Relevance of C1 and C2 Epitopes for Hemopoietic Stem Cell Transplantation: Role for Sequential Acquisition of HLA-C-Specific Inhibitory Killer Ig-Like Receptor[1]

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Killer Ig-like receptors (KIR) and HLA class I ligands were studied in unrelated hemopoietic stem cell transplantation for chronic myeloid leukemia ($n = 108$). Significantly improved overall survival was observed in patients, which were homozygous for HLA-C-encoded group 1 (C1) ligands compared with those with group 2 (C2) ligands. Favorable outcome in the former patient group was an early effect that was highly significant in patients transplanted with G-CSF-mobilized peripheral blood and patients with advanced disease stages. In contrast, presence of C1 ligands in the donor was associated with significantly reduced patient survival. The differential roles of the two HLA-C ligands are explained in the context of a biased NK cell reconstitution, which is generally dominated by the presence of C1- but absence of C2-specific NK cells. The clinical observations are corroborated by in vitro experiments showing that NK cells derived from hemopoietic progenitor cells generally acquire the C1-specific inhibitory KIR2DL2/3 at earlier time points and with higher frequency than the C2-specific KIR2DL1. These findings define a novel determinant for understanding the role of NK cells in clinical hemopoietic stem cell transplantation. *The Journal of Immunology,* **2007, 178: 3918–3923.**

Natural killer cells are increasingly acknowledged as important mediators of graft-vs-leukemia responses in hemopoietic stem cell transplantation (HSCT).[3] The structural basis for NK cell-mediated allorecognition are MHC class I-specific inhibitory receptors, namely killer Ig-like receptors (KIR) and the lectin-like CD94:NKG2A heterodimer, which are both expressed in a clonally distributed mode (1). The clonal distribution of NK cell receptors leads to a repertoire, which is highly diverse in terms of receptor combinations on different clones. The majority of NK cells in peripheral blood express at least one inhibitory receptor for self-MHC class I and is functionally competent to recognize and eliminate target cells that have down-regulated the respective MHC class I ligands (2, 3). Additionally, a subpopulation of NK cells exists that lacks inhibitory receptors for self-MHC class I and is generally hyporesponsive to target cells that are deficient in MHC class I expression (4, 5). In this regard, it was recently shown that the acquisition of functional competence, a process called "licensing," is mediated through interaction of inhibitory NK cell receptors with cognate class I ligands (6).

Three different inhibitory KIR with specificity toward different HLA class I-encoded ligands seem to play a major role in allorecognition: HLA-C allotypes with asparagine at position 80 (C1 ligands) are recognized by KIR2DL2/3, HLA-C allotypes with lysine on position 80 (C2 ligands) are recognized by KIR2DL1, and HLA-B allotypes with a polymorphic sequence motif at position 77–83 are recognized by KIR3DL1 (Bw4 ligand). In addition, CD94:NKG2A represents a receptor of broad class I specificity, which recognizes leader peptides of most HLA-A, -B, and -C molecules in the context of the ubiquitous HLA-E. Based on these considerations, knowledge of *HLA* genotype as well as KIR expression on the clonal level enabled the reliable prediction of NK cell alloreactivity in vitro (2).

The clinical relevance of NK cell alloreactivity was originally demonstrated in the setting of haploidentical HSCT where patients receive a transplant from a related donor, which is matched for one but mismatched for the other *HLA* haplotype (7). A strong graft-vs-leukemia effect against myeloid leukemias was observed when the recipient was lacking at least one of the three major KIR ligands that were present in the donor. In subsequent studies of related or unrelated HSCT, NK cell-mediated effects were less clear-cut. Whereas a beneficial influence of KIR ligand mismatching on leukemic relapse as well as survival was observed in some studies (8, 9), similar effects were not seen in others (10, 11). Recently, a combined analysis of KIR ligands together with DNA- or RNA-based *KIR* typing was suggested as better predictors of clinical outcome (12, 13).

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[3] Abbreviations used in this paper: HSCT, hemopoietic stem cell transplantation; KIR, killer cell Ig-like receptor; aGvHD, acute graft-vs-host disease; SSP, sequence-specific primer; RR, risk ratio; CI, confidence interval; OS, overall survival; EFS, event-free survival; TRM, transplantation-related mortality; PBSC, peripheral blood stem cell; BM, bone marrow; CML, chronic myeloid leukemia; NCAM, neural cell adhesion molecule.

