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Role of Donor Activating KIR–HLA Ligand–Mediated NK Cell Education Status in Control of Malignancy in Hematopoietic Cell Transplant Recipients

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Some cancers treated with allogeneic hematopoietic stem cell transplantation (HSCT) are sensitive to natural killer cell (NK) reactivity. NK function depends on activating and inhibitory receptors and is modified by NK education/licensing effect and mediated by coexpression of inhibitory killer-cell immunoglobulin-like receptor (KIR) and its corresponding HLA I ligand. We assessed activating KIR (aKIR)-based HLA I–dependent education capacity in donor NKs in 285 patients with hematological malignancies after HSCT from unrelated donors. We found significantly adverse progression-free survival (PFS) and time to progression (TTP) in patients who received transplant from donors with NKs educated by C1:KIR2DS2/3, C2:KIR2DS1, or Bw4:KIR3DS1 pairs (for PFS: hazard ratio [HR], 1.70; $P = .0020$, $P_{corr} = .0039$; HR, 1.54; $P = .020$, $P_{corr} = .039$; HR, 1.51; $P = .020$, $P_{corr} = .040$; and for TTP: HR, 1.82; $P = .049$, $P_{corr} = .096$; HR, 1.72; $P = .096$, $P_{corr} = .18$; and HR, 1.65; $P = .11$, $P_{corr} = .20$, respectively). Reduced PFS and TTP were significantly dependent on the number of aKIR-based education systems in donors (HR, 1.36; $P = .00031$, $P_{corr} = .00062$; and HR, 1.43; $P = .019$, $P_{corr} = .038$). Furthermore, the PFS and TTP were strongly adverse in patients with missing HLA ligand cognate with educating aKIR-HLA pair in donor (HR, 3.25; $P = .00022$, $P_{corr} = .00045$; and HR, 3.82; $P = .027$, $P_{corr} = .054$). Together, these data suggest

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important qualitative and quantitative role of donor NK education via aKIR-cognate HLA ligand pairs in the outcome of HSCT. Avoiding the selection of transplant donors with high numbers of aKIR-HLA-based education systems, especially for recipients with missing cognate ligand, is advisable.

INTRODUCTION

Natural killer (NK) cells are critical to the innate immune system because they mediate cytotoxic lysis to cancer and virally infected cells [1,2]. In hematopoietic stem/progenitor cell transplantation (HSCT), NK cells play an important role mediating the graft-versus-tumor (GVT) effect [3-7]. Among NK cell receptors, inhibitory killer cell immunoglobulin-like receptors (iKIRs) are of paramount importance because of their ability to recognize ubiquitously expressed host major histocompatibility complex (MHC) class I ligands [8,9] and their regulatory role due to NK cell education or licensing [10]. Apparently, the extracellular domains of some activating killer cell immunoglobulin-like receptors (aKIRs), such as KIR2DS1-4 and KIR3DS1, are highly homologous to their inhibitory counterparts, probably resulting from both the evolution by gene duplication [11] and functional selection. Moreover, the ligation capacity of these activating KIRs and HLA class I ligands has been suggested [8,12]. Despite this homology and ligation to HLA class I, the functionality of aKIRs is not fully assessed, considering their relatively low affinity to the cognate MHC class I ligands [13] and assumed negligible functionality in allo-HSCT. Indeed, except KIR2DS1, which has been shown to interact in education fashion with group 2 HLA-C molecules (C2 group) in vitro and in HSCT settings [14,15], no further functional effects were demonstrated between interacting aKIRs and HLA class I molecules.

HLA class I mismatch between hematopoietic stem/progenitor cells (HSC) in the donor and recipient may lead to "missing self" recognition, defined as the NK cell's capacity to attack allogeneic cells when an HSC donor-recipient pair is MHC class I mismatched [16-18]. This hypothesis assumes that NK cells expressing iKIR cannot be inhibited if the cognate HLA class I ligand is missing or extinguished from target cells. However, some subsets of NK cells lack all known self-MHC–specific inhibitory receptors, yet they are nevertheless self-tolerant [19]. The lack of data on potential role of aKIRs in HSCT prevented their incorporation into the current hypothesis explaining NK cell allorecognition, including the "missing self" recognition model.

It is well established that NK cell inhibition mediated by iKIRs is overseen by the NK cell licensing, mediated by the coexpression of iKIR and its cognate self MHC class I ligand [10,20]. Through the licensing process, only licensed NK cells become fully competent to be triggered through activating receptors, while remaining tolerant to the normal tissues [21,22]. This observation implies that mature NK cell subsets of different killing potential are present in a host depending on stochastic expression of iKIRs and the specificity of ubiquitously expressed HLA ligands. Support for a mirroring role of activating KIRs in NK cell education was demonstrated in an in vitro study where freshly isolated NK cells from donors coexpressing KIR2DS1 and HLA-C2 in homozygous dose were hyporesponsive to cellular targets [23]. In clinical HSCT for patients with acute myeloid leukemia, it was demonstrated that coexpression of activating KIR2DS1 with homozygous C2 ligand in the donor did not provide any antileukemic effect, compared with C1 expressing donors [15], indicating hyporesponsiveness of C2/C2:KIR2DS1 coexpressing NK cells in HSCT setting. Thus, although the idea of the NK cell education function of C2:KIR2DS1 system was supported, it remains elusive for other aKIR genes specific for self-MHC class I. It seems unlikely that the killing capacity of those highly active educated NK cells can be assessed by the association study with only 1 KIR or HLA genetic system considered.

Using a model of germline-encoded activating KIRs and their cognate self MHC class I ligand gene pairs in HSC donors, we investigated whether the NK cell expression of different activating KIRs in the presence of their cognate HLA ligands in donors could affect the clinical outcome in patients with malignant diseases who underwent HSCT. Subsequently, we assessed a potential dose effect of the aKIR-HLA ligand systems in donors, in terms of education capacity and the clinical effect of missing single educating HLA ligand in HSC recipients.

METHODS

Patients and Donors

We evaluated 285 patients with lympho- and myeloproliferative malignancies who received HSC between 2002 and 2010 from unrelated donors matched for 7 to 10 of the possible 10 alleles at HLA-A, -B, -C, -DRB1, and -DQB1 loci. HLA genotyping data at allele level were provided and verified by the Lower Silesian Center for Cellular Transplantation with National Bone Marrow Donor Registry, Institute of Hematology and Transfusion Medicine, and Medical University of Warsaw search centers. Transplantations were facilitated and clinical data and informed consent from the patients were provided by the 9 clinical transplantation centers mentioned in the affiliations. All of the transplantation centers were active European Group for Blood and Marrow Transplantation member teams [24] and adhered to the contemporary European Group for Blood and Marrow Transplantation practice guidelines and recommendations for HSCT procedures. The study was approved by the Wroclaw Medical University ethics committee.

Characteristics of Patients and Donors

Characteristics of the 285 patients with malignant diseases and their donors are listed in Table 1. We compared 2 groups of patients: those with and without aKIR-HLA I ligand education systems. Patient groups were similar with respect to age, diagnosis, risk level, year of transplantation, conditioning regimen, graft-versus-host disease (GVHD) prophylaxis, donor's ethnic group, graft type, degree of HLA matching, and class of HLA mismatch. Patient groups whose donors were sufficient or devoid of aKIR-HLA education systems and those patients with and without HLA ligand for aKIR-HLA education system in donors were evaluated for HSCT outcome and the incidence of acute GVHD (aGVHD) grade II to IV.

