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Status of Donor-Recipient HLA Class I Ligands and Not the KIR Genotype Is Predictive for the Outcome of Unrelated Hematopoietic Stem Cell Transplantation in Beta-Thalassemia Patients

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ABSTRACT

Several studies have investigated the role played by killer immunoglobulin-like receptors (KIRs) and their ligands on the outcome of hematopoietic stem cell transplantation (HSCT) in patients affected by oncohematologic diseases. However, the interpretation of the results of these studies is considerably hampered by the heterogeneity of the diseases, disease status at transplantation, and the different protocols employed for both conditioning and graft-versus-host disease (GVHD) prophylaxis. To better define the role of KIRs in HSCT, we studied KIR genotypes and HLA class I ligands in a homogeneous group of 45 thalassemia patients transplanted with bone marrow cells from an HLA-identical, unrelated donor. Patients that were heterozygotes for HLA-Cw groups 1 (HLA-Cw^{Asn80}) and 2 (HLA-Cw^{Lys80}) had a higher risk of developing acute GVHD than C1/C1 or C2/C2 homozygotes (relative risk [RR] = 8.75; 95% confidence interval [CI]: 1.63-46.76; P = .007). Vice versa, all patients who experienced primary/secondary graft failure were C1/C1 or C2/C2 homozygotes (RR = 20.45; 95% CI = 1.08-384.24; P = .009). Moreover, the presence of the HLA-A11 antigen conferred protection against GVHD (0% versus 35%, P = .02). Our results suggest that C1/C2 heterozygosity, may favor the development of donor alloreactivity and thereby increase the risk of GVHD. Conversely, C1/C1 and C2/C2 homozygosity seems to reduce the risk of GVHD but may increase the incidence of graft rejection. These data may be helpful in tailoring the intensity of GVHD prophylaxis and conditioning regimens in thalassemia patients receiving HSCT from an HLA-identical volunteer donor.

KEY WORDS

KIRS • KIR ligands • KIR genotypes • NK cell alloreactivity • Unrelated BMT • Thalassemia

INTRODUCTION

The family of killer immunoglobulin-like receptor (KIR) genes currently consists of 15 functional genes (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1, KIR3DL2, KIR3DL3, KIR3DS1) and 2 pseudogenes (KIR2DP1 and KIR3DP1) encoded within a 100-200-kb region of the Leukocyte Receptor Complex (LRC) on chromosome 19q13.4 [1]. KIR genes are organized within the LRC as haplotypes, which have been shown to exhibit extensive variation in the number and type of KIR genes present [2].

The receptors encoded by these genes are a cluster of polymorphic immunoglobulin-like molecules that modulate the cytotoxic activity of natural killer (NK) cells through interaction with HLA class I classical and nonclassical molecules [3].

HLA-Cw is the predominant ligand for the 2DL1, 2DL2, and 2DL3 inhibitory KIRs and the 2DS1 and 2DS2 activatory KIRs [4]. The replacement of an amino acid residue at positions 77 and 80 of the HLA-C α-helix makes it possible to divide HLA-Cw molecules into 2 groups, C1 (HLA-Cw^{Asn80}) and C2 (HLA-Cw^{Lys80}), each 1 selectively recognized by a specific pair of activatory/inhibitory KIRs [5-10]. The molecules belonging to the HLA-Bw4 group present epitopes recognized by the 3DL1 inhibitory KIR, whereas HLA-A3 and HLA-A11 molecules present epitopes recognized by the 3DL2 inhibitory KIR [11-15]. HLA-G Class I molecules have been reported to inhibit NK cytotoxicity at the maternal-fetal interface by binding with the 2DL4 inhibitory KIR, and could play an important role in the development/maintenance of maternal-fetal tolerance [16,17]. Despite the recent insights into KIR-ligand interactions, not all KIR ligands are known, particularly those involving activatory KIRs.

