

## ORIGINAL ARTICLE

# Influence of killer immunoglobulin-like receptor/HLA ligand matching on achievement of T-cell complete donor chimerism in related donor nonmyeloablative allogeneic hematopoietic stem cell transplantation

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**Achievement of complete donor chimerism (CDC) after allogeneic nonmyeloablative hematopoietic stem cell transplantation (NMHSCT) is important for preventing graft rejection and for generating a graft-vs-malignancy effect. The alloreactivity of NK cells and some T-cell subsets is mediated through the interaction of their killer immunoglobulin-like receptors (KIRs) with target cell HLA/KIR ligands. The influence of KIR matching on the achievement of T-cell CDC after NMHSCT has not been previously described. We analyzed 31 patients undergoing T-cell replete related donor NMHSCT following fludarabine and 200 cGy TBI. Recipient inhibitory KIR genotype and donor HLA/KIR ligand matches were used to generate an inhibitory KIR score from 1 to 4 based upon the potential number of recipient inhibitory KIRs that could be engaged with donor HLA/KIR ligands. Patients with a score of 1 were less likely to achieve T-cell CDC ( $P=0.016$ ) and more likely to develop graft rejection ( $P=0.011$ ) than those with scores greater than 1. Thus, patients with lower inhibitory KIR scores may have more active anti-donor immune effector cells that may reduce donor chimerism. Conversely, patients with greater inhibitory KIR scores may have less active NK cell and T-cell populations, which may make them more likely to achieve CDC.**

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## Introduction

Nonmyeloablative hematopoietic stem cell transplantation (NMHSCT) is an effective treatment modality for many patients who are not candidates for traditional myeloablative allogeneic HSCT.<sup>1–5</sup> Achievement of T-cell complete donor chimerism (CDC) after NMHSCT has been considered of critical importance for generating a graft-vs-malignancy (GVM) effect.<sup>3,6</sup>

The GVM effect has been attributed to donor-derived alloreactive immune cells including T-lymphocytes and natural killer (NK) cells.<sup>7–10</sup> The interaction of killer immunoglobulin-like receptors (KIRs) with target cell HLA-class I molecules regulates these NK cells and some T-lymphocyte subsets.<sup>10</sup> KIRs exist in either an activating or inhibitory form, both of which share a common two or three subunit extracellular domain that binds to identical ligands. The inhibitory KIRs are characterized by a cytoplasmic tail containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs), whereas the activating KIRs lack a cytoplasmic tail but possess an adaptor protein (DAP12) with immunoreceptor tyrosine-based activating motifs (ITAMs).<sup>10–12</sup> Engagement of a ligand with the KIR of an immune effector cell causes the cell to generate either an activating or inhibitory signal depending on the functional status of the receptor. Interactions through these receptors control different activation/inhibitory signaling pathways, and their balance regulates the behavior of the NK cell.<sup>13</sup> Inhibitory KIRs have higher binding affinity to cognate ligands as compared to their activating counterparts.<sup>14</sup> Therefore, when concurrent engagement of both activating and inhibitory KIRs occurs, the inhibitory signal predominates with the potential to prevent NK cell-mediated target cell lysis.

HLA-Cw is the main ligand for most inhibitory KIRs. Two HLA-Cw groups exist, designated C1 and C2, which can be distinguished by their amino acid residues at positions 77 and 80 in the  $\alpha 1$  helix of the HLA-C

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molecule.<sup>15</sup> The C1 group is characterized by a serine at position 77 and an asparagine at position 80 and includes HLA-Cw1, -Cw3, -Cw7 and -Cw8. The corresponding inhibitory receptors for the C1 group are KIR2DL2 and KIR2DL3. In contrast, the C2 group has an asparagine at position 77 and a lysine at position 80 and includes HLA-Cw2, -Cw4, -Cw5 and -Cw6. The respective inhibitory receptor for the C2 group is KIR2DL1. Other human inhibitory KIRs with known ligands include KIR3DL1 that binds to HLA-Bw4 epitopes<sup>16</sup> and KIR3DL2 that binds to HLA-A3 or HLA-A11.<sup>10</sup>