In this study, *KIR* and *HLA class I* genotypes were studied in a cohort of chronic myeloid leukemia (CML) patients, which underwent HSCT from unrelated donors. Our findings demonstrate a strong, previously undescribed influence of HLA-C ligands on patient survival supporting a new, experimentally supported model, which is based on the sequential expression of HLA-C-specific inhibitory KIR during reconstitution of the NK cell repertoire.

Materials and Methods

Patient population

All patients were of Caucasoid origin and underwent transplantation at the University Hospital (Essen, Germany). They fulfilled the following inclusion criteria: 1) unrelated HSCT for CML; 2) first transplantation (i.e., no prior allo- or autotransplantation); 3) institutional standard myeloablative regimen (2.5 Gy cobalt 60 total body irradiation per day administered on 4 consecutive days followed by 120 mg/kg cyclophosphamide); 4) institutional graft-vs-host disease (GvHD) prophylaxis using short-course methotrexate and cyclosporine; 5) unmanipulated grafts. The median patient age was 38 years (range 17–55). The mean follow-up time of patients was 1545 days (51.5 mo). The study was approved by the Institutional Review Board of the Department of Bone Marrow Transplantation at the University Hospital (Essen, Germany). Written informed consent was obtained for all aspects of the transplantation procedure in accordance with the Declaration of Helsinki.

Genotyping for HLA and KIR

HLA-A and *-B* polymorphisms were determined by low- and intermediate-resolution DNA-based typing using PCR-sequence-specific primer (SSP) typing as described previously (9). Additionally, to enable unambiguous assignment of *HLA-C*-encoded KIR ligands, *HLA-C* alleles were determined by DNA-based direct sequencing in all donors and recipients included in this study. HLA class II polymorphisms were assessed by high-resolution PCR-SSP as previously described (14). Assessment of *KIR* polymorphisms was done as previously described (15). In brief, presence or absence of the 14 known functional *KIR* genes—*KIR2DL1–5*, *KIR2DS1–5*, *KIR3DL1–3*, and *KIR3DS1*—was tested by PCR-SSP. Additionally, a common allelic variant of *KIR2DS4*, carrying a 22-bp deletion in exon 5, was detected using an alternative forward primer KIR2DS4del: 5′-GGT TCA GGC AGG AGA GAA T-3′ in combination with the original reverse primer for *KIR2DS4*.

Clinical study end points

The patient cohort was divided into two groups: an early disease group including all patients transplanted in first chronic phase and an advanced disease group including all other patients. Overall survival was defined as survival without lethal event from any cause. Event-free survival was defined as survival in complete remission without lethal event from any cause. Transplantation-related mortality included lethal events from any cause other than hemologic relapse. Hemologic relapse was diagnosed based on standard hematologic criteria. Diagnosis and grading of acute and chronic GvHD was performed according to the published standard criteria.

Statistical analysis

Cumulative estimates of survival were calculated by the Kaplan-Meier method. Differences between time-to-event distribution functions were compared by a log-rank test. Interactions of different covariates on the analytical end point overall survival were evaluated by stepwise proportional hazards general linear model (PHGLM) analysis using the Cox regression method (16). Conditional risk ratios (RR) and their 95% confidence intervals (95% CI) were derived from PHGLM analysis after adjustment for significant covariates in the model. For models, where survival was not the outcome of interest, competing risks were accounted for in the Cox models according to Fine and Gray (17).

Differentiation of NK cells from hemopoietic progenitors

Human $CD34^+/CD38^-/Lin^-$ hemopoietic progenitor cells were enriched from cord blood by using the CD34 Progenitor Cell Isolation kit (Miltenyi Biotec) and subsequent flow cytometric cell sorting. $CD34^+/CD38^-/lin^-$ cells were plated in 24-well plates in direct contact with irradiated AFT024 stromal cells and differentiated into mature NK cells as described (18). KIR expression was analyzed by flow cytometry using CD3 (no. 347347), CD158a (anti-KIR2DL1/S1; no. 556062), KIR-NKAT2 (anti-KIR2DL2/L3/S2; no. 556070), and NKB1 (anti-KIR3DL1; no. 555967) (all BD Pharmingen), as well as KARp50.3 (anti-KIR2DS4; no. PNIM3337) and CD56 (A07789) (both Beckman Coulter).