In T cell-depleted allogeneic hematopoietic stem cell transplantation (HSCT) from an HLA-haploidentical family donor, it has been demonstrated that donor NK alloreactive cells exert a graft-versus-leukemia effect, which reduces the risk for relapse of myeloid leukemia, and confer protection against graft-versus-host disease (GVHD) and rejection through lysis of recipient antigen-presenting cells and T lymphocytes, respectively [18-20]. Numerous studies have investigated the influence of KIRs on the outcome of unmanipulated related and unrelated HSCT for hematological malignancies, but the results remain controversial [21-25]. Disease heterogeneity, disease status at transplantation, as well as differences in conditioning regimens and GVHD prophylaxis, may at least partly explain the discrepancies in the results of these studies. Thalassemia patients undergoing HSCT have a competent immune system that has not been compromised by previous chemotherapy and constitute a homogeneous cohort in terms of disease, stem cell source, conditioning regimen, and GVHD prophylaxis. For this reason HLA-identical unrelated HSCT for thalassemia represents an ideal model for the evaluation of the role of immunogenetic factors in the outcome of transplantation.

To further contribute to the comprehension of the role of KIR-ligand interactions in HSCT, we investigated the KIR gene content and the currently known HLA class I ligands in a group of 45 thalassemia patients transplanted in 3 Italian Bone Marrow Transplantation (BMT) Centers.

PATIENTS AND METHODS

Patients and Donors

KIR gene content was retrospectively evaluated in 45 β-thalassemia major patients transplanted from an unrelated donor from September 1993 to December 2005. The study was approved by the local institutional review board of each participating Center, and informed consent was obtained from all patients or from their parents or legal guardians. The clinical characteristics of donor-recipient pairs are reported in Table 1. Median time of follow-up for surviving patients was 4 years and 7 months (range: 1.5-14 years). Twenty patients were females (44%) and 25 males (56%), the age range being 2-26 years (median age

Table 1. *Clinical Data and Transplantation Outcome of 45 Beta-Thalassemia Patients*

No. of patients	**45**
Median patient age, years (range)	**12.4 (2-26)**
Median donor age, years (range)	**33 (19-47)**
Patient/donor sex	
Male patient with female donor, n (%)	**14 (31.1%)**
Other combinations, n (%)	**31 (68.9%)**
Risk class*	
1, n (%)	**14 (31.1%)**
2, n (%)	**18 (40%)**
3, n (%)	**13 (28.9%)**
Patient/donor CMV serology	
Negative/negative, n (%)	**5 (11.1%)**
Other combinations, n (%)	**40 (88.9%)**
Patient/donor HLA compatibility	
Class I (-A, -B, -C) and II (-DRB, -DQB1) match, n (%)	**44 (97.8%)**
Single Class I mismatch, n (%)	**1 (2.2%)**
HLA-DPB1 match or permissive mismatch†, n (%)	**27 (60%)**
HLA-DPB1 nonpermissive mismatch → HVG†, n (%)	**11 (24.4%)**
HLA-DPB1 nonpermissive mismatch → GVH†, n (%)	**7 (15.6%)**
Conditioning regimen	
BU-TT-CY	**28 (62.2%)**
BU-TT-FLU	**17 (37.8%)**
Median cell dose, $\times 10^8$/kg (range)	**4.5 (2-15)**
Transplantation outcome	
Rejection, n (%)	**7 (15.6%)**
aGvHD grade II-IV, n (%)	**12 (31.5%)**
Overall survival, n (%)	**39 (86.7%)**
Thalassemia-free survival, n (%)	**32 (71.1%)**
Transplantation-related mortality, n (%)	**6 (13.3%)**

HVG indicates host-versus-graft; GVH, graft-versus-host; CMV, cytomegalovirus; BU, Busulfan; TT, Thiotepa; CY, Cyclophosphamide; FLU, Fludarabine.

*Risk class according to the Pesaro classification [26].

†Classification of HLA-DPB1 alleles was performed according to the algorithm described by Zino et al [30] and Fleischhauer et al [31].

12.5). Twenty-two unrelated donors were female (48%) and 23 male (52%), age range being 19 to 47 years (median 33 years). Fourteen male recipients (31%) had been transplanted from a female donor.

Prior to transplantation, the patients had been assigned to 1 of 3 risk classes according to the criteria proposed by Lucarelli et al [26]. The patients had a homogeneous distribution among the 3 risk classes: 14 were assigned to Risk Class 1 (low risk), 18 to Risk Class 2 (intermediate risk), and 13 to Risk Class 3 (high risk).