KIR interactions have been suggested to influence outcomes after haploidentical,<sup>9,17</sup> matched unrelated donor<sup>18</sup> and matched related donor<sup>19–21</sup> myeloablative allogeneic hematopoietic stem cell transplantation (HSCT). However, in the NMHSCT setting where donor and recipient hematopoiesis may coexist, the effect of KIR interactions is not known. Since the loci for HLA and KIRs are on different chromosomes that segregate independently, disparities may exist between recipient KIRs and donor HLA class I KIR-ligands in related donor HSCT. This may allow recipient NK cells to become alloreactive against donor T cells that lack corresponding KIR ligands. This is the first report to suggest that KIR–HLA ligand matching may influence the achievement of T-cell CDC in related donor NMHSCT.

## Patients and methods

### *Patient characteristics*

Between May 2000 and November 2003, 38 patients underwent T-cell replete related donor NMHSCT at the Cleveland Clinic (Cleveland, OH, USA). Of these, 31 had specimens available for KIR genotyping and they comprise the patient population for this analysis. All patients and donors were treated on NMHSCT protocols that were reviewed and approved by the Cleveland Clinic's Institutional Review Board with signed informed consent obtained from all patients prior to the transplant procedure.

### *Treatment*

All patients received fludarabine 30 mg/m<sup>2</sup> per day on days –5, –4 and –3, followed by TBI 200 cGy on day –1 and infusion of allogeneic peripheral blood stem cells on day 0. Donors received G-CSF 10 µg/kg subcutaneously daily for peripheral blood stem cell mobilization. Leukapheresis began on the fifth day of G-CSF administration and continued for 1 or 2 days to provide a minimum of 2.0 × 10<sup>6</sup> CD34+ cells/kg for transplant. All transplants were performed using T-cell-replete allogeneic peripheral blood stem cells. All patients received cyclosporine 200 mg/day starting day –1 and mycophenolate mofetil 1500 mg/day starting day +1 for GVHD prophylaxis. In the absence of GVHD, these agents were discontinued on day +56. However, with the development of GVHD these immunosuppressant medications were continued often with the addition of corticosteroids. G-CSF was administered for neutropenia post transplant until the absolute

neutrophil count recovered to >500/µl. On day +5 post transplant prophylactic antimicrobial therapy, consisting of itraconazole, amoxicillin and acyclovir, was begun.

### *T-cell chimerism analysis*

Short tandem repeat analysis for T-cell chimerism was performed on peripheral blood samples that were collected weekly starting on day +14 post transplant until 1 month, then every 2 weeks for 3 months, then monthly for 3 months and then every 3 months. DNA was extracted from a T-cell enriched fraction of peripheral blood prepared using Rosette-Sep T-cell enrichment cocktail (StemCell Technologies, Vancouver, British Columbia, Canada), and amplified using the PowerPlex 16 kit (Promega Corp., Madison, WI, USA) according to the manufacturer's directions. Allele designations and peak areas were determined using the PowerType 16 Macro (Promega Corp., Madison, WI, USA). Calculation of the percentage of recipient DNA in a mixed chimeric specimen after transplantation was determined by dividing areas of recipient-specific peaks by the sum of the areas of recipient-plus donor-specific peaks. Results were reported as an average of the percent recipient-specific DNA for all informative loci identified. CDC was defined as achievement of ≥95% DNA of donor origin, and mixed chimerism was defined as ≥1% and <95% DNA of donor origin in the T-cell-enriched fraction. Graft rejection was defined as either failure to ever achieve any donor-derived hematopoiesis or the complete loss of donor-derived hematopoiesis after complete or mixed chimerism was achieved.