KIR Genotyping Method

KIR genotyping was performed on genomic DNA from unrelated HSC donors with the use of PCR amplification with sequence-specific primers. KIR genotyping kit (Inno-Train Diagnostik GmbH, Kronberg, Germany) was used according to the manufacturer's protocol, with minor modifications as detailed previously [25]. Twelve blind samples were KIR retyped for quality control and the results were consistent with primary data.

KIR-ligand Groups and NK Cell Licensing Systems

Regular association study of KIRs or their HLA ligand groups is insufficient to assess the educated NK cells that are likely be more responsive, compared with uneducated NK cells. The novelty of this model of association study depends on 2 level assignments of each donor according to (1) aKIR gene specificity, and (2) cognate HLA class I ligand group (2-level-selection). This assignment can give an insight into potential aKIR-based NK cell education capacity. Further, 3-level-selection according to (1) aKIR gene specificity, (2) cognate HLA class I ligand group in the donor, and (3) HLA class I ligand group in the recipient can give an insight in potential role of

Table 1
Characteristics of Patients with Malignant Diseases and Stem-Cell Donors, according to the HLA-aKIR Education Capacity in Donor (N = 285)

Characteristic	HLA-aKIR Education Capacity in Donor		
	Present (n = 240)	Deficient (n = 45)	*P*
Patient age, median (range), yr	31.7 (1.6-59.5)	28.5 (3.4-55.7)	.56
Diagnosis			
AML	114 (48)	19 (42)	.13
ALL (including BCR-ABL positive)	57 (24)	12 (27)	
Acute leukemia (unspecified)	5 (2)	1 (2)	
Chronic leukemia	51 (21)	10 (22)	
Lymphoma	6 (3)	3 (7)	
Multiple myeloma	7 (3)	0	
Risk level at time of transplantation*			
High	50 (21)	6 (13)	.12
Intermediate	45 (19)	7 (16)	
Low	126 (53)	30 (67)	
Missing data	19 (8)	2 (4)	
Year of transplantation			
2002-2007	113 (47)	27 (60)	.11
2008-2010	127 (53)	18 (40)	
Conditioning regimen			
Myeloablative	145 (60)	29 (64)	.23
Reduced intensity	84 (35)	12 (27)	
Missing data	11 (5)	4 (9)	
GVHD prophylaxis			
Cyclosporine	196 (82)	38 (84)	.19
Methotrexate	102 (43)	19 (42)	
T cell depletion	39 (16)	8 (18)	
Other	10 (4)	1 (2)	
Ethnic group of donor†			
Polish	50 (21)	8 (18)	.64
Other European	190 (79)	37 (82)	
Source of stem cells			
Bone marrow	58 (24)	13 (29)	.30
Peripheral blood	171 (71)	31 (69)	
Missing data	11 (5)	1 (2)	
Degree of HLA matching			
10 of 10 alleles matched	158 (66)	36 (80)	.26
9 of 10 alleles matched	65 (27)	8 (18)	
8 of 10 alleles matched	16 (7)	1 (2)	
7 of 10 alleles matched	1 (<1)	0	
Class of HLA mismatch			
Class I	62 (26)	7 (16)	.30
Class II	14 (6)	2 (4)	
Class I and II	6 (3)	0	

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; BCR-ABL, breakpoint cluster region-Abl 1 (Abelson) fusion gene.
Data presented are n (%) unless otherwise indicated. Present denotes presence of at least 1 aKIR-based HLA ligand-mediated NK cell education system in donor. Deficient denotes aKIR-null donors.
* Low risk indicates first complete remission or chronic phase; intermediate risk, second or higher complete remission or chronic phase; and high risk, primary induction failure, relapse or progression.
† All patients reported ethnic group of European Polish ancestry.

aKIR-educated donor NK cells in immunosurveillance upon malignant cells in recipients. Specifically, analogous to iKIR-based NK cell licensing, the lack of this particular HLA ligand in the recipient may be potentially associated with "downward resetting" of aKIR-based education [26]. Alleles of HLA-A,-B, and-C loci were assigned to 4 aKIR-ligating groups (C1, C2, Bw4, and A11/C2/4/5/16), according to 77 to 83 amino acid residue sequence of the HLA class I heavy chain [27], as listed by Dorak [28] and Graef et al. [12]. Because inhibitory function of those infrequent HLA-A alleles with Bw4 epitope was proven, they were incorporated into the Bw4-activating KIR ligand group [29]. NK cell education status was defined as discovery of germline-encoded and potentially coexpressed aKIR and its self MHC I ligand gene pair in a donor. By analogy to experimental data for iKIRs, we assessed the clinical role of NK cell subsets educated via aKIR-HLA pairs in such donors [14,23]. Functional cognate HLA-aKIR gene pairs were assigned according to the classification described by Long and Rajagopalan [8] and Graef et al. [12], who described ligation capacity of these receptors and ligands. Generally, the following 4 HLA-aKIR cognate gene pair types (aKIR-based NK cell education systems) were assigned in different donors in different combinations: C1:KIR2DS2 and/or C1:KIR2DS3 (C1:KIR2DS2/3), C2:KIR2DS1, Bw4:KIR3DS1, and A11/C2/4/5/16:KIR2DS4 (2-level-selection). In HLA class I–mismatched donor-recipient pairs, the status of patients missing HLA ligands cognate with donors' aKIR-educated NK cells was established (3-level-selection) and clinical outcomes were compared with that of patients with the HLA ligand.

Statistical Analysis

Cox regression was used to examine the hazard ratios of failure for time-to-event outcomes of HSCT (progression-free survival [PFS] and time to progression [TTP]) with HLA-aKIR education status in donor. *Failure for PFS* was defined as relapse, progression, or death, whichever occurred first. *TTP* was defined as time to relapse or progression. *Relapse* was defined as hematological, cytological, or molecular relapse, whichever was diagnosed first, of acute leukemia in complete remission or chronic leukemia in chronic phase. *Progression* was defined as progression to more advanced stage of malignant disease without complete remission or chronic phase at transplantation. Logistic regression was used to assess the association of donor aKIR-HLA education status with the probability of aGVHD grade II to IV. Wherever possible, models were adjusted for the patient's age, diagnosis, level of risk (low or intermediate/high), and conditioning regimen (myeloablative or reduced-intensity conditioning). As we have analyzed similar data as iKIR-based NK cell licensing in the prior publication [30], adjustments were made for multiple comparisons using the Bonferroni inequality method, according to the formula $P_{corr} = 1-(1-P)^k$, where k equals the number of comparisons. Estimates of PFS were obtained with the Kaplan-Meier method, and cumulative incidence estimates were used to summarize the probability of time-to-event outcomes. Death without relapse or progression was regarded as a competing risk for purposes of estimating the TTP. Statistics were estimated and Kaplan-Meier survival analyses were made using Statistica 9.1 package (StatSoft, Tulsa, OK).

