NKG2D expression with KIR, as all NKp46$^+$ 2B4$^+$ NK cells express NKG2D independently of KIR expression (data not shown). No difference was observed for NKp30 and NKp44 expression considering the type of conditioning, GvHD, relapse, or inh. KIR/HLA inc. (data not shown). Overall, NK cells from patients developing GvHD featured a differentiated and activated status in haploidentical RIC PBSC HSCT with PTCy.

NK cells from patients developing GvHD have a lower ex vivo degranulation activity

To evaluate the cytotoxic function of NK cells, we assessed the cell-surface mobilization of CD107a on NK cells. The degranulation potential of NK cells was assessed by testing ex vivo the spontaneous lysis (32) exerted by patient NK cells collected around day 30 and day 100. The two patients who developed a late GvHD (days 112 and 122) were excluded from the functional study performed at day 30. Neither the type of conditioning nor GvH

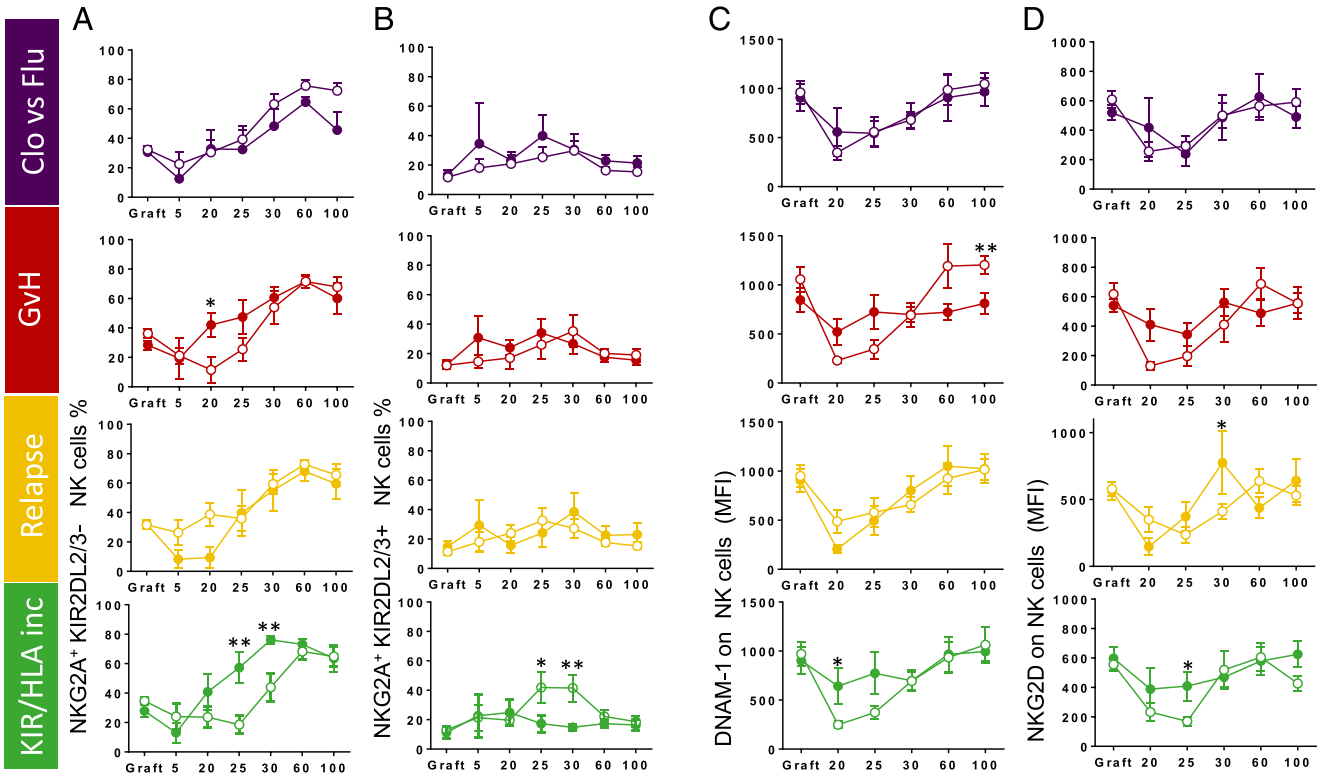

FIGURE 5. High expression of NKG2A in the absence of KIR2DL2/3 and the evolution of activating DNAM-1 and NKG2D expression reflect the evolution of NKp46$^+$ 2B4$^+$ NK cells. (**A**) NKG2A$^+$ KIR2DL2/3$^-$ NK cell subset frequencies and (**B**) NKG2A$^+$ KIR2DL2/3$^+$ NK cell subset frequencies were represented following the posttransplantation kinetics (days 5, 20, 25, 30, 60, and 100) according to clofarabine (open purple circle) versus fludarabine (filled purple circle) treatment, GvHD (open red circle for no GvHD and filled red circle for GvHD), relapse occurrence (open yellow circle for no relapse and filled yellow circle for relapse), and inh. KIR/HLA inc. (open green circle for no inc. and filled green circle for inc.). Mean fluorescence intensity (MFI) of (**C**) DNAM-1 and (**D**) NKG2D expressed on all NK cells following posttransplantation kinetics (days 5, 20, 25, 30, 60, and 100) according to clofarabine (open purple circle) versus fludarabine (filled purple circle) treatment, GvHD (open red circle for no GvHD and filled red circle for GvHD), relapse occurrence (open yellow circle for no relapse and filled yellow circle for relapse), and inh. KIR/HLA inc. (open green circle for no inc. and filled green circle for inc.). *$p < 0.05$, **$p < 0.01$, Student t test.

occurrence nor relapse nor inh. KIR/HLA inc. had any impact on NK cell degranulation at day 30 (Fig. 6A). However, NK cells from patients developing GvHD displayed a significantly lower degranulation rate compared to those of patients without GvHD at day 100 ($p = 0.01$) (Fig. 6B).