### *HLA and KIR typing*

All patients and donors were HLA typed by serologic and DNA-based methods. Serologic HLA classes I and II typing was performed by standard lymphocytotoxicity assays. HLA class I (HLA-A\*, -B\* and -Cw\*) and class II (DRB1\*, DQB1\*, DPB1\*) molecular-based typing was performed, using commercial kits, by PCR-reverse sequence specific oligonucleotide probe (rSSOP) (Lifecodes Corp., Stamford, CT, USA or One Lambda Inc., Los Angeles, CA, USA) and/or PCR-sequence specific priming (SSP) (Pel Freez, Brown Deer, WI, USA or Genovision, West Chester, PA, USA) according to the manufacturer's instructions. These methods provided intermediate-resolution typing, and in some cases high-resolution allele assignment as well, which allowed assessment of KIR ligands. HLA-DRB1\* typing was also performed by direct sequencing with PCR-sequence-based typing as previously described.<sup>22,23</sup>

KIR genotyping was performed by PCR-SSOP (One Lambda) and/or PCR-SSP (Pel Freez) using commercial kits according to the manufacturer's instructions. This typing provided data for the presence or absence of KIR genes and limited information about particular KIR alleles or variants.

Patients were categorized according to their HLA inhibitory KIR ligand groups by determining whether or not they expressed: (1) HLA-A3 or -A11; (2) HLA-Bw4 and (3) HLA-Cw groups (homozygous C1, homozygous C2

or heterozygous C1/C2).<sup>10,15,16</sup> All of the donor-recipient pairs had DNA samples available from which KIR genotyping was performed retrospectively to determine the recipient inhibitory KIR (KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1 and KIR3DL2). Recipient inhibitory KIR genotype and donor HLA/KIR ligand matches were used to generate an inhibitory KIR score from 1 to 4 based upon the potential number of recipient inhibitory KIRs that could be engaged with donor HLA/KIR ligands (see Table 1 for representative examples).

**Statistical analysis**

Categorical variables are summarized as frequency counts and percentages. Continuous variables are summarized as the median and range. Comparisons of continuous variables between more than two groups were made using the Kruskal–Wallis test, while comparisons between two groups were made using the Wilcoxon’s rank-sum test. The primary purpose of the analysis was to compare rates of achieving T-cell CDC and graft rejection based upon patient inhibitory KIR scores. The Kaplan–Meier method was used to estimate each of these outcomes and groups were compared using the log-rank test. All analyses were performed using SAS software (SAS Institute Inc., Cary, NC, USA).

**Results**

Patient characteristics are shown in Table 2. The median number of prior chemotherapy regimens was 2 (range, 0–5). Eight patients (26%) had undergone autologous peripheral blood stem cell transplants performed a median of 456 days (range, 83–1425 days) before their NMHSCT. The median CD3+ and CD34+ cell doses infused for NMHSCT were  $3.92 \times 10^8$ /kg (range,  $1.74$ – $8.10 \times 10^8$ /kg) and  $6.64 \times 10^6$ /kg

(range,  $2.29$ – $12.51 \times 10^6$ /kg), respectively. Three patients had HLA disparities with their related donor: one patient with AML had an HLA-B mismatch, one with Hodgkin’s lymphoma had an HLA-DPB1 mismatch and one multiple myeloma patient had an HLA-DPB1 mismatch.

A total of 23 (74%) patients developed CDC, which initially occurred at a median of 49 days (range, 14–210 days) post transplant. Of the patients who achieved CDC, 11 (48%) had lack of a donor KIR ligand-Cw group for the recipient inhibitory KIR.

Patients were then analyzed with further KIR/ligand matching that also included an assessment of the donor’s HLA-A3, -A11 and -Bw4 ligands in order to determine their inhibitory KIR score. A total of 4 (13%) patients had a score of 1, 16 (52%) a score of 2, 10 (32%) a score of 3 and 1 (3%) a score of 4. Compared to those patients with an inhibitory KIR score of >1, patients with a score of 1 were less likely to achieve CDC (Figure 1). This difference could not be attributed to distinct diagnostic subsets, the number of prior therapies or to the CD3+ or CD34+ cell doses infused (see Table 3). All four patients with an inhibitory KIR score of 1 had donors who were homozygous for the C1 group, but did not express any other HLA/KIR ligands. In addition, each of these recipients had a KIR genotype that expressed 2DL1, 2DL3, 3DL1 and 3DL2 while only two of them also expressed 2DL2 (see Table 1, example 1).

One patient transplanted with an HLA-B disparity had an inhibitory KIR score of 2 and achieved durable CDC by day 28 post transplant. The two patients with HLA-DPB1 disparities had inhibitory KIR scores of 3 and achieved durable CDC by days 119 and 130 post transplant.