RESULTS

Distribution of aKIR-based NK Cell Education Systems

To assess aKIR-HLA–mediated education in clinical transplantation, we first evaluated the distribution of HLA ligand-aKIR-based education systems in 285 transplant donors for patients with malignant diseases. The frequencies of HLA class I ligand groups and aKIR genes in the patients were consistent with gene frequencies in previously published studies [31,32]. The majority of donors (n = 240, 84%) encoded from 1 to 4 putative aKIR-HLA education systems (C1:KIR2DS2/3, C2:KIR2DS1, Bw4:KIR3DS1, and/or A11/C2/4/5/16:KIR2DS4 systems) (Table 2). However, in 45 (16%) donors (aKIR-HLA null donors) the HLA-A–, B–, and C–dependent aKIR-based education systems were not detected. In 113 (40%) donors, a single aKIR education system was revealed, and in the remaining 128 (44%) donors, 2 or more systems overlapped. The level of overlapping is characterized in Table 2.

aKIR-based Education Systems in Donor and Clinical Outcome

To clarify the contribution of different aKIRs to the HLA-ligand dependent functionality in NK cell education, we compared PFS and TTP in HSCT recipients who underwent transplantation from donors with coexpression of aKIR and cognate HLA ligand versus recipients who underwent transplantation from donors without a corresponding aKIR-based education system. We found a significantly reduced PFS and increased rate of progression, estimated as TTP, in patients who underwent transplantation from donors who coexpressed C1:KIR2DS2/3 molecules, compared with patients who underwent transplantation from donors negative for C1-based system (42.4% versus 56.9%; hazard ratio [HR], 1.70; 95% confidence interval [CI], 1.22 to 2.38; $P = .0020$, $P_{corr} = .0039$; median time to event, 185.5 versus 421 days;

Table 2
Distribution of HLA-aKIR–based Education Systems in Donors for Patients who Underwent Transplantation with Malignant Diseases (N = 285)

HLA-aKIR–based Education Systems in Donors	Distribution
Presence of aKIR-HLA-based education systems	
C1:KIR2DS2 or C1:KIR2DS3, n (%)	137 (48)
C2:KIR2DS1, n (%)	71 (25)
Bw4:KIR3DS1, n (%)	88 (31)
A11/C2/4/5/16:KIR2DS4, n (%)	137 (48)
Number of aKIR-HLA based education systems, n (%)	
0	45 (16)
1	113 (40)
2	78 (27)
3	32 (11)
4	17 (6)
Overlapping of aKIR-HLA based education systems, no./n (%)	
C1:KIR2DS2 or C1:KIR2DS3, no./n (%)	
Single	41/137 (30)
Overlapped	96/137 (70)
C2:KIR2DS1, no./n (%)	
Single	3/71 (4)
Overlapped	68/71 (96)
Bw4:KIR3DS1, no./n (%)	
Single	8/88 (9)
Overlapped	80/88 (91)
A11/C2/4/5/16:KIR2DS4, no./n (%)	
Single	60/137 (44)
Overlapped	77/137 (56)
Total, no./n (%)	
Single	113/240 (47)
Overlapped	127/240 (53)
At least 1 of C1 and C2 education systems	169/285 (59)
At least 1 out of C1, C2 and Bw4 education systems	179/285 (63)
At least 1 of C1, C2, Bw4 and A11/C2/4/5/16 education systems	240/285 (84)

Education system denote coexpression of C1 and KIR2DS2 or KIR2DS3, C2 and KIR2DS1, Bw4 and KIR3DS1, or A11/C2/4/5/16 and KIR2DS4 genes in donor.

and 21.4% versus 15.3%; HR, 1.82; 95% CI, 1.00 to 3.31; $P = .049$, $P_{corr} = .096$; median time to event, 186 versus 402.5 days; for PFS and TTP, respectively) (Figure 1A,B). Similarly, significantly reduced PFS and TTP were found for C2:KIR2DS1 and Bw4:KIR3DS1 systems in donors (39.7% versus 53.6%; HR, 1.54; 95% CI: 1.07 to 2.23; $P = .020$, $P_{corr} = .039$; median time to event, 173.5 versus 356 days; and 23.3% versus 16.5%; HR, 1.72; 95% CI, .91 to 3.24; $P = .096$, $P_{corr} = .18$; median time to event, 176.5 versus 351 days; for C2:KIR2DS1 system, respectively; and 40.5% versus 54.4%; HR, 1.51; 95% CI, 1.07 to 2.14; $P = .020$, $P_{corr} = .040$; median time to event, 183.5 versus 359 days; and 27.7% versus 16.2%; HR, 1.65, 95% CI, .90 to 3.02; $P = .11$, $P_{corr} = .20$; median time to event, 199 versus 356 days; for Bw4:KIR3DS1 system, respectively) (Figure 1C-F). These results suggest functionality of C1:KIR2DS2/3, C2:KIR2DS1, and Bw4:KIR3DS1 aKIR-HLA–based systems in education of NK cells. The adverse clinical outcomes in patients with malignancy who underwent HSCT suggests that NK cells educated by these systems are hyporesponsive against tumors. However, for patients who underwent transplantation from A11/C2/4/5/16:KIR2DS4-positive donors, the clinical outcome was similar to patients with donors negative for this system (48.1% versus 52.1%; HR, 1.03; 95% CI .74 to 1.44; $P = .85$, $P_{corr} = .96$; median time to event, 335 versus 292.5 days; and 21.7% versus 15.0%; HR, 1.43; 95% CI, .78 to 2.59; $P = .24$, $P_{corr} = .43$; median time to event, 297 versus 349 days; for PFS and TTP, respectively in patients with and without A11/C2/4/5/16:KIR2DS4 system in donor) (Figure 1G,H).

Quantity of aKIR-based Education Systems

Given the overlapping of different aKIR-HLA education systems (from 0 to 4 systems in a donor) (Table 2), we sought to determine whether the number of the aKIR-based education systems in the donor affected the clinical outcome in HSCT recipients. We found highly significantly reduced PFS and TTP with an increasing number of aKIR-based education systems. For 0 to 2 systems in a donor (including C1:KIR2DS2/3 and C2:KIR2DS1 systems) we found decreased median time to event being highly associated with increased number of systems (median time to event: 463, 218, and 157.5 days; HR, 1.60; 95% CI, 1.26 to 2.04; $P = .00013$, $P_{corr} = .00026$; and median time to event, 421, 225, and 157.5 days; HR, 1.76; 95% CI, 1.15 to 2.70; $P = .0098$, $P_{corr} = .019$; for PFS and TTP, respectively) (Figure 2A,B). For 0 to 3 aKIR-based systems in a donor (including C1:KIR2DS2/3, C2:KIR2DS1, and Bw4:KIR3DS1 systems), the PFS and TTP were similarly adversely associated with the number of aKIR-based education systems in donor (HR, 1.36; $P = .00031$, $P_{corr} = .00062$; and HR, 1.43; $P = .019$, $P_{corr} = .038$; for PFS and TTP, respectively) (Figure 2C,D). These data suggest that coexpression of multiple aKIRs and cognate HLA ligands in donors (education system overlapping) can increase the tolerization of antitumor NK cell subsets and worsen the clinical course in quantitative manner.

We did not find a difference in clinical outcome among malignant patients after HSCT from aKIR-expressing donors with homozygous or heterozygous cognate HLA ligand background (data not shown).

Quality of aKIR-based Education System

To assess qualitative variability among different education systems, we compared clinical outcomes in patients who underwent transplantation from aKIR-HLA null donors to those with single positive (sp) C1:KIR2DS2/3 and spA11/C2/4/5/16:KIR2DS4 donors when bias from overlapping systems in donors was excluded. Given that HLA-C*02, C*04, and C*05 molecules (but not A*11 and C*16 molecules) potentially ligate both KIR2DS4 and KIR2DS1 (receptor for C2 group) [8,12] and might produce a partial functional overlapping, we excluded donor-recipient pairs with C2:KIR2DS1-positive donors from the comparison. Additionally, patients who underwent transplantation from donors positive for HLA-C*16:01, a member of C1 group, were excluded to avoid potential partial overlapping between KIR2DS4- and KIR2DS2/3-based NK cell education. In this comparison, the bias of overlapping systems was reduced as far as possible, and a role of spC1- and spA11/C2/4/5-based aKIR education was compared with patients who underwent transplantation from aKIR-HLA null donors. In patients who underwent transplantation from donors with spC1:KIR2DS2/3, the PFS was the adverse of that of patients who underwent transplantation from aKIR-null and spA11/C2/4/5-KIR2DS4-positive donors, but these differences were of limited significance (51.2%, 55.6%, and 68.4%; median time to event, 359, 365, and 553 days; HR, .86; 95% CI, .75 to 1.00; $P = .052$, $P_{corr} = .10$; for spC1:KIR2DS2/3, aKIR-HLA null, and spA11/C2/4/5-KIR2DS4 systems, respectively) (Figure 3A). For TTP, the differences were insignificant (16.7%, 7.7%, and 12.5%; median time to event, 357.5, 348, and 522.5 days; HR, .99; 95% CI, .75 to 1.31; $P = .93$, $P_{corr} = 1.00$; for spC1:KIR2DS2/3, aKIR-null, and spA11/C2/4/5-KIR2DS4 systems, respectively) (Figure 3B). These outcomes may indicate the low education capacity of single aKIR-HLA–based system and are in line with quantitative role of multiple systems. Likely, the A11/C2/4/5-KIR2DS4 system is weakly involved in NK cell education.