Transplantation Regimen and GVHD Prophylaxis

All patients were prepared for the allograft using a myeloablative conditioning regimen. In 30 cases the conditioning regimen included Busulfan (BU), 3.5 mg/kg by mouth daily in divided doses for 4 days (total dose 14 mg/kg), Thiotepa (TT), 10 mg/kg i.v. administered in 2 doses on the same day, followed by Cyclophosphamide (CY), 50 mg/kg i.v. once daily for 4 days (total dose 200 mg/kg) or CY 60 mg/kg i.v. once daily for 2 days (total dose 120 mg/kg) depending upon the risk class according to Lucarelli et al [26]. The remaining 15 patients were given a modified conditioning regimen with BU14, TT10, and Fludarabine (FLU), 40 mg/m^2 i.v. administered once daily for 4 days (total dose 160 mg/m^2) [27].

To prevent any risk related to persistent cytopenia in patients with poor graft function, an autologous rescue of bone marrow cells was harvested and cryopreserved before transplantation for all patients. All patients received unmanipulated bone marrow cells. Marrow was infused 48-72 hours after the last dose of CY/FLU. All patients received GVHD prophylaxis with cyclosporine-A (Cs-A), 3 mg/kg/day i.v. starting from day −2, and short-term methotrexate (MTX), 15 mg/m^2 on day +1 and 10 mg/m^2 on days +3, +6, and +11. Cs-A was switched to 6 mg/kg/day orally as soon as oral administration could be tolerated; starting from day +90, the dose was tapered, until discontinuation at 1 year. Engraftment was documented by in situ Y chromosome hybridization of bone marrow or blood samples in sex-mismatched donor/recipient pairs and by analysis of short tandem repeats on blood and/or bone marrow samples in sex-matched pairs.

Acute GVHD (aGVHD) was graded according to internationally accepted criteria [28,29]. Graft rejection was defined as either the absence of hematopoietic reconstitution of donor origin on day +45 after the allograft (primary graft rejection) or as loss of donor cells after transient engraftment of donor-origin hematopoiesis, with return to transfusion dependence (secondary graft rejection).

HLA Typing

All patients and donors were HLA typed using high-resolution molecular techniques. DNA for molecular typing was extracted from whole blood using standard methods. The alleles at the HLA-A, -B, -C, -DRB1, -DRB3, -DRB4, -DRB5, -DQA1, -DQB1, and -DPB1 loci were identified by polymerase chain reaction-sequence-specific primers (PCR-SSP) (Dynal, Oslo, Norway) and sequence-based typing.

Forty-four donor/recipient pairs were matched at molecular level for the HLA-A, -B, -C, -DRB1, -DRB3, -DRB4, -DRB5, -DQA1, and -DQB1 loci. One donor/recipient pair had a disparity at the HLA-C locus (Cw*0501 versus Cw*0202), but both HLA-Cw molecules belonged to the C2 ligand group (Cw^{Lys80}). Twenty-seven patients (60%) were matched or permissively mismatched with their donors for the HLA-DPB1 alleles, 11 (24%) had at least 1 nonpermissive DPB1 disparity in host-versus-graft direction, and 7 (16%) had a nonpermissive DPB1 disparity in GVHD direction [30,31].

Typing of KIR Genes

The KIR genes 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, 3DL3, 2DS1, 2DS2, 2DS3, 2DS4, and 3DS1 were typed using the KIR-gene-specific primers described by Uhrberg et al with minor modifications [32]. The KIR gene 2DS5 was typed using the primers described by Gagne et al [33].