Three (10%) patients had graft rejection after NMHSCT. These included one CML patient (secondary graft rejection on day 140 post transplant, after achieving mixed T-cell chimerism—67% donor on day 55) and one CLL patient

**Table 1** Representative examples for calculating an inhibitory KIR score

	A3/11	Bw4	C1	C2	Score
<i>Example 1<sup>a</sup></i>					
Donor HLA/KIR ligand	–	–	+	–	1
Recipient KIR genotype	+3DL2	+3DL1	+2DL2/3	+2DL1	
<i>Example 2<sup>b</sup></i>					
Donor HLA/KIR ligand	–	+	+	–	2
Recipient KIR genotype	+3DL2	+3DL1	+2DL2/3	+2DL1	
<i>Example 3<sup>c</sup></i>					
Donor HLA/KIR ligand	+	+	+	+	3
Recipient KIR genotype	+3DL2	+3DL1	+2DL2	–	

Abbreviation: KIR = killer immunoglobulin-like receptor.

<sup>a</sup>This recipient’s KIR genotype expressed all inhibitory KIRs. However, her donor only expressed 1 HLA/KIR ligand (C1) that could potentially engage the receptor and therefore her inhibitory KIR score is 1.

<sup>b</sup>Although this recipient’s KIR genotype expressed all inhibitory KIRs, his donor only expressed 2 HLA/KIR ligand matches (Bw4, C1) that could potentially engage the receptors and therefore his inhibitory KIR score is 2.

<sup>c</sup>This donor expressed all four inhibitory HLA/KIR ligands. However, given the recipient’s KIR genotype only three KIRs (3DL2, 3DL1, 2DL2) are available for engagement with the donor’s HLA/KIR ligands and therefore his inhibitory KIR score is 3.

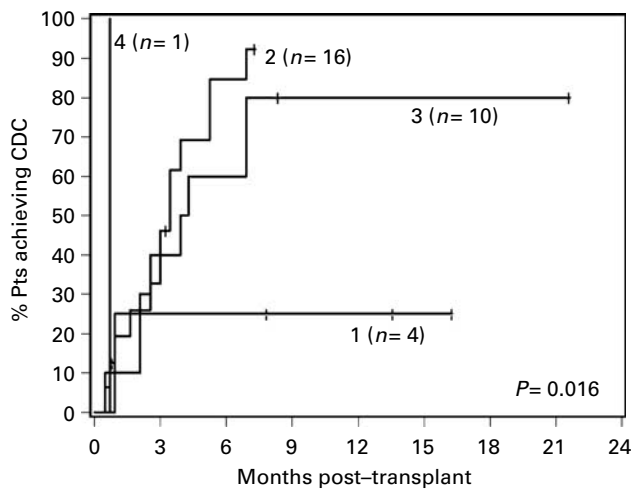
**Table 2** Patient characteristics

Median age (years)	51 (range, 21–63)
Females/males (%)	19(61)/12(39)
<i>Race (N)</i>	
Caucasian	26
African-American	5
<i>Diagnoses (N)</i>	
NHL	5
Multiple myeloma	5
MDS	4
AML	4
CML chronic phase	4
Renal cell carcinoma	4
Hodgkin’s lymphoma	2
Myelofibrosis	2
CLL	1
<i>No. of prior chemotherapies:</i>	
0	6 (19%)
1	7 (23%)
2	6 (19%)
≥3	12 (39%)
Prior radiation therapy	6 (19%)

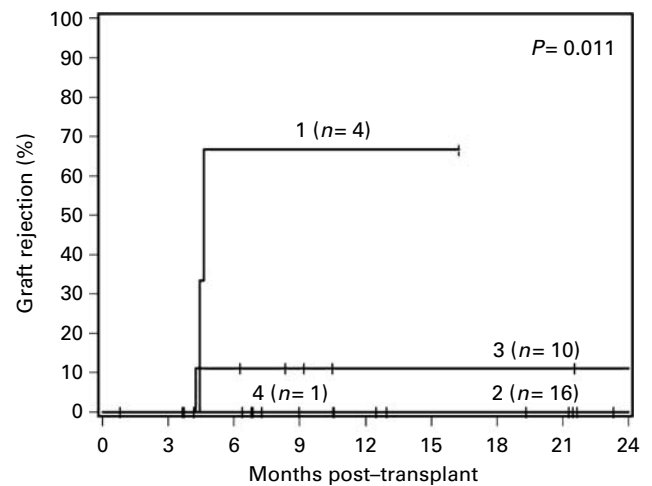
Abbreviation: MDS = myelodysplastic syndromes.