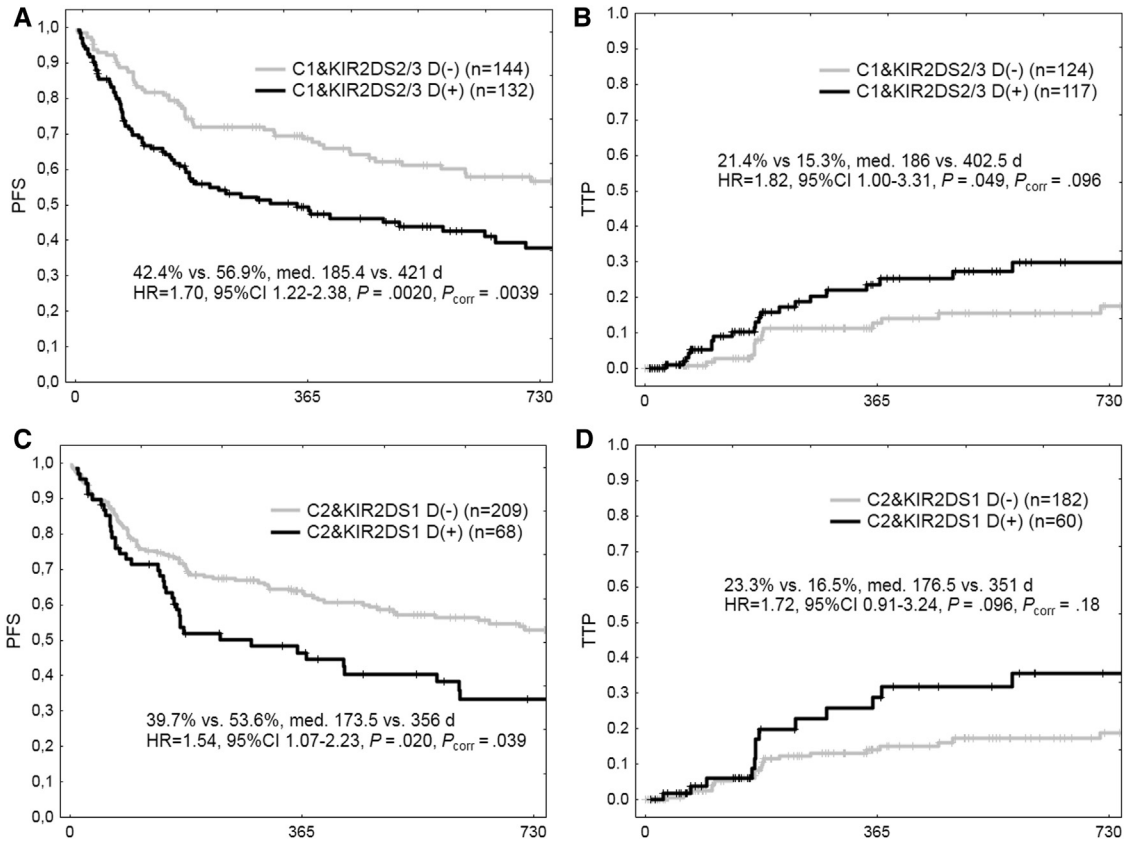

Figure 1. Clinical outcome in transplant recipients with malignant diseases depending on activating KIR-cognate HLA ligand-mediated NK cell education in donor. The cumulative PFS (left panels) and TTP (right panels) are shown for patients with (A,B) C1:KIR2DS2/3, (C,D) C2:KIR2DS1, (E,F) Bw4:KIR3DS1, and (G,H) A11/C2/4/5/16:KIR2DS4 NK cell education systems in donors, compared with patients whose donors were deficient with the corresponding system. D(+) denotes positive in donor; D(−), negative in donor. P_{corr} denotes P value corrected using Bonferroni inequality method according to the formula $P_{corr} = 1-(1-P)^k$ where k equals the number of comparisons.

This effect may be attributed to the complex functionality of KIR2DS4 activating receptor or its low affinity to cognate HLA ligands [12,33-35], as discussed further.

aKIR-based NK Cell Education Effect was Not Associated with HLA Disparity and aGVHD

To evaluate a potential bias from HLA mismatch that alone may influence the clinical outcome in HSC recipients, we compared the PFS and TTP in patients who underwent transplantation from donors with and without aKIR-HLA–based education system when the study group was restricted to HLA-mismatched donor-recipient pairs. As in the total group, the PFS and TTP of the corrected group remained adverse upon aKIR-based education in donors, suggesting no influence of the HLA mismatch itself (Table 3). Additionally, a kind of hierarchy of the clinical effect was confirmed in this comparison, with the adverse effect becoming weaker, starting from C1:KIR2DS2/3 through C2:KIR2DS1 and Bw4:KIR3DS1 to A11/C2/4/5/16:KIR2DS4 system (for PFS, from HR, 3.17, $P = .00024$, $P_{corr} = .00048$; HR, 2.03; $P = .021$, $P_{corr} = .042$; HR, 1.89, $P = 034$, $P_{corr} = .067$; to HR, .84; $P = .57$, $P_{corr} = .82$, respectively; and for TTP, from HR, 5.97; $P = .0071$, $P_{corr} = .014$; HR, 2.82; $P = .065$, $P_{corr} = .13$; HR, 1.73; $P = .34$, $P_{corr} = .56$; to HR, .85, $P = .77$, $P_{corr} = .95$, respectively) (Table 3). By this hierarchy, a qualitative variability of aKIR-based education systems seems to be supported.

To assess the involvement of the acute GVHD (the major factor of morbidity and mortality in HSCT) in the clinical outcome in patients with aKIR-HLA–mediated NK cell education, we compared the incidence of grade II to IV aGVHD in patients who underwent transplantation from donors with and without an aKIR-based education systems. No significant associations of aGVHD grade II to IV were found in patients whose donors were sufficient versus deficient with either of an aKIR-based education system tested (for detailed results see, Table 4). These results suggest that clinical effects in patients who received a transplant from donors with an aKIR-HLA–based educated NK cells were independent of the degree of HLA matching and not associated with the incidence of aGVHD of intermediate/high grade.