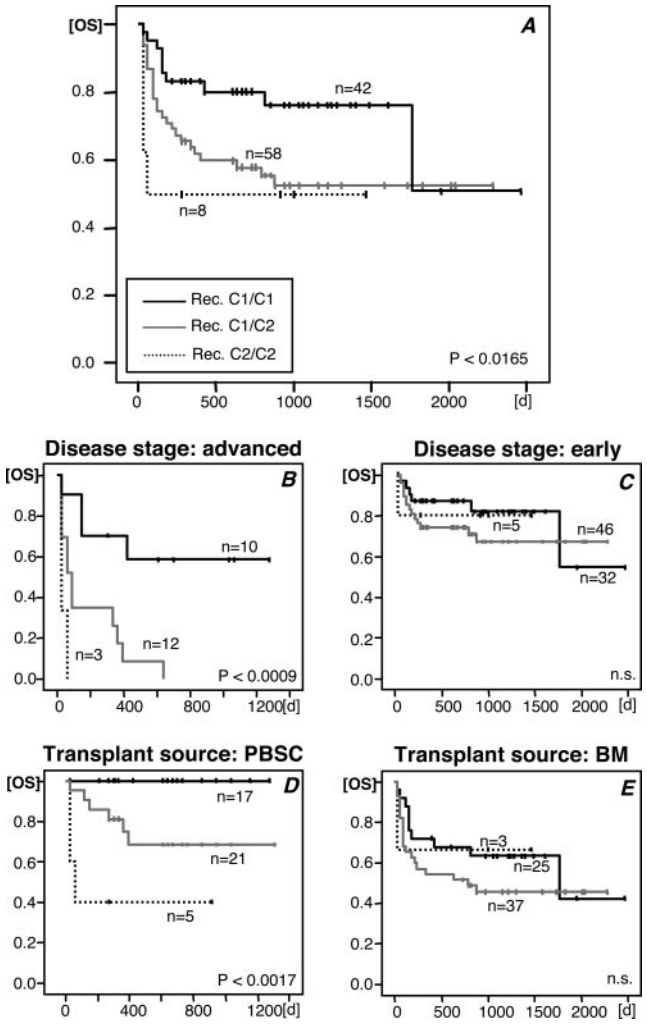

FIGURE 1. Influence of HLA class I-encoded KIR ligands of the recipient on overall survival. Kaplan-Meier estimates for probability of OS of HSCT recipients with (*A*) HLA-C-encoded KIR ligands C1/C1, C1/C2, or C2/C2 stratified for disease stages (*B*) advanced disease and (*C*) early disease as well as transplant sources (*D*) peripheral blood stem cells and (*E*) bone marrow. Numbers of patients in each subgroup are indicated. Significance for heterogeneity between study groups was tested by log-rank statistics. The *p* values were either calculated for all patients (*A*) or without further stratification for the subgroups indicated (*B–D*).

Analysis of KIR expression by real-time RT-PCR

RNA extractions and RT reactions were done as described previously (19). Real-time PCR analyses of KIR expression was performed using *KIR* gene-specific primers (19) as well as primers for neural cell adhesion molecule (NCAM)/CD56: 5′-TTGTGAATGTGCCACCTACC-3′ and 5′-TTCTTCGCTGCTGATGTTCC-3′ (size: 437 bp), and β-actin: 5′-GAAAGTCCTCACCGAGCGC-3′ and 5′-AGGGTACATGGTGGTGCCG-3′ (size: 352 bp). Real-time PCR was performed in a volume of 25 μl, with 60 ng of template cDNA, 12.5 pM of each primer and 12.5 μl of SYBR Green Mastermix (Qiagen). After initial enzyme activation for 15 min at 95°C, 40 cycles were performed, consisting of 20 s at 96°C, 45 s at 62°C, and 45 s at 72°C for the first five cycles and then 20 s at 95°C, 45 s at 60°C, and 45 s at 72°C for the next 35 cycles. Relative mRNA levels were calculated using β-actin as endogenous reference and normalized to day 0 of culture using the $2^{-\Delta\Delta CT}$ method according to the manufacturer's instructions, where CT is the cycle threshold. Each PCR was run in triplicate.