Similarly, at day 100 the degranulation potential of patient NK cells was investigated in the presence of the acute leukemia H9 cell line and the acute myeloid KG1 cell line to evaluate their potential alloreactivity and antileukemia effect at this later point (Fig. 6B). Lower degranulation of NK cells from patients with GvHD was observed in the presence of H9 cells ($p = 0.01$) but not KG1 cells, which are basically less recognized by NK cells (33, 34). However, the degranulation potential of recipient NK cells was significantly higher than that of healthy donor NK cells against H9 ($p = 0.04$) and KG1 ($p = 0.0006$) (Fig. 6C), suggesting that recipient NK cells present an efficient potential of degranulation. No difference in terms of granzyme and perforin expression in recipient NK cells was observed at days 30 and 100 between patients developing GvHD or not (data not shown). However, at day 100, KIR2DL2/3$^+$ NK cells from patients developing GvHD presented a lower degranulation against 221 ($p = 0.01$) and H9 ($p = 0.03$), and KIR2DL2/3$^+$ NK cells from patients with KIR/HLA inc. presented a lower degranulation against KG1 ($p = 0.03$) (Figs. 6D, 7).

Discussion

Haploidentical HSCT has been increasingly used in recent years in patients with no suitable donors. Indeed, the PTCy administration following donor cell infusion is able to kill donor alloreactive T cells without deteriorating the graft itself, allowing for a normal rate of engraftment and low incidence of severe acute or chronic GvHD (35). These results are also currently associated with relatively good survivals, as reported in this article, which represents a new step and revolution in the long history of allogeneic HSCT. However, relapse remains a major concern in the setting of haploidentical HSCT. Because many haplodonors can be identified in the patient's family and because of the HLA disparity between donors and recipients, the prediction of NK cell alloreactivity could be of crucial importance for patient outcome and donor selection. The results reported here show that, in the context of RIC PBSC haplotransplant using PTCy, genetic inh. KIR/HLA inc. are associated with an improvement of the clinical outcome and particularly with a lower relapse incidence and better survivals for patients presenting this profile. KIR2DL/HLA-C inc. were observed to be more frequently associated with GvHD occurrence and lower relapse rates. Moreover, KIR2DL2/3$^+$ and KIR3DL1$^+$ NK cell recoveries at day 30 postgraft were inversely impacted by KIR/HLA inc., suggesting that inh. KIR2DL/HLA-C inc. is the most beneficial and may improve patient outcome in terms of relapse and survival. A deep investigation of KIR expression should be important to document mono-KIR$^+$ NK cells, excluding NK cells coexpressing other inh. KIR. Thus, it seems possible to propose a new model highlighting the impact of inh. KIR/HLA inc. on GvHD and relapse after RIC PBSC haplotransplant using PTCy, as illustrated in Fig. 7.