Missing Patient HLA Ligand for Donor aKIR-HLA–based Education System

To assess the effectory stage of aKIR-HLA–educated NK cells, we compared PFS and TTP in patients with and without an HLA ligand cognate for aKIR involved in education in the donor (3-level selection, see Methods). We found deeply reduced PFS and increased relapse/progression rate among patients with malignant diseases with the missing single HLA ligand cognate with aKIR involved in NK cell education in donors, compared with patients possessing all cognate HLA ligands (100% versus 50.2%; HR, 3.25; 95% CI, 1.74 to 6.08; $P = .00022$, $P_{corr} = .00045$; median time to event, 119 versus 286 days; and 33.3% versus 19.2%; HR, 3.82; 95% CI,

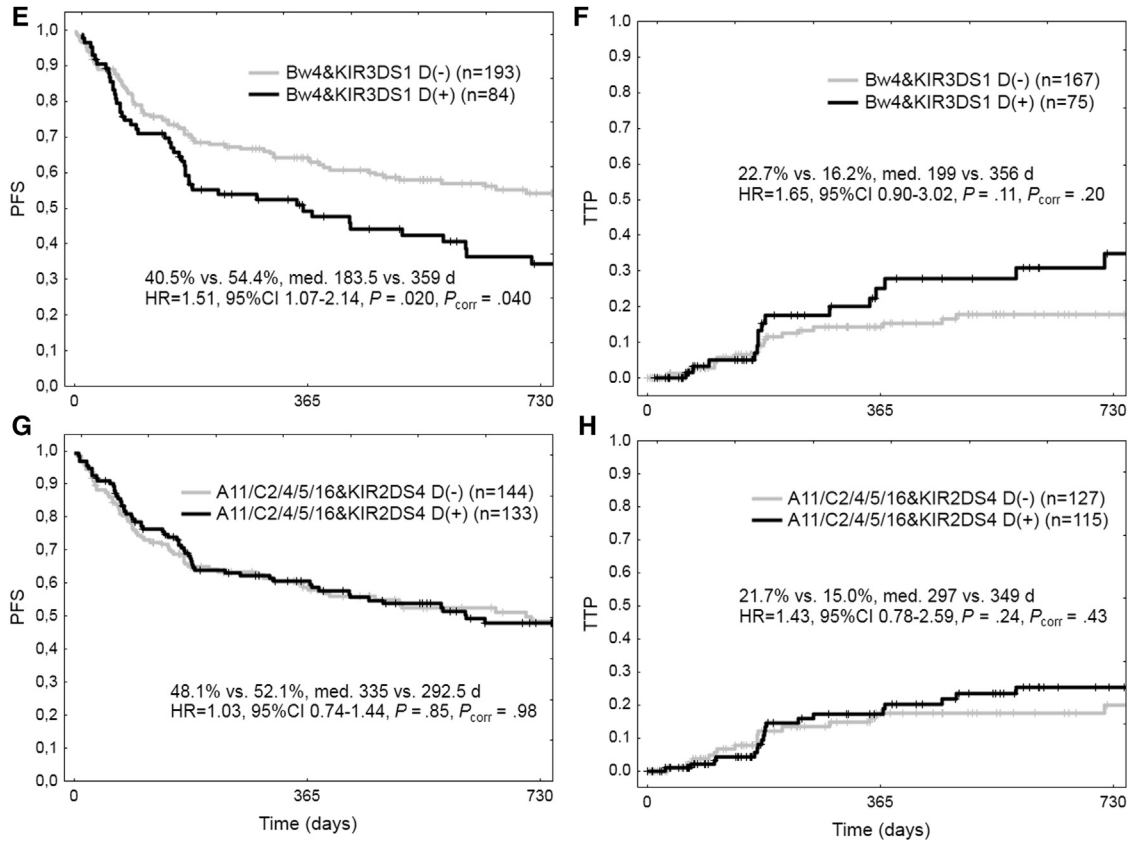

Figure 1. (*continued*).

1.16 to 12.58; $P = .027$, $P_{corr} = .054$; median time to event, 119 versus 284 days; for PFS and TTP, respectively) (Figure 4A,B). The effect was similar if compared groups were restricted to HLA-mismatched donor-recipient pairs (100% versus 48.5%; HR, 3.45; 95% CI 1.67 to 7.13; $P = .00083$, $P_{corr} = .0017$; median time to event, 106.5 versus 381 days; and 37.5% versus 16.9%; HR, 4.26; 95% CI, 1.14 to 15.90; $P = .031$, $P_{corr} = .061$; median time to event, 105 versus 372 days; for PFS and TTP, respectively) (Table 3). These findings strongly suggest that the NK cell anticancer function is abrogated if the HLA ligand cognate with donor's educating aKIR is missing in patients with malignancy. The functionality of a missing education ligand in patients seems to be independent of the level of HLA mismatch itself. For cancer immunosurveillance, these results may indicate that the education of NK cells by aKIR-cognate HLA ligand pairs is as important as licensing via iKIR-HLA ligand pairs and the lack of ligation of aKIRs expressed specifically by educated NK cells can decompose the immunosurveillance.

The incidence of grade II to IV aGVHD was similar in patients deficient with a single HLA ligand cognate with any aKIR-HLA–based education system in donors, and those sufficient with all cognate HLA ligands (36.4% versus 30.2%; odds ratio [OR], 1.32; 95% CI, .37 to 4.68; $P = .67$, $P_{corr} = .89$; and 36.4% versus 39.1%; OR, .89; 95% CI, .24 to 3.36; $P = .87$, $P_{corr} = .98$; for all donor-recipient pairs and the subset of patients with HLA-mismatched donor, respectively) (Table 4). The incidence of intermediate/high-grade aGVHD seems to be independent of whether the missing HLA ligand in patient was cognate or not with the aKIR-HLA–mediated NK cell education system in the respective donor.

DISCUSSION

To our knowledge, this is the first study showing systematic clinical relevance of NK cell education via different aKIRs and cognate HLA ligands in patients with malignant diseases after HSCT. Coexpression of C1:KIR2DS2/3, C2:KIR2DS1, and/or Bw4:KIR3DS1 aKIR-HLA pairs in donors is functional in NK cell education, can implicate NK cell hyporesponsiveness to malignant cells in the recipient, and can worsen both survival and relapse/progression rate in patients with malignancy after HSCT. These data are consistent with education effects observed earlier for C2:KIR2DS1 axis, both in vitro and in the HSCT setting [15,23].

The NK cell education via aKIRs results in quantitative effect where a higher number of aKIR-cognate HLA pairs in donors implicates larger hyporesponsiveness to the recipients' malignant cells. This finding is in line with some of the association studies in acute myeloid leukemia/myelodysplastic syndrome patients who received transplants from donors with a low number of aKIR genes or donors with KIR group A haplotype, known to carry only 1 aKIR. In these patients, a reduced relapse rate and improved disease-fee survival were found [36,37]. Although these studies did not consider NK cell education and were 1-level selection association studies, the aKIR-based education was less probable when the number of aKIR genes was low in the donor. Due to the independent segregation of HLA and KIR genes and preferential nonstochastic mode of aKIR expression [38,39], it is likely that in the presence of multiple aKIR-HLA–based education systems encoded in the donor, multiple NK cell clones could be hyporesponsive to the patient's malignant cells, and relapse or progression can occur more frequently.