Table I. HLA-C-*encoded KIR ligands in the recipient and major clinical end points*

		Recipient KIR Ligands								
		Overall		C1/C1		C1/C2		C2/C2		
		(n = 108)		(n = 42)		(n = 58)		(n = 8)		
		n	%	n	%	n	%	n	%	p^a (Log rank)
OS	Total	68/108	62.9	32/42	76.2	32/58	55.2	4/8	50.0	0.0164
Graft type	Bone marrow	34/65	52.3	15/25	60.0	17/37	45.9	2/3	66.7	NS
	Peripheral blood	34/43	79.1	17/17	100	15/21	71.4	2/5	40.0	0.0017
Disease stage	Early	62/83	74.7	26/32	81.3	32/46	69.6	4/5	80.0	NS
	Advanced	6/25	24.0	6/10	60.0	0/12	0.0	0/3	0.0	0.0009
EFS	Total	62/108	56.9	30/42	71.4	28/58	48.3	4/8	50.0	0.0268
Graft type	Bone marrow	28/65	43.1	13/25	52.0	13/37	35.1	2/3	66.7	NS
	Peripheral blood	34/43	79.1	17/17	100	15/21	71.4	2/5	40.0	0.0012
Disease stage	Early	57/83	68.7	25/32	78.1	28/46	60.9	4/5	80.0	NS
	Advanced	5/25	20.0	5/10	50.0	0/12	0.0	0/3	0.0	0.0010
TRM	Total	31/108	28.7	7/42	16.7	21/58	34.2	3/8	37.5	0.0216
Graft type	Bone marrow	25/65	38.5	7/25	28.0	17/37	45.9	1/3	33.3	NS
	Peripheral blood	6/43	13.9	0/17	0.0	4/21	19.0	2/5	40.0	0.0112
Disease stage:	Early	19/83	22.9	5/32	15.6	13/46	28.3	1/5	20.0	NS
	Advanced	12/25	48.0	2/10	20.0	8/12	66.7	2/3	66.7	0.0010
Relapse	Total	15/108	10.2	5/42	11.9	9/58	15.5	1/8	12.5	NS
Graft type	Bone marrow	12/65	18.5	5/25	20.0	7/37	18.9	0/3	0.0	NS
	Peripheral blood	3/43	6.9	0/17	0.0	2/21	9.5	1/5	20.0	0.108
Disease stage	Early	7/83	8.4	2/32	6.3	5/46	10.9	0/5	0.0	NS
	Advanced	8/25	32.0	3/10	30.0	4/12	33.3	1/3	33.3	0.0375
aGvHD, grade 3–4	Total	23/108	21.3	6/42	14.3	14/58	24.1	3/8	37.5	0.084
Graft type	Bone marrow	18/65	27.7	6/25	24.0	11/37	29.7	1/3	33.3	NS
	Peripheral blood	5/43	11.6	0/17	0.0	3/21	14.3	2/5	40.0	0.0259
Disease stage	Early	16/83	19.3	6/32	18.8	9/46	19.6	1/5	20.0	NS
	Advanced	7/25	28.0	0/10	0.0	5/12	41.7	2/3	66.6	0.0297

[a] Test statistics for equality of survival distributions were performed for the three groups C1/C1, C1/C2, and C2/C2. Values of p represent the linear trend of the log-rank calculation.

Results

Differential contribution of C1 and C2 ligands in donor and recipient

The clinical relevance of polymorphisms in KIR and HLA class I ligands for HSCT was retrospectively analyzed in a cohort of 108 CML patients treated with the same transplant protocol in a single center. All transplant pairs were matched for HLA-A (A3/A11) and B (Bw4)-derived KIR ligands, whereas HLA-C (C1/C2) ligands were mismatched in 21% of cases. Consistent with previous reports (7), a significant reduction of leukemic relapse was observed ($p < 0.029$) when C1 and C2 ligands were mismatched between donor and patient (data not shown). However, no significant differences were seen in overall survival (OS), event-free survival (EFS), or transplant-related mortality (TRM) (for definition of clinical study end points, see *Materials and Methods*).