(secondary graft rejection on day 135 post transplant, after achieving CDC on day 49) each with an inhibitory KIR score of 1, as well as one CML patient (secondary graft rejection on day 129, after achieving mixed T-cell chimerism—36% donor on day 83) with an inhibitory KIR score of 3. The CD3+ and CD34+ cell doses were not different for those with or without graft rejection (median CD3+ cell dose  $3.30 \times 10^8/\text{kg}$  (range,  $2.78\text{--}3.98 \times 10^8/\text{kg}$ ) vs  $3.92 \times 10^8/\text{kg}$  (range,  $1.74\text{--}8.10 \times 10^8/\text{kg}$ ), respectively,  $P = 0.46$ ; median CD34+ cell dose  $7.03 \times 10^6/\text{kg}$  (range,  $3.13\text{--}7.04$ ) vs  $6.61 \times 10^6/\text{kg}$  (range,  $2.29\text{--}12.51 \times 10^6/\text{kg}$ ) respectively,  $P = 0.51$ ). Patients with an inhibitory KIR score of 1 were more likely to develop graft rejection than those with a score of  $\geq 2$  (Figure 2).

Three patients received donor lymphocyte infusions (DLI) after their NMHSCT. One myelodysplastic syndrome (MDS) patient with an inhibitory KIR score of 2 previously achieved CDC, but required a DLI on day 223 post transplant for relapsed disease. Another patient had chronic phase CML with an inhibitory KIR score of 1. This patient never achieved CDC post transplant and a DLI on day 111 was unsuccessful with subsequent graft rejection. The last patient had MDS with an inhibitory KIR score of 1. This patient also never achieved CDC, and, in the setting of relapsed disease post transplant, a DLI was performed on day 105. Following the DLI the patient demonstrated persistent T-cell mixed chimerism and experienced further progression of disease.



**Figure 1** Kaplan-Meier curves for the percentage of patients achieving T-cell complete donor chimerism (CDC) after NMHSCT based upon patients' inhibitory KIR scores.



**Figure 2** Kaplan-Meier curves for the percentage of patients developing graft rejection after NMHSCT based upon patients' inhibitory KIR scores.

**Table 3** Demographic data and T-cell complete donor chimerism results for NMHSCT patients based upon their inhibitory KIR score

Inhibitory KIR score	N	Number that achieved durable CDC (%)	Median number of days to achieve CDC (range)	Median number of prior chemotherapy regimens (range)	Diseases	Median CD3+ cell dose ( $\times 10^8$ per kg) (range)	Median CD34+ cell dose ( $\times 10^6$ per kg) (range)
1	4	1 (25)	28	1 (1-4)	1 CLL 1 MDS 1 RCC 1 CML	3.44 (2.78-3.92)	6.98 (6.58-7.04)
2	16	13 (81)	49 (14-160)	2.5 (0-5)	4 MM 2 MDS 1 HL 3 AML 1 MFB 2 NHL 1 CML 2 RCC	3.92 (2.06-6.56)	6.69 (3.46-12.51)
3	10	8 (80)	56 (14-210)	1 (0-4)	1 MDS 1 HL 1 AML 2 CML 1 RCC 2 NHL 1 MFB 1 MM	4.16 (2.19-8.10)	6.10 (2.29-7.21)
4	1	1 (100)	21	4	1 NHL	1.74	6.42
(P-value)		0.016 <sup>a</sup>		0.31 <sup>b</sup>		0.16 <sup>b</sup>	0.42 <sup>b</sup>

Abbreviations: HL = Hodgkin's lymphoma; MFB = myelofibrosis; MM = multiple myeloma; RCC = renal cell carcinoma.