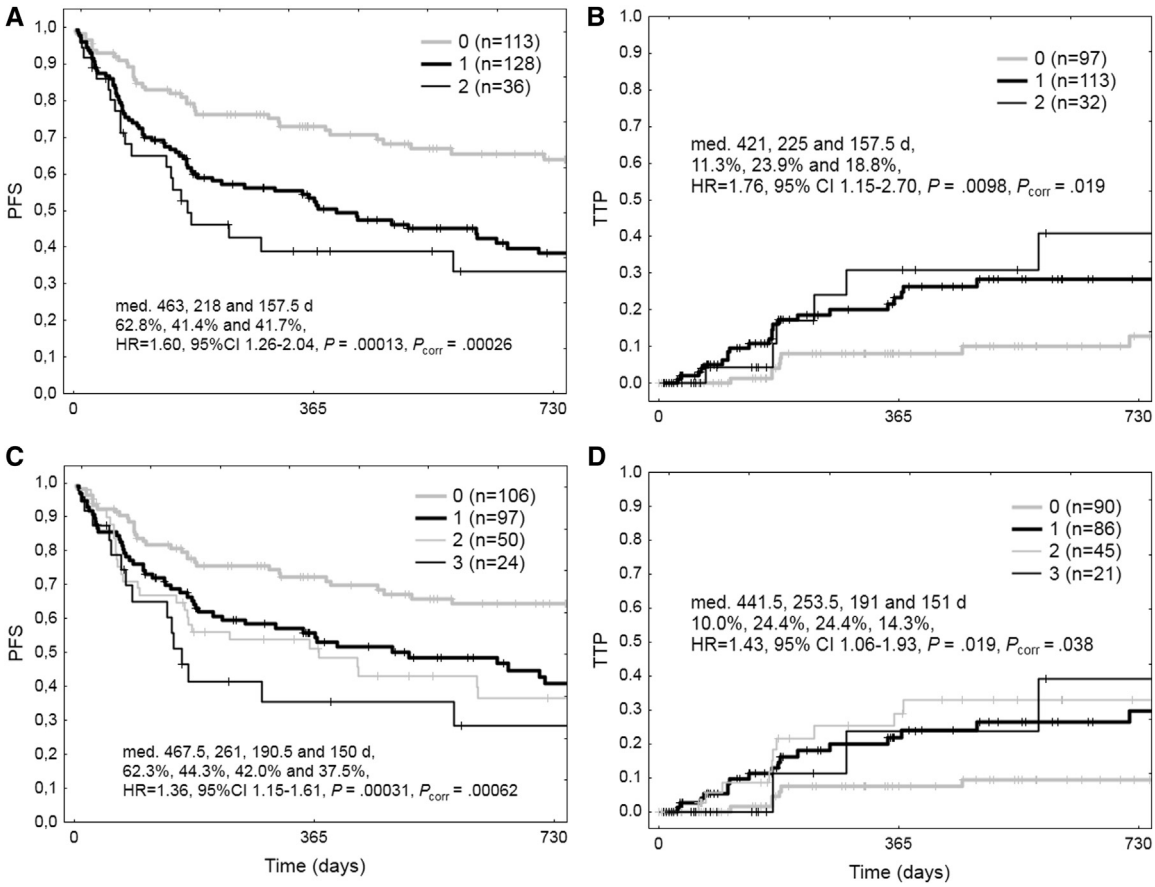

Figure 2. Clinical outcome in transplant recipients with malignant diseases depending on the number of activating KIR-cognate HLA ligand NK cell education systems in the donors. The cumulative PFS (left panel) and TTP (right panel) are shown for patients who received transplants from donors with (A,B) 0, 1, or 2 systems out of C1:KIR2DS2/3 and C2:KIR2DS1 groups, and (C,D) 0, 1, 2, or 3 systems of C1:KIR2DS2/3, C2:KIR2DS1, and Bw4:KIR3DS1. Abbreviations are explained in Figure 1.

It appeared that the clinical effect was different for different NK cell education systems, with strongest effect for C1:KIR2DS2/3 and gradually weaker effect for C2:KIR2DS1, Bw4:KIR3DS1, and A11/C2/4/5/16:KIR2DS4 systems. These data point to qualitative mode of aKIR-HLA–mediated NK cell education, as well. For qualitative functionality, it might be important that different aKIR genes and their alleles inherently show various NK cell expressions and affinities to

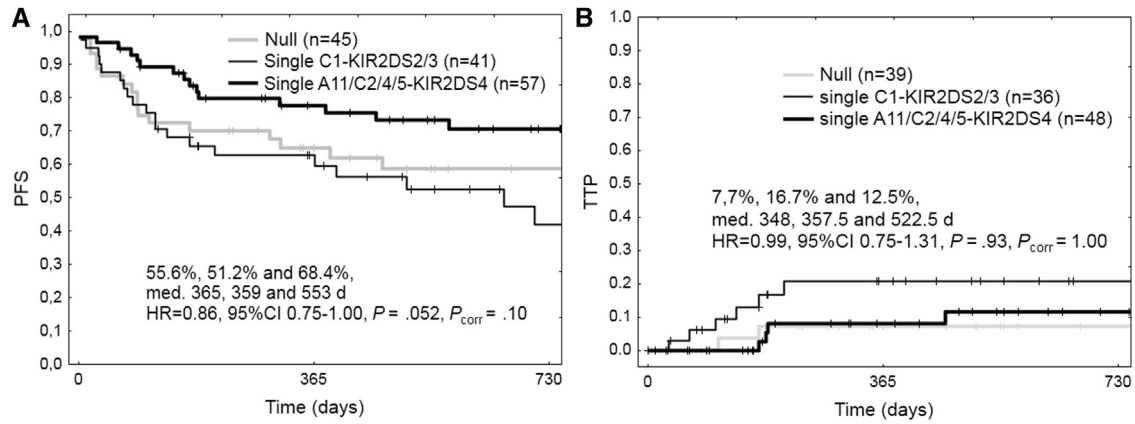

Figure 3. Clinical outcome in transplant recipients with malignant diseases depending on the presence of single positive (sp)A11/C2/4/5/16-KIR2DS4 or spC1-KIR2DS2/3 education system or the lack (aKIR-HLA–null donors) of aKIR-mediated NK cell education. Donor-recipient pairs with overlapping education systems in donor were excluded. (A) The cumulative PFS and (B) TTP in patients with single A11/C2/4/5-KIR2DS4 NK cell education system in donors, single C1-KIR2DS2/3, or deficient for the education system (null donors). In these comparisons, patients who received transplants from C2-KIR2DS1–positive donors were excluded because HLA-C*02, C*04, and C*05 molecules can ligate both KIR2DS4 and KIR2DS1 (the receptor for group C2 ligands) and induce a partial functional overlapping with C2-based education systems. Patients who received transplants from HLA-C*16:01-positive donors were also excluded because HLA-C*16:01 molecule can ligate both KIR2DS4 and KIR2DS2/3 (the receptors for group C1 ligands) and induce a partial functional overlapping with C1-based education system. Abbreviations are explained in Figure 1.

Table 3
PFS and TTP in HSC Recipients with Malignant Diseases Depending on aKIR-Mediated NK Cell Education in Donor or Missing Ligand in Recipient Cognate with Educating aKIR in Donor