Statistical Analysis

Patient, disease, and transplant-related variables were expressed as median and range or as percentage, as appropriate. The following variables were analyzed for their potential impact on outcome: donor and recipient sex, donor and recipient age, Pesaro risk class at HSCT, cytomegalovirus (CMV) serology, conditioning regimen, marrow cell dose infused, HLA Class I mismatch, HLA-DPB1 disparity, classified in 1 of 3 categories according to the identity, or degree of permissivity in case of disparity [30,31]. Patients were censored at the time of rejection, death, or last follow-up. Probabilities of overall survival (OS) and survival with transfusion independence (thalassemia-free survival) were estimated by the Kaplan-Meier method and expressed as percentage with a 95% confidence interval (CI) [34]. The probability of developing aGVHD, experiencing rejection or transplantation-related mortality (TRM) was expressed as cumulative incidence curves to adjust the analysis for competing risks, namely death and graft failure for GVHD and death for graft failure [35,36]. The significance of differences between curves was estimated by the log-rank test. Furthermore, the differences in percentages of events in the groups of patients were compared using the 2-sided Fisher's exact P test or a χ^2, as

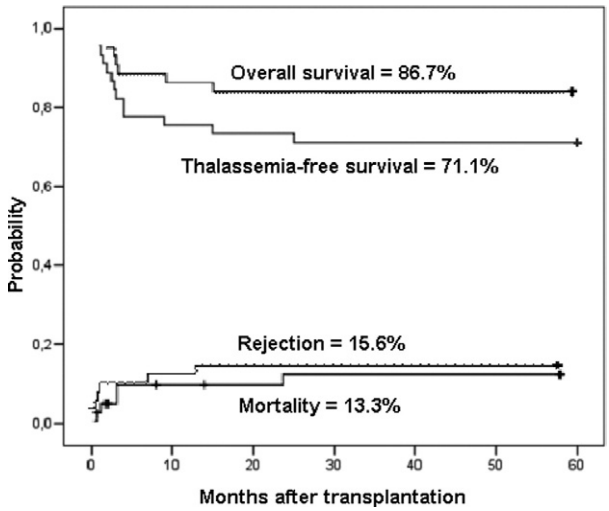

Figure 1. Kaplan-Meier probabilities of overall survival, thalassemia-free survival and cumulative incidence of mortality and rejection in 45 thalassemia patients transplanted from an unrelated donor.

appropriate. *P*-values of $<.05$ were considered as significant. Due to the limited size of the cohort analyzed, no correction was made for multiple comparisons. Variables with a *P*-value $<.5$ in univariate analysis were included in a multivariate analysis performed using the Cox proportional hazard regression model [37,38]. Statistical analysis was performed using the SAS System (SAS, Cary, NC) and the NCSS computer program (J. Hintze, 2001, NCSS and PASS, Number Cruncher Statistical System, Kaysville, UT).

RESULTS

Clinical Outcome

The results obtained in our patient cohort are reported in Table 1. Figure 1 shows the 3-year Kaplan-Meier estimates of survival (86.7%), thalassemia-free survival (71.1%), the cumulative incidence of rejection (15.6%), and TRM (13.3%) for the 45 patients studied. Twelve (31%) and 5 (13%) patients developed grade II-IV and grade III-IV aGVHD, respectively. Seven patients rejected the allograft and 6 died of transplant-related complications: 4 patients died of aGVHD, 1 of liver failure secondary to chronic GVHD (cGVHD), and 1 of CMV-related interstitial pneumonia.

KIR Gene Frequencies

Comparisons of KIR gene frequencies among donors, recipients, and a panel of healthy blood donors did not yield significant differences in the KIR gene distribution. The gene frequencies were similar to those reported in other Caucasoid populations [32,39]. Of the 14 KIR genes analyzed, the framework genes 2DL4, 3DL2, and 3DL3 were present in all donor and recipient pairs and 2DL1 was present in 88 (98%) of the 90 subjects examined. Most variations were observed for the number and specificity of activatory KIR genes, particularly 2DS1, 2DS3, and 2DS5, which were present in 47%, 31%, and 36% of the total subjects, respectively. Interestingly, 5 of the 7 patients (71%) who rejected had 1-2 additional activatory KIR genes compared to their donors (2DS2 and 2DS3 in 1 case, 2DS1 in 2 cases, and 2DS3 in the remaining 2 cases). There were no differences between the KIR gene profile of patients that developed grade II-IV aGVHD and their donors.