We next assessed whether KIR ligands affect clinical outcome in ways, which are independent of mismatching between donor and recipient. Recipients with C1/C1 ligands (i.e., C1 epitope present on both HLA-C alleles) had a significantly improved OS compared with patients having either C1/C2 or C2/C2 ligands (Fig. 1*A*). A similar analysis of A3/A11 and Bw4-associated clinical outcome did not reveal any significant associations. Improved clinical outcome of the C1/C1 patient group was independent of the *KIR* genotype of the donor. In general, none of the *KIR* genes alone nor the frequency of group A and B haplotypes represented a confounding factor in Cox logistic regression analysis (data not shown). A lack of cognate interaction due to absence of an inhibitory KIR was found in only two cases (one still in remission, one TRM) where *KIR2DL1* was absent in the donor of a C2/C2 patient.

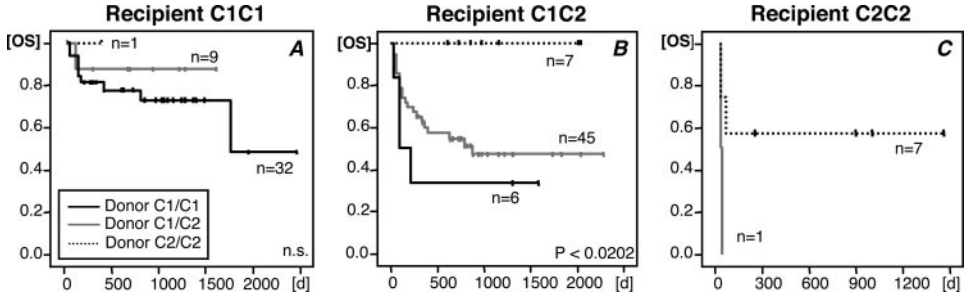

FIGURE 2. Influence of HLA-C-encoded KIR ligands of the donor on clinical outcome. Kaplan-Meier estimates for probability of OS of HSCT recipients with (*A*) C1/C1, (*B*) C1/C2, or (*C*) C2/C2 ligands having donors with C1/C1 (solid line), C1/C2 (gray line), or C2/C2 (dotted line) ligands. Number of patients in each subgroup is indicated. Significance for heterogeneity between study groups was tested by log-rank statistics. The p values were calculated without further stratification for the three patient subgroups indicated in each panel except in (*C*), where no donors with C1/C1 were present.

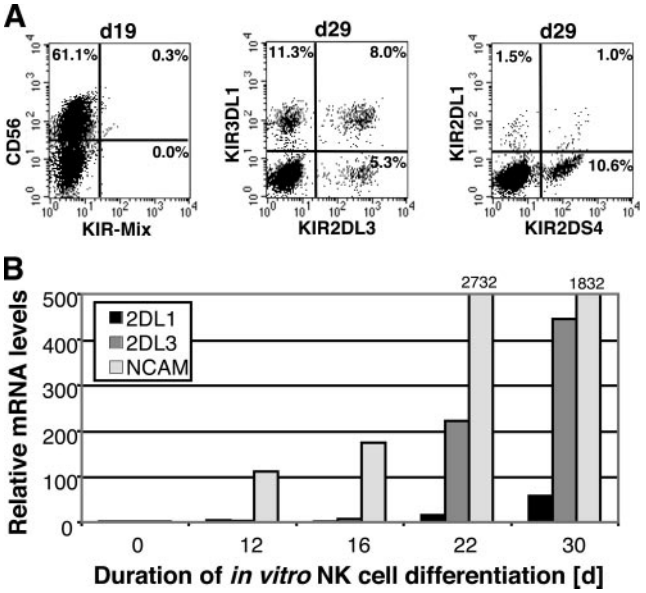

FIGURE 3. Delayed acquisition of C2-specific KIR2DL1 during NK cell differentiation. Cord blood-derived $CD34^+/CD38^-/Lin^-$ hemopoietic progenitor cells were differentiated into mature NK cells in a stroma cell-supported culture system. *A*, A sample with homozygous group A haplotype was analyzed for expression of the indicated KIR by flow cytometry after 19 days (*left dot plot*, gated on live cells) and 29 days (*middle* and *right dot plot*, gated on CD56) in culture. *B*, Using a different cord blood sample, the relative increase in expression levels of KIR2DL1, KIR2DL3, and NCAM (CD56) mRNA compared with day 0 were analyzed by real-time RT-PCR. NCAM levels >500 are indicated above each column.