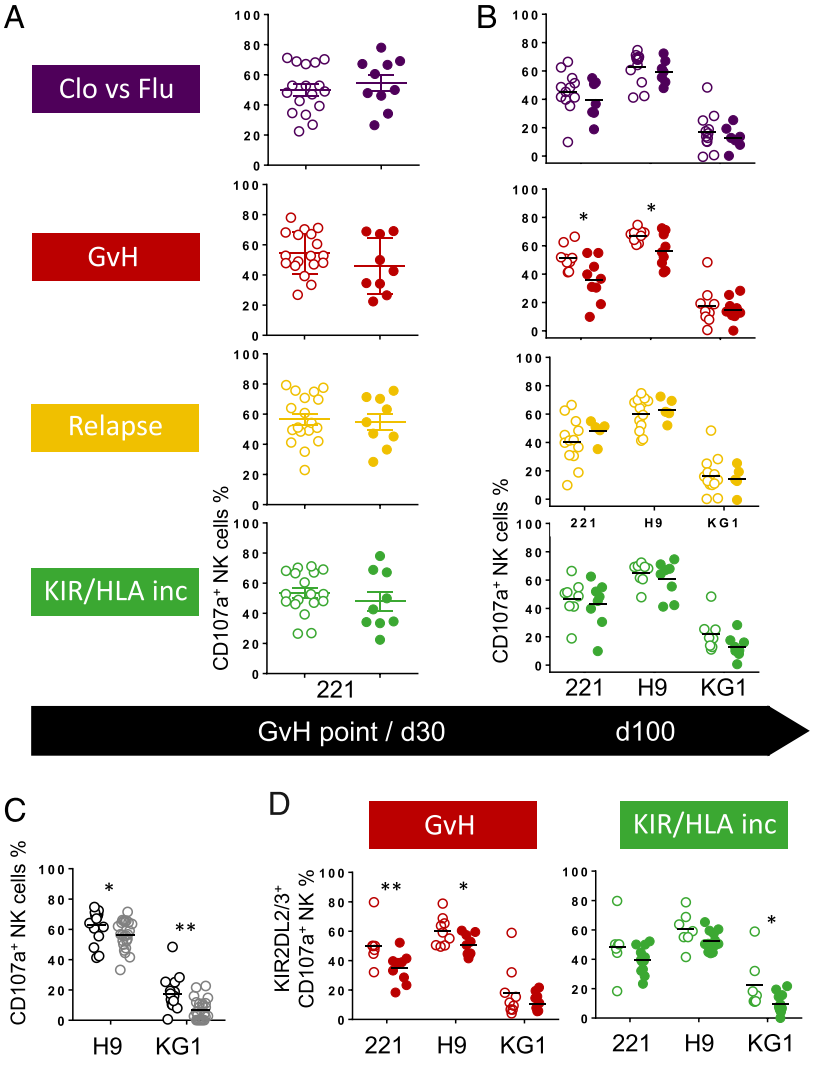

FIGURE 6. Lower ex vivo degranulation of NK cells from patients developing GvHD. (**A**) The degranulation of recipient NK cells at day 30 (n = 27) was assessed against 221 cells and compared according to clofarabine (open purple circle) versus fludarabine (filled purple circle) treatment, GvHD (open red circle for no GvHD and filled red circle for GvHD), relapse occurrence (open yellow circle for no relapse and filled yellow circle for relapse), and inh. KIR/HLA inc. (open green circle for no inc. and filled green circle for inc.). (**B**) The degranulation of recipient NK cells at day 100 (n = 18) was assessed against 221 cells, the lymphoid acute leukemia H9 cell line, and the myeloid acute leukemia KG1 cell line and compared according to clofarabine versus fludarabine treatment, GvHD, relapse occurrence and KIR/HLA inc. (**C**) The degranulation of recipient NK cells (n = 18) at day 100 was assessed against H9 and KG1 cell lines in comparison with healthy blood donor NK cells (n = 24). (**D**) The degranulation of recipient KIR2DL2/3$^+$ NK cells at day 100 was assessed against H9 and KG1 cell lines and compared according to GvHD (open red circle for no GvHD, n = 9; and filled red circle for GvHD n = 9) and KIR/HLA inc. (open green circle for no KIR/HLA inc., n = 8; and filled green circle for KIR/HLA inc., n = 12). $*p < 0.05$, $**p < 0.01$, Student t test.

So far, data regarding the impact of alloreactive NK cells after T-replete haploidentical transplantation are very scarce (9–11, 36). Using high doses of thymoglobulin instead of PTCy as GvHD prophylaxis, Huang and coworkers (36) have shown a deleterious effect of KIR ligand inc. on clinical outcomes after haploidentical HSCT, characterized by more acute GvHD and relapse and shorter survival. However, these authors suggested that the large dose of T cells contained in the graft and infused in patients may have affected NK cell function and KIR expression in vivo, with the consequence of inhibiting the beneficial role of alloreactive NK cells in terms of GvL effect.