Table 2. *HLA Class I KIR Ligands and KIR Gene Profiles in 45 Beta-Thalassemia Patients and Their Donors*

No. of patients	**45**
Patient KIR ligands	
Patient C1/C1*	**9 (20%)**
Patient C2/C2*	**14 (31.1%)**
Patient C1/C2*	**22 (48.9%)**
Patient HLA-Bw4 present, n (%)	**30 (66.6%)**
Patient HLA-Bw4 absent, n (%)	**15 (33.4%)**
Patient HLA-A11 present, n (%)	**11 (24.4%)**
Patient HLA-A11 absent, n (%)	**34 (75.6%)**
Donor/recipient KIR gene profile†	
D < R, n (%)	**13 (28.8%)**
D ≥ R, n (%)	**16 (35.6%)**
D ≠ R, n (%)	**16 (35.6%)**
Patient KIR ligand/donor activatory KIR‡	
Patient-C1-absent/donor-KIR2DS2-present, n (%)	**9 (20%)**
Other 3 combinations, n. (%)	**36 (80%)**
Patient-C2-absent/donor-KIR2DS1-present, n (%)	**3 (6.6%)**
Other 3 combinations, n (%)	**42 (93.4%)**
Patient KIR ligand/donor inhibitory KIR§	
Patient-C1-absent/donor-KIR2DL2-present, n (%)	**9 (20%)**
Other 3 combinations, n (%)	**36 (80%)**
Patient-C1-absent/donor-KIR2DL3-present, n (%)	**13 (28.8%)**
Other 3 combinations, n (%)	**32 (71.2%)**
Patient-C2-absent/donor-KIR2DL1-present, n (%)	**9 (20%)**
Other 3 combinations, n (%)	**36 (80%)**
Patient-Bw4-absent/donor 3DL1 present, n (%)	**15 (35.7%)**
Patient-Bw4-present/donor 3DL1 present, n (%)	**27 (64.3%)**

*C1 = HLA-Cw^{Asn80} KIR ligand.

*C2 = HLA-Cw^{Lys80} KIR ligand.

†Donor/recipient groups according to Gagne et al [33]: D < R = donor KIR genotype included in the recipient KIR genotype; D ≥ R = recipient KIR genotype identical or included in the donor KIR genotype; D ≠ R = different KIR genotypes in the donor and the recipient.

‡Combinations of donor 2DS2 and 2DS1 activatory KIRs and patient C1 and C2 KIR ligands, according to the model proposed by Cook et al [23].

§Combinations of donor 2DL2, 2DL3, 2DL1, and 3DL1 inhibitory KIRs with patient C1, C2, and Bw ligands, according to the model proposed by Hsu et al [24].

HLA KIR Ligands and Their Influence on Transplantation Outcome

The HLA-Cw KIR ligand was studied in the 45 donor/recipient pairs (Table 2): 20% (9 of 45) were C1/C1 homozygotes, 31% (14 of 45) were C2/C2 homozygotes, and 49% (22 of 45) were C1/C2 heterozygotes. The HLA-Cw^{Asn80} and HLA-Cw^{Lys80} molecules expressed by donor/recipient pairs had a significant impact on transplantation outcome in our cohort (Table 3). Univariate analysis showed that when donor/recipient pairs were C1/C2 heterozygotes, patients had a higher risk of developing grade II-IV aGVHD (10 of 22; 45.5%) than patients of the C1/C1 or C2/C2 homozygous groups (2 of 23; 8.7%); RR = 8.75; 95% CI = 1.63-46.76; P = .007; log rank = .004; Figure 2). On the contrary, the 7 patients who rejected the allograft were all C1/C1 or C2/C2 homozygotes (7 of 23; 30.4%) whereas none of the C1/C2 heterozygous patients experienced graft failure (0 of 22); RR = 20.45; 95% CI = 1.08-384.24; P = .009; log rank = .005; Figure 3). The increased risk of aGVHD observed in C1/C2 heterozygotes as well as the increased risk of rejection observed in C1/C1 and C2/C2 homozygotes were confirmed by multivariate analysis (RR 6.28 [95% CI = 1.49-31.2], P = .01) and (RR 6.50 [95% CI = 1.32-6.15], P = .05), respectively. No significant correlation was observed for OS, and thalassemia-free survival, even if the latter was better in the C1/C2 group (Figure 4).