aKIR-Based NK Cell Education Status	n	Education D+	Education D(−)	HR	95% CI	*P* Value	P_{corr}
		Events/n (%) median time to event, d					
PFS							
KIR2DS2/3 & C1 D(+) versus KIR2DS2/3 & C1 D(−)	87	31/43 (72.1) 138	16/44 (36.4) 662	3.17	1.71-5.86	.00024	.00048
KIR2DS1 & C2 D(+) versus KIR2DS1 & C2 D(+)	87	17/22 (77.3) 261,5	30/65 (46.2) 356	2.03	1.11-3.69	.021	.042
KIR3DS1 & Bw4 D(+) versus KIR3DS1 & Bw4 D(−)	87	18/23 (78.3) 349	29/64 (45.3) 352	1.89	1.05-3.42	.034	.067
KIR2DS4 & A11/C5/2/4/16 D(+) versus KIR2DS4 & A11/C5/2/4/16 D(−)	87	28/52 (53.8) 387	19/35 (54.3) 225	.84	.47-1.51	.57	.82
TTP							
KIR2DS2/3 & C1 D(+) versus KIR2DS2/3 & C1 D(−)	75	10/37 (27.0) 138	3/38 (7.9) 618.5	5.97	1.63-21.91	.0071	.014
KIR2DS1 & C2 D(+) versus KIR2DS1 & C2 D(+)	75	6/21 (28.6) 237	7/54 (13.0) 352.5	2.82	.94-8.46	.065	.13
KIR3DS1 & Bw4 D(+) versus KIR3DS1 & Bw4 D(−)	75	5/21 (23.8) 349	8/54 (14.8) 292.5	1.73	.56-5.34	.34	.56
KIR2DS4 & A11/C5/2/4/16 D(+) versus KIR2DS4 & A11/C5/2/4/16 D(−)	75	8/45 (17.8) 384	5/30 (16.7) 198.5	.85	.28-2.59	.77	.95
aKIR-Based NK Cell Education in Donor and Missing Cognate Ligand in Recipient		Education D+; Lig R(−)	Education D+; Lig R(+)				
PFS							
aKIR Edu. D(+) HLA ligand R(−) versus aKIR Edu. D(+) HLA Ligand R(+)	78	10/10 (100) 106.5	33/68 (48.5) 381	3.45	1.67-7.13	.00083	.0017
TTP							
aKIR Edu. D(+) HLA Ligand R(−) versus aKIR Edu. D(+) HLA Ligand R(+)	67	3/8 (37.5) 105	10/59 (16.9) 372	4.26	1.14-15.90	.031	.061

D+/D(−), positive or negative status in donor, respectively; R+/R(−), positive or negative status in recipient, respectively; aKIR Edu., denotes activating killer cell immunoglobulin-like receptor-cognate HLA ligand education system.
Data restricted to HLA mismatched donor-recipient pairs. Comparisons were adjusted for patient's age (lower inclusive, or higher than 18), diagnosis (lympho- or myeloproliferative disease), level of risk (low or intermediate/high), and conditioning regimen (myeloablative or reduced intensity).

HLA ligands [8,12,38-40]. In this cohort, the biological effect of NK cell education was strong for C1:KIR2DS2/3, C2:KIR2DS1, and Bw4:KIR3DS1 education systems in donors, whereas the A11/C2/4/5/16:KIR2DS4 system showed negligible biological impact. The explanation for this low education functionality can stem from the relative frequency of full-length and dysfunctional 22-del KIR2DS4 gene variants with 17% and 63% gene frequencies, respectively, in ethnically matched healthy population [31]. The product of deleted variant of KIR2DS4 is not anchored to the cell membrane but encodes a soluble form of the protein that is potentially secreted [41]. It is likely that the shedding phenomenon can eliminate the education capacity of KIR2DS4-del molecule. It is also possible that non-HLA class I–dependent activating receptors that are present in aKIR-HLA-null donors and their stress-induced ligands (such as NKG2D-MICA/B system and NKp30, NKp44, and/or NKp46 of uncertain ligands) [42] are potentially functional in NK cell education in patients with malignancy. This suggestion is in line with the data that, in contrast to clonal expression of

Table 4
Incidence of Grade II to IV aGVHD in HSC Recipients with and without aKIR-HLA Ligand-Dependent NK Cell Education in Donors and Missing Cognate HLA Ligand in Recipients

aKIR-Based NK Cell Education Status	n	aGVHD Incidence/n (%)		OR	95% CI	*P*	P_{corr}
		Edu. D(+) events/n (%)	Edu. D(−) events/n (%)				
All pairs							
KIR2DS2/3 & C1 D(+) versus KIR2DS2/3 & C1 D(−)	262	36/125 (28.8)	42/137 (30.7)	.91	.54-1.56	.74	.93
KIR2DS1 & C2 D(+) versus KIR2DS1 & C2 D(+)	263	25/65 (38.5)	53/198 (26.8)	1.71	.95-3.10	.075	.14
KIR3DS1 & Bw4 D(+) versus KIR3DS1 & Bw4 D(−)	263	22/79 (27.8)	56/184 (30.4)	.88	.49-1.58	.67	.89
KIR2DS4 & A11/C5/2/4/16 D(+) versus KIR2DS4 & A11/C5/2/4/16 D(−)	263	37/127 (29.1)	41/136 (30.1)	.95	.56-1.62	.86	.98
		Edu. D(+) Lig R(−)	Edu. D(+) Lig R(+)				
aKIR Edu. D(+) HLA Ligand R(−) versus aKIR Edu. D(+) HLA Ligand R(+)	213	4/11 (36.4)	61/202 (30.2)	1.32	.37-4.68	.67	.89
HLA mismatched pairs		Edu. D(+)	Edu. D(−)				
KIR2DS2/3 & C1 D(+) versus KIR2DS2/3 & C1 D(−)	83	15/41 (36.6)	15/42 (35.7)	1.04	.42-2.54	.93	1.00
KIR2DS1 & C2 D(+) versus KIR2DS1 & C2 D(+)	83	10/20 (50.0)	20/63 (31.7)	2.15	.77-5.10	.14	.26
KIR3DS1 & Bw4 D(+) versus KIR3DS1 & Bw4 D(−)	83	9/22 (40.9)	21/61 (34.4)	1.32	.48-3.59	.59	.83
KIR2DS4 & A11/C5/2/4/16 D(+) versus KIR2DS4 & A11/C5/2/4/16 D(−)	83	20/50 (40.0)	10/33 (30.3)	1.53	.60-3.90	.37	.60
		Edu. D(+) Lig R(−)	Edu. D(+) Lig R(+)				
aKIR Edu. D(+) HLA ligand R(−) versus aKIR Edu. D(+) HLA ligand R(+)	75	4/11 (36.4)	25/64 (39.1)	.89	.24-3.36	.87	.98

Lig, cognate HLA ligand in recipient; aKIR Edu., activating killer cell immunoglobulin-like receptor-cognate HLA ligand education system in donor depending on coexpression of *C1 and KIR2DS2/3, C2 and KIR2DS1, Bw4 and KIR3DS1* or *A11/C2/4/5/19 and KIR2DS4* gene pairs. Further abbreviations are explained in Table 3.

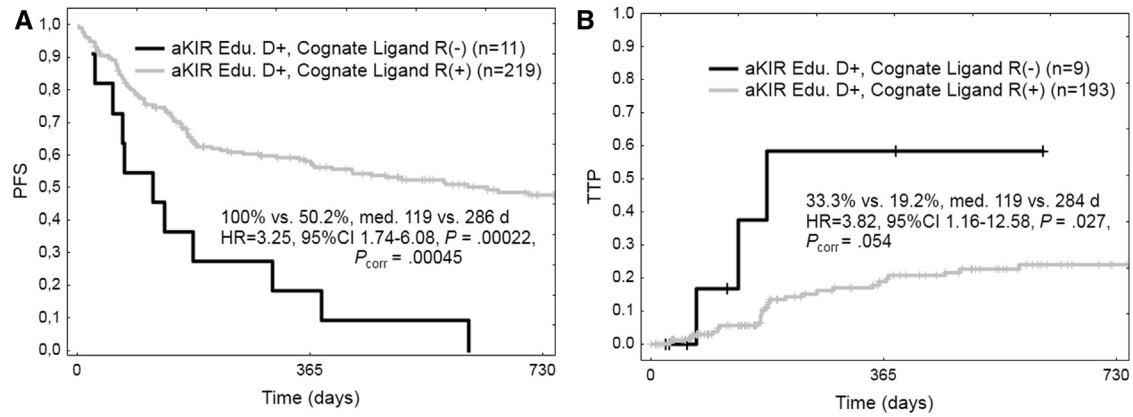

Figure 4. Clinical outcome in transplant recipients with malignant diseases depending on the missing HLA ligand in patients cognate with activating KIR involved in NK cell education in donor. (A) The cumulative PFS and (B) TTP are shown for patients with missing HLA ligand for C1:KIR2DS2/3, C2:KIR2DS1, or A11/C2/4/5/16:KIR2DS4 NK cell education system in donors, compared with patients who were sufficient with all corresponding HLA ligands. aKIR Edu., denotes activating KIR-HLA ligand education system in donor; R(+), positive in recipient; R(−), negative in recipient. Further abbreviations are explained in Figure 1.

aKIRs, the expression of other activating non-KIR receptors is common on nearly all NK cells in steady state conditions [43] and upon tumor challenge can outweigh a weak aKIR-type education function of A11/C2/4/5/16:KIR2DS4 system [44].