Three previous studies have reported the impact of KIR/HLA inc. after haplotransplant using PTCy but with contradictory data (9–11). Symons et al. (9) showed in a series of 86 patients with bone marrow as the source of graft that inh. KIR gene mismatches between donor and recipient, or KIR haplotype AA recipients from KIR B+ donors, were associated with fewer relapses and improved OS and DFS. Conversely, a recent retrospective multicenter analysis including 444 acute leukemia patients having received a T cell–replete haplotransplant with PTCy, using either bone marrow or PBSC as stem cell source and MAC or RIC as the conditioning regimen, reported a deleterious impact of KIR ligand

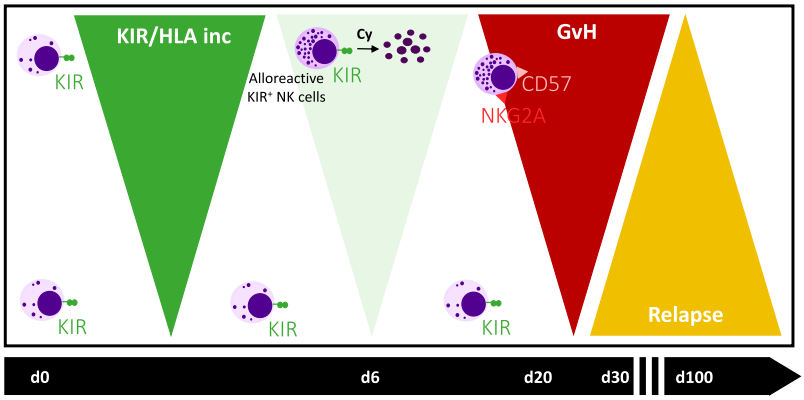

FIGURE 7. Cartoon illustrating the impact of KIR/HLA inc. on GvHD and relapse. KIR/HLA inc. promote activation and proliferation of alloreactive NK cells, which are quickly targeted by PTCy. This activated context seems to promote the differentiation and activation of NK cells associated with GvHD and limiting relapse. In parallel, in the absence of KIR/HLA inc., quiescent KIR NK cells are not eliminated by PTCy and are not efficient to limit relapse.

mismatches (defined according to HLA typing) (10). Different points can explain these discordant results compared with ours. Indeed, donor KIR genotyping was not available in this series (10) to analyze properly KIR/HLA inc. Also, in this missing self/KIR-ligand mismatch model, the presence of respective inh. KIR in the donor was assumed but not verified. This is problematic, as KIR genes harbor a broad allelic polymorphism that leads to unexpressed membrane KIR, as documented for KIR3DL1 (37). In addition, bone marrow and PBSC were both considered in this study, whereas we only included patients receiving PBSC. Finally, Russo et al. (11) showed no impact of NK cell alloreactivity after haplotransplant with PTCy but in a context of MAC regimen using bone marrow as the stem cell source.