HLA-Bw4 molecules are recognized by the KIR3DL1 receptor [14]. The frequency of KIR3DL1 was 93% in both donors and recipients. No significant differences were observed for the incidence of aGVHD or rejection related to the presence or absence of the HLA-Bw4 ligands in our donor/recipient pairs. In fact, in 30 HLA-Bw4 positive patients, the incidence of aGVHD was 27% (8 of 30) and the incidence of rejection was 13% (4 of 30), while in 15 HLA-Bw4-negative patients the incidence of aGVHD and rejection was 27% (4 of 15) and 20% (3 of 15), respectively (Tables 2 and 3).

HLA-A11 molecules present epitopes that are recognized by the 3DL2 inhibitory KIR framework gene [15]. Among the 45 donor/recipient pairs examined, 24% (11 of 45) expressed HLA-A11 molecules. None of the patients expressing HLA-A11 molecules developed grade II-IV aGVHD (RR = 0.08; 95% CI: 0.004-1.44; P = .02; log rank = .03) (Figure 5 and Tables 2 and 3). The HLA-A11-positive patients were evenly distributed among the 3 groups of C1 or C2 homozygotes and C1/C2 heterozygotes (2 of 9, 6 of 14, and 3 of 22, respectively).

KIR Genotype Analysis

Characterization of KIR genotypes made it possible to subdivide the 45 donor/recipient pairs into 3 groups according to 3 different combination patterns, as described by Gagne et al [33] (Table 2). In 13 pairs (28%), the donor KIR genotype was included in the recipient KIR genotype [ie, the recipient had additional KIR genes absent in the corresponding donor (D < R)]. In 16 pairs (36%), the recipient KIR genotype was either included or identical to the corresponding donor KIR genotype (D ≥ R). In the remaining 16 pairs (36%), the donor and the recipient had different KIR genotypes (D ≠ R). The last group was not considered for statistical analysis. The incidence and grade of aGVHD were evaluated in the above-defined groups of donors and recipients (Table 3). When the donor KIR genotype was included in the recipient KIR genotype (D < R), 31% (4 of 13) of patients developed II-IV grade aGVHD compared to 25% (4 of 16) when the pair pattern was D ≥ R (P = ns). The incidence of rejection was higher, although not statistically significant, in the D < R group with 3 of 13 cases of rejection (23%), compared to 0 of 16 cases in the D ≥ R group. No differences were observed between the 3 groups for OS, thalassemia-free survival, and TRM (Table 3).

Interaction between Donor KIRs and Recipient HLA Class I Ligands

We analyzed the 45 donor/recipient pairs to test whether donor 2DS1 and 2DS2 activatory KIRs and the presence or absence of C1 and C2 ligands in the recipients had an influence on the outcome of transplantation [23]. We also investigated the effect of the "missing KIR ligand" for the inhibitory KIRs 2DL1, 2DL2, 2DL3, and 3DL1 [24] (Tables 2 and 3).

In donor/recipient pairs with the combination patient-C1-absent/donor-KIR2DS2-present (9/45), the incidence of aGVHD was 11% (1/9) and the incidence of rejection was 33% (3/9). In the patient-C1-present/donor-KIR2DS2-present group (13 of 45), the incidence of aGVHD was 31% (4 of 13) with no cases of rejection. In donor/recipient pairs that were patient-C1-absent/donor-KIR2DS2-absent (5 of 45), we observed 1 case of aGVHD (20%) and a single case of rejection (20%). In 18 of 45 pairs that were patient-C1-present/donor-KIR2DS2-absent, the incidence of aGVHD was 33% (6 of 18) and the incidence of rejection was 17% (3 of 18). A comparison of the patient-C1-absent/donor-KIR2DS2-present group with those of the other combinations did not yield significant differences for aGVHD or rejection. The analysis performed to evaluate if the presence of KIR2DS1 in the donor had an influence on the outcome of patients lacking the C2 ligands did not reveal any significant association (Table 3).