Furthermore, our findings suggest that the NK cell antitumor function in HSCT setting strongly depends on the presence of educating HLA ligand in recipient cells. Although generally withstanding the Bonferroni correction, this set of data is hindered by a small strata rendering the data on PFS and TTP comparisons unsettled. We present them to show some intriguing directions for further study. The effect of the recipient's missing ligand cognate with donor's NK cell clones educated via aKIR-HLA is strongly adverse and results in lower PFS and shorter TTP. This observation contrasts with the favorable role of the model "missing self" recognition that involves the missing ligand for NK cell clones unselected for their education status [17,45]. Our results may suggest the dysregulation of the NK cell–mediated cancer immunosurveillance when aKIR-HLA–educated NK cells of transplant origin can no longer be engaged by the cognate HLA ligand in the recipient malignant cells. Although aKIR-HLA–based NK cell education leads to hyporesponsiveness, the cognate-educating HLA ligand of the cancer patient appears to be involved in triggering the tumor-killing function in the educated donor NK cells. This observation is in line with in vitro data where patients' HLA-C2 homozygous leukemia blasts and Epstein-Barr virus–transformed B cell lines were very efficiently killed by donor NK cell clones educated via C2:KIR2DS1 [6]. The clone was cytolytic against the target cells, and lysis was strongly inhibited by blocking KIR2DS1, indicating a positive role of aKIR even in hyporesponsive, educated NK cells. It seems likely that, compared with uneducated NK cells, these NK cells educated via aKIRs and cognate HLA may play a crucial role in cancer immunosurveillance.

The adverse impact of the recipient's missing HLA ligand, the 1 cognate with donor's aKIR, was similar to our previous results for donor iKIRs [30], wherein we observed an adverse impact of missing ligands in recipients cognate with an iKIR-HLA licensing system in the donor. These 2 harmful receptor-ligand constellations together can be explained by the finding that multiple receptors have been implicated in the cocreation of immunological synapses and close cooperation in natural cytotoxicity. In immunological synapse, the inhibitory and activating KIR-HLA signaling is integrated with additional components (eg, accessory molecules and non-KIR inhibitory and activating receptors after engagement by transformation-induced ligands) [46]. These molecules with their ligands regulate 2 main effector functions of NK cells: the adhesion to the target cell and the release of cytotoxic granules. Although little is known about the mechanism of NK cell education, it was shown that the engagement of activating receptors with associated DAP-12 immunoreceptor by the cognate ligand induces the increased NK cell conjugation to target cell due to the elevated inside-out signaling from their immunoreceptor to β2 integrin LFA-1 [20,47]. The adhesion to the target cell is promoted by each activating receptor (and some accessory molecules) separately, whereas the degranulation requires synergistic activation through coengagement of several activating receptors [48]. It is crucial that engaged iKIRs in educated NK cells can efficiently inhibit the degranulation and not inhibit the adhesion [48]. Therefore, it seems likely that educating HLA I ligands cognate with aKIRs take part in degranulation and those HLA cognate with licensing iKIRs can increase the adhesion of licensed/educated NK cell to cancer cells and stabilize the immunological synapse. Consequently, the NK cell killer function can be impaired by the lack of either activation or inhibitory HLA class I ligands on target cancer cells. It is likely that, without iKIR-HLA engagement, NK cells become "not licensed to kill" and without aKIR-HLA engagement this operational defect does not help different activating signals overcome the inhibition. These functional pathways inherent in NK cells are likely to be intensified in fully responsive licensed/educated NK cells as they all depend on KIR-HLA ligation [20,21,48].

Although it is likely and biologically plausible that the iKIR and aKIR systems described in our previous paper [30] and here have independent influence on relapse-free survival, we cannot definitely confirm this independence for lost education status because of highly limited and partly overlapping strata. Further independent study will elucidate whether or not the lost NK cell education mediated by the missing recipient HLA ligand cognate with donor inhibitory and aKIR education repertoire have independent impacts on outcome.

Interestingly, our comparisons in patients after HSCT show similar incidence of grade II to IV acute GVHD for patients who received a transplant from donors with and

without aKIR-based–educated NK cells. This suggests that aGVHD is independent of aKIR-based education status in a donor. Along with the finding that the GVT effect was quantitatively dependent on aKIR-based NK cell education, these observations may offer insight into the dissection between the GVHD and GVT effects in HSCT therapy. However, further study should be undertaken considering educated and uneducated NK cell status before reaching a conclusion.

Taken together, our data complement the model of NK cell education where iKIR-mediated NK cell reactivity is upregulated [10,20] and aKIR-mediated education induces hyporeactivity to tissues, and both are dependent on the recognition of ubiquitously expressed self MHC class I. It is essential that the 2 immune pathways of NK cell activation and education/hyporeactivity intersect at the aKIR-HLA interface, and activation of NK cell can be prevented by the target cell expressing self HLA. Similar immune pathway intersection is the case for inhibition and licensing/hyperresponsiveness pathways at the iKIR-HLA interface [10]. Importantly, as demonstrated for the C2:KIR2DS1 axis, the aKIR-based education reduces the responsiveness of NK cells to stimulation with cellular targets, but not with cytokines alone [23]. Apparently, the intrinsic cytolytic pathways are potentially active and are kept silent in NK cells educated via aKIR-HLA. This activation-inhibition-education crosstalk creates a platform to tolerize the otherwise highly reactive NK cells against normal cells expressing self HLA. Simultaneously, the tolerization to normal cells redirects NK cell responsiveness to stressed or malignant cells, expressing induced ligands for non-HLA specific–activating receptors that are unlikely to be involved in NK cell education in the steady state [49]. The discovery of the quantitative additive function of aKIR-HLA systems in NK cell education is consistent with the finding of a synergistic role of activating receptors in NK cell immunological synapse [48] and warrants its important role in clinical transplantation.

In conclusion, upon engagement with cognate HLA ligands in the HSC donor, activating KIRs are involved in NK cell education that quantitatively depends on the number of aKIR-HLA systems and leads to hyporesponsiveness of NK cells. In clinical HSCT, these data suggest supplemental selection of fully HLA matched donors with lowest possible number of those aKIRs involved in NK cell education. This can be fulfilled by the 2-level-selection of those donors with defined aKIR-HLA based NK cell education status. In partially mismatched donor-recipient pairs, avoiding the selection of HSC donors with aKIR-mediated education status, especially when cognate HLA ligand is missing in the recipient, is advisable. To avoid these donors, 3-level-selection is necessary. The modulation of aKIR-HLA-based immune response can also be considered in different models of adoptive NK cell–based cancer immunotherapy [50,51], as cancer immunosurveillance greatly depends on the aKIR-HLA–mediated NK cell education.

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