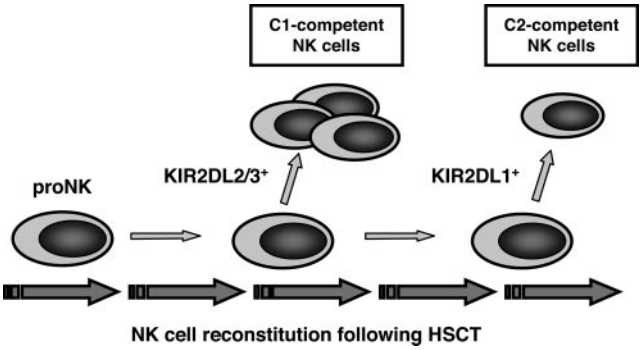

FIGURE 4. Model of NK cell reconstitution following HSCT. In the course of maturation of NK cells from hemopoietic progenitors (proNK), the C1-specific KIR2DL2/3 is generally expressed earlier and at higher frequency than the C2-specific KIR2DL1. In the setting of clinical HSCT, this bias in immune reconstitution might lead to an early functional repertoire, which is dominated by C1-specific NK cells. At subsequent stages, KIR2DL1-expressing NK cells with specificity for C2 ligands are generated and the final NK cell repertoire is established.

The main reason for superior OS as well as EFS of the C1/C1 patient group was reduced TRM ($p < 0.022$; Table I). Next, the patient cohort was divided into two groups: an early disease group ($n = 83$) including all patients transplanted in first chronic phase and remission, respectively, and an advanced disease group ($n = 25$) including all other patients. The absence of C2 ligands was strongly associated with improved OS in patients with advanced disease (Fig. 1*B*) but not early disease (Fig. 1*C*). Furthermore, a significant decrease of severe acute GvHD (aGvHD) ($p < 0.029$) was observed in the advanced disease group having C1/C1 ligands (Table I). When the analysis was stratified for transplant source, C1/C1 ligands were associated with improved OS in patients transplanted with peripheral blood stem cells (PBSC; $n = 43$; *Fig.* 1*D*) but not bone marrow (BM; $n = 65$; Fig. 1*E*). Moreover, a decrease of severe (grade 3–4) aGvHD was observed ($p < 0.026$) in the PBSC group having C1/C1 ligands. The transplant source was evenly distributed among disease groups (advanced disease group: 15 BM and 10 PBSC; early disease group: 50 BM and 33 PBSC). For both disease groups, there was a statistical trend toward better OS of C1/C1 patients that were transplanted with PBSC (data not shown).

In the donor, C1 and C2 ligands had opposite effects on clinical outcome (Fig. 2): when stratified for HLA-C ligands of the patient, C2/C2 donors were associated with improved OS compared with recipients with C1/C2 donors and recipients with C1/C1 donors ($p < 0.011$; adjusted for recipient groups C1/C1 and C1/C2). In multivariate logistic regression analysis, the status of C1 and C2 ligands in the recipient (RR: 5.9; 95% CI: 1.8–19.3; $p < 0,0001$) as well as in the donor (RR: 3.2; 95% CI: 1.1–9.5; $p < 0,01$) both constituted major risk factors for OS. Using backward sequential exclusion (as well as forward sequential inclusion) methods, both variables remained in a Cox model together with the variables "disease status" (RR: 6.7; 95% CI: 3.4–13.3; $p < 0,0001$), "transplant source" (RR: 2.2; 95% CI: 1.0–4.7; $p < 0,014$), and "HLA class I A/B mismatch" (RR: 1.6; 95% CI: 1.0–2.5; $p < 0,022$). The C1/C2 ligand mismatch (7) was excluded from the model (RR: 1.3; CI: 0.4–4.1; $p < 0,052$). Other variables like "donor age," "patient age," and "sex mismatch" were tested and excluded from the model (data not shown).