<sup>a</sup>Log-rank test from Kaplan-Meier curves for inhibitory KIR score groups 1 vs 2 vs 3 vs 4.

<sup>b</sup>Kruskal-Wallis test for inhibitory KIR score groups 1 vs 2 vs 3 vs 4.

## Discussion

Assessment of donor chimerism after allogeneic NMHSCT has been used to determine whether residual disease persists as well as to monitor donor hematopoietic engraftment. Obtaining complete donor T-cell chimerism post transplant has also been considered important to achieve a GVM effect.<sup>3,6</sup> Several factors have been associated with the development of CDC after NMHSCT, including the type of hematologic malignancy, the amount of pretransplant therapy, the source of hematopoietic stem cells, the composition of the hematopoietic graft and the type of donor (related vs unrelated).<sup>24–26</sup> Baron *et al.*<sup>24</sup> reported a higher level of T-cell donor chimerism in patients whose grafts contained greater numbers of T-cells, NK cells and monocytes. The kinetics of achieving donor T-cell chimerism after NMHSCT have also been predictive for graft rejection.<sup>5,24,27</sup> However, the influence of KIR/ligand matching on the achievement of T-cell CDC after NMHSCT has not been previously reported.

The alloreactivity of donor-derived immune effector cells, including NK cells and some T-cell subsets, is mediated through the interaction of their KIRs with host HLA/KIR ligands. KIR interactions appear to influence outcomes after myeloablative HSCT,<sup>9,17–21</sup> however, their importance in NMHSCT is not well known. Although donors and recipients have identical HLA types in matched related donor allogeneic HSCT, the loci for HLA and KIRs are on different chromosomes that segregate independently. Therefore, disparities between the recipient KIRs and the donor HLA class I KIR-ligands occur. This may allow recipient NK cells to become alloreactive against donor T cells that lack corresponding KIR ligands.

The current report utilized a strategy of KIR/ligand matching to quantitate the potential number of recipient inhibitory KIRs that could be engaged with donor HLA/KIR ligands to derive an inhibitory KIR score. Patients with a score of 1 were significantly less likely to achieve T-cell CDC and were more likely to develop graft rejection than those with scores greater than 1. Thus, patients with lower inhibitory KIR scores may have more active anti-donor immune effector cells (NK cells and T-cell subsets) that may reduce donor chimerism. Conversely, those patients with greater inhibitory KIR scores may have less active NK cell and T-cell populations, which may therefore make them more likely to achieve CDC.

Since all of the transplant recipients had at least one inhibitory KIR gene specific for an HLA/KIR ligand present in their donor, the genotypic potential existed to inhibit all NK cells. However, KIR expression may be variable among different immune effector cell clones, which may affect the development of CDC. Discrepancies between KIR genotyping and phenotyping have been reported to occur in approximately 25% of cases.<sup>28</sup> Therefore, further investigation of KIR expression at the cellular level, rather than by KIR genotyping alone, is warranted.<sup>28–30</sup> Moreover, in a subsequent analysis the presence of single or multiple activating KIRs was not found to influence the development of CDC between the different inhibitory KIR score groups in our patients (data not shown).

Preclinical data has demonstrated that host NK cells may mediate graft rejection<sup>31–33</sup> and this may be further anticipated after NMHSCT when increased numbers of these cells are still present. Strategies to enhance engraftment for these patients have included intensification of the transplant conditioning regimen such as escalation of TBI doses.<sup>34,35</sup> We have observed that escalating the TBI dose from 200 cGy to 400 cGy resulted in more rapid achievement of durable CDC and less graft rejection for NMHSCT patients.<sup>36</sup>

Assessment of interactions between KIRs and their HLA class I ligands has important implications not only for myeloablative HSCT, but also for patients undergoing NMHSCT. The finding that patients with an inhibitory KIR score of 1 are less likely to achieve CDC and more likely to have graft rejection suggests that further strategies to intensify therapy (for example, escalation of TBI doses or the administration of prophylactic DLIs for patients without GVHD) may be appropriate for such patients. Further investigation of KIR/ligand matching including KIR phenotypic analyses may be useful to help optimize conditions for achievement of CDC and prevention of graft rejection after NMHSCT.

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