One of the major interests of our study was also that we were able to thoroughly study NK cell recovery. Inh. KIR/HLA inc. were associated with a loss of KIR2DL2/3$^+$ NK cells at day 30. Moreover, a significant proportion of patients with inh. KIR/HLA inc. developed acute GvHD around day 30 with a more differentiated phenotype of NK cells. Thus, in accordance with the recent results of Russo et al. (11), we observed that PTCy seems to eliminate early alloreactive KIR2DL NK cells for D/R pairs with genetic inh. KIR/HLA inc. This was particularly compelling for KIR2DL2/3$^+$ NK cells. It has been shown that the high levels of serum IL-15 observed early after HSCT are associated with a more differentiated status of NK cells in recipients developing GvHD (11). It can be hypothesized that the alloreactive context promotes a strong activation of responsive NK cells that are subsequently targeted by the immunosuppressive PTCy treatment. This activation appears to emphasize efficient antileukemic responses. However, further investigations are needed to determine the nature of cell effectors directly engaged in the antileukemic responses.

Immunophenotypic NK cell investigation throughout the kinetics of immune reconstitution in this series led us to highlight different statuses of NK cell differentiation based on NKp46 and 2B4 expression, as recently described by Roberto et al. (38). At an early stage, immature NK cells are NKp46$^-$ 2B4$^+$ and acquire progressively NKp46 around day 30, then lose 2B4 to finally coexpress both markers around day 6 on infused NK cells. Interestingly, these three NK cell subsets defined on NKp46 and 2B4 expression match NK cell subsets identified by the level of CD56 expression (high, intermediate, and low). Moreover, these three NK cell subsets harbor a different phenotype, and NKp46$^+$ 2B4$^+$ (CD56^{int}) NK cells express differentiation-associated NK receptors such as NKG2A, KIR, DNAM-1, and NKG2D. The evolution of this population is significantly different in patients with relapse occurrence and patients developing GvHD, who share a similar profile with those with KIR/HLA inc. This NKp46$^+$ 2B4$^+$ NK subset appears earlier in patients developing GvHD or those with KIR/HLA inc., whereas this NK subset appears later in patients with relapse occurrence. It is possible that the kinetics of appearance of this NK cell subset matches the beneficial GvL effect early during immune reconstitution. Patients developing GvHD harbored more differentiated NK cells (2B4$^-$, NKp46$^+$, CD57$^+$, and NKG2A$^+$). Functionally, it can be suggested that the lower degranulation of NK cells observed ex vivo at day 100 from patients with GvHD could reflect stronger in vivo activation of NK cells due to the high cell booster of the GvHD context.

The results reported in this study show that inh. KIR/HLA inc. are associated with an improvement of the clinical outcome and particularly with a lower relapse incidence and better survivals for patients presenting this profile in the context of RIC PBSC haplotransplant using PTCy. We propose a model in which KIR/HLA inc. promote activation and proliferation of alloreactive NK cells, which are quickly targeted by PTCy. This activated context seems to promote the differentiation and activation of NK cells associated with GvHD and limiting relapse. In contrast, in the absence of KIR/HLA inc., quiescent KIR NK cells are not eliminated by PTCy and are not efficient to limit relapse. Because many haplodonors can be identified in the patient's family and because of the HLA disparity between donors and recipients, the prediction of NK cell alloreactivity based on KIR/HLA inc. could be of crucial importance for patient outcome and donor selection. Also, further investigations are needed to identify the NK cell subsets that are beneficial for triggering the GvL effect in this context.

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Disclosures

The authors have no financial conflicts of interest.

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