Sequential acquisition of HLA-C-specific inhibitory KIR

The differential role of C1 and C2 ligands was a time-dependent function: when the analysis was restricted to the first 500 days, a beneficial role of recipient C1 ligands for clinical outcome (OS) was evident (log rank: $p < 0.015$), whereas this effect was no longer measurable in later time segments. We thus reasoned that the observed effects might be related to the specific reconstitution of NK cells, which takes place during the early post-HSCT phase. Here, as an in vitro model for NK cell reconstitution, cord blood-derived $CD34^+CD38^-$ hemopoietic progenitor cells were differentiated into $CD3^-CD56^+$ NK cells using a similar protocol as described by Miller et al. (18). Typically, surface expression of KIR was detectable after a culture period of 2–3 wk. Flow cytometric analysis of HLA-C-specific inhibitory KIR was performed in samples with homozygous group *A* haplotypes, which do not possess cross-reactive stimulatory KIR (19). Generally, after the onset of de novo KIR expression on differentiating NK cells, C1-specific KIR2DL3 was detectable at higher frequencies than C2-specific KIR2DL1 (Fig. 3*A*). In line with these observations, KIR2DL3 mRNA (and also KIR2DL2 mRNA, when present on a given KIR haplotype) was consistently found at earlier time points and at higher levels in developing NK cells than KIR2DL1 mRNA (Fig. 3*B*). This observation was true for samples with group A as well as group B haplotypes (data not shown). These data, together with previous observations of clinical NK cell reconstitution (20, 21), suggest a model of sequential order of KIR expression for the HLA-C-specific inhibitory KIR that might be genetically predetermined (Fig. 4).

Discussion

In the present study, the clinical importance of KIR and KIR ligand polymorphisms in unrelated HSCT was analyzed. To reduce the number of confounding factors, analysis was restricted to CML patients transplanted in a single center using a standardized clinical

treatment protocol. The HLA-C-encoded KIR ligands C1 and C2 were identified as major factors differentially influencing clinical outcome of HSCT in a mismatch-independent way. Whereas the presence of C2 ligands in the patient was associated with substantially decreased overall survival, the opposite effect was observed for C2 ligands of the donor: overall survival was better when donors with C2/C2 ligands were taken.

Recently, two groups identified C2/C2 recipients as high-risk group in unrelated HSCT. Whereas in one study, C2 ligands were associated with an increased relapse rate (22), in another study epistatic interaction between C2 in the recipient and the stimulatory KIR2DS2 in the donor was found (23). In the present study, no influence of C2 ligands on the relapse rate was found. The differential effects of HLA-C ligands on clinical outcome were also independent of donor *KIR* genotype. Specifically, no correlation was found between donor-derived HLA-C-specific *KIR* genes (*KIR2DL1, KIR2DL2/3, KIR2DS1, KIR2DS2*) and recipient-derived HLA-C ligands. Unfortunately, the subgroup of C2/C2 recipients was too small to make definitive statements concerning the influence of *KIR* genes on their cognate KIR ligands.

How could the C1/C2 status influence clinical outcome in allogeneic HSCT in ways that are independent of KIR genotype and KIR ligand mismatch? A previously unrecognized factor, which might contribute to the differential role of HLA-C ligands in donor and recipient, is the biased reconstitution of the NK cell repertoire upon engraftment. In this regard, previous clinical studies analyzing reconstitution of KIR repertoires demonstrated that during the early phase of HSCT NK cells with C1-specific KIR2DL2/3 or KIR2DS2 receptors are found at higher frequencies than NK cells expressing C2-specific KIR2DL1 or KIR2DS1 (20, 21). Furthermore, analysis of NK cell differentiation in vitro showed a consistent bias toward early expression of KIR2DL2/3 or KIR2DS2 on the surface of developing NK cells (18). Notably, these studies did not differentiate between stimulatory and inhibitory KIR variants, thus making the functional significance of these observations unclear. Our in vitro analysis of KIR expression during NK cell development, which was performed on the genetic background of group A haplotypes, indicates that the C1-specific KIR2DL3 is indeed expressed substantially earlier during development than the C2-specific KIR2DL1. It thus appears that the sequential order of KIR expression (at least concerning the HLA-C-specific inhibitory KIR) might be genetically "hardwired" rather than influenced by environment and HLA class I (Fig. 4).

The early lack of NK cells expressing the C2-specific inhibitory KIR2DL1 might have several clinical consequences: in C1/C1 recipients, NK cells expressing the C1-specific KIR2DL2/3 are present already at early time points post-HSCT and thus be able to perform adequate surveillance of HLA-C expression, for example on leukemic or virus-infected cells. In C1/C2 heterozygous recipients, only C1 expression would be properly surveyed whereas in C2 homozygous patients no early NK cell-mediated control of HLA-C expression would be possible while KIR2DL1-expressing NK cells are missing. To establish NK cell tolerance, the C2-specific "hole" in the repertoire might be filled by other inhibitory receptors with broad HLA class I specificity like CD94:NKG2A or LILRB1. Thus, in C2 homozygous and to a lesser degree also in C1/C2 heterozygous recipients, the reconstituting NK cell repertoire will be governed by broadly specific NK cells, which are not able to recognize changes in single HLA class I allotypes. In this regard, it was recently shown in vitro as well as in the setting of haploidentical transplantation that NKG2A expression on NK cells is associated with impaired immune responsiveness and antileukemic reactivity (21). The increase in transplant-related mortality in recipients with C2 ligands might thus partly be due to less efficient control of infections caused by an early general deficiency in immunocompetent NK cells.

It was recently shown that not all NK cells express an inhibitory receptor for self-MHC class I (4, 5) and that according to the "licensing" model these cells have not acquired functional competence (6). As these cells are hyporesponsive toward MHC class I-deficient target cells, their alloreactive potential should be limited. These considerations speak against a prominent role of "unlicensed" NK cells in clinical HSCT. In contrast, the requirement for functional "licensing" of NK cells might be bypassed under conditions of chronic inflammation as previously speculated (6). Thus, in the setting of clinical stem cell transplantation, patient conditioning regimen as well as occurrence of GvHD might indeed provide an environment that promotes activation of "unlicensed" NK cells. Their contribution to NK cell alloreactivity should thus be considered in future studies.

A second factor contributing to the poor performance of HLA-C2-expressing recipients is related to an increase in acute GvHD, which is probably not an NK cell-, but a T cell-mediated process. The lack of KIR2DL1 expression in the early phase following HSCT is probably not restricted to the NK cell compartment but is equally relevant for the subset of KIR-expressing T cells, which frequently appear at considerable frequencies post-HSCT (24). Notably, in a previous study of T cells from peripheral blood of two donors, T cell clones expressing KIR2DL2/3 were frequently found, whereas KIR2DL1 expression was rare (25). The KIR-expressing T cell subset was previously shown to mainly consist of Ag-experienced effector/memory T cells (26), which might play a significant role in control of acute GvHD. The lack of KIR2DL1 expression could thus lead to a lack of inhibition of the alloreactive T cell subset in recipients with C2 ligands. This effect would explain the increased frequency of severe aGvHD in C2-expressing recipients transplanted with PBSC (Table I), containing an ~10-fold higher amount of T cells compared with bone marrow grafts.

Why do HSCT recipients with C2 ligands benefit from donors that are homozygous for C2 ligands? This observation might be due to the fact that KIR2DL1 is the only HLA-C-specific inhibitory receptor in C2/C2 donors. Thus, the KIR repertoire of C2/C2 donors is expected to contain a high frequency of KIR2DL1-expressing C2-specific NK cells. In contrast, C1/C2 donors will provide lower frequencies of C2-specific NK cells, because their NK cells can "choose" between C1 and C2-specific inhibitory KIR for tolerance induction. Finally, C1/C1 donors will provide the lowest frequency of C2-specific NK cells, because these NK cells would not be inhibited by self ligands. Donor-derived C2-specific NK cells could thus close the temporary "hole" in the repertoire of recipients with C2 ligands and in this way increase the functionality of the NK cell compartment. Indeed, the beneficial effect of C2/C2 donors was significant in recipients with C2 ligands but not in recipients lacking C2 ligands (Fig. 2). As a consequence, prospective selection of a C2/C2 donor might improve clinical outcome of patients having C2 ligands.

In conclusion, the present study suggests an important role for reconstitution of NK cells in clinical HSCT based on the sequential acquisition of inhibitory KIR for the two HLA-C-encoded epitopes. The distribution of C1 and C2 epitopes in donor and recipient had a major influence on clinical outcome, which was independent of KIR ligand mismatching. Whereas the effect of KIR ligand mismatching is based on the generation of a high frequency of alloreactive NK cells, the matching algorithm emerging from the present study is based on the frequency of immunocompetent NK cells that can recognize cognate HLA-C ligands. Those C1/C2 constellations that enable reconstitution of high numbers of immunocompetent NK cells show substantially improved clinical

outcome. Further studies are needed to more closely monitor KIR expression patterns on NK as well as T cells during the reconstitution phase of HSCT, which might become relevant for the prospective selection of suitable donors.

Disclosures

The authors have no financial conflict of interest.

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