Analysis of donor 2DL1, 2DL2, 2DL3, and 3DL1 inhibitory KIRS showed that donors/recipients of the C1-absent/donor-KIR2DL2-present group (9 of 45) only had 1 case of aGVHD (11%) and 3 cases of rejec-

Table 3. *Univariate Analysis for the Risk of aGVHD (Grade II-IV), Rejection, and Transplantation-Related Mortality and the Probability of Thalassemia-Free Survival in 45 Transplanted Thalassemia Patients*

	Total no. Patients	Grade II-IV aGVHD		Rejection		Thalassemia-Free Survival		Transplantation-Related Mortality	
No. of Patients (%)	45 (100%)	12 (26.6%)	*P*	7 (15.5%)	*P*	32 (71.1%)	*P*	6 (13.3%)	*P*
Median patient age, years (range)	12.4 (2-26)	12.5 (4-23)	.38	13 (3-15)	ns	12 (2-26)	ns	14.1 (9-19)	.42
Median donor age, years (range)	33 (19-47)	34 (22-47)	ns	36 (26-44)	.21	34 (19-47)	ns	30 (22-45)	.43
Patient/donor sex			ns		.40		ns		.35
Male patient with female donor, n (%)	14 (31.1%)	3 (25%)		1 (14.3%)		10 (31.2%)		3 (50%)	
Other combinations, n (%)	31 (68.9%)	9 (75%)		6 (85.7%)		22 (68.8%)		3 (50%)	
Risk Class*			ns		ns		ns		.33
1, n (%)	14 (31.1%)	4 (33.3%)		2 (28.6%)		11 (34.4%)		1 (16.7%)	
2, n (%)	18 (40%)	5 (41.7%)		3 (42.8%)		13 (40.6%)		2 (33.3%)	
3, n (%)	13 (28.9%)	3 (25%)		2 (28.6%)		8 (25%)		3 (50%)	
Patient/donor CMV serology			ns		ns		ns		ns
Negative/negative, n (%)	5 (11.1%)	1 (8.3%)		0 (0%)		4 (12.5%)		1 (20%)	
Other combinations, n (%)	40 (88.9%)	11 (91.7%)		7 (100%)		28 (87.5%)		5 (80%)	
Patient/donor HLA compatibility									
Class I (-A, -B, -C) and II (-DRB, -DQB1) match, n (%)	44 (97.8%)	12 (100%)	ns	7 (100%)	ns	31 (96.9%)	ns	6 (100%)	ns
Single Class I mismatch, n (%)	1 (2.2%)	0 (0%)		0 (0%)		1 (3.1%)		0 (0%)	
HLA-DPB1 match or permissive mismatch†, n (%)	27 (60%)	6 (50%)	ns	3 (42.8%)	.41	22 (68.8%)	.09	2 (33.3%)	.19
HLA-DPB1 nonpermissive mismatch → HVG†, n (%)	11 (24.4%)	5 (41.7%)		3 (42.8%)		6 (18.6%)		2 (33.3%)	
HLA-DPB1 nonpermissive mismatch → GVH†, n (%)	7 (15.6%)	1 (8.3%)		1 (14.4%)		4 (12.6%)		2 (33.3%)	
Conditioning regimen			ns		ns		.05		.02
BU-TT-CY	28 (62.2%)	6 (50%)		4 (57.2%)		14 (43.7%)		6 (100%)	
BU-TT-FLU	17 (37.8%)	6 (50%)		3 (42.8%)		18 (56.3%)		0 (0%)	
Median cell dose, $\times 10^8$/kg (range)	4.5 (2-15)	5.2 (2.7-9)	ns	4.5 (2.6-7.5)	.46	4.7 (2.1-15)	.24	4.0 (3.4-7.5)	.45
Patient KIR ligands									
Patient C1/C1 or C2/C2	23 (51.1%)	2 (16.7%)	.007	7 (100%)	.009	14 (43.7%)	.18	2 (33.3%)	.41
Patient C1/C2	22 (48.9%)	10 (83.3%)		0 (0%)		18 (43.7%)		4 (66.7%)	
Patient HLA-Bw4 present, n (%)	30 (66.6%)	8 (66.7%)	ns	4 (57.2%)	ns	22 (68.7%)	ns	4 (66.7%)	ns
Patient HLA-Bw4 absent, n (%)	15 (33.4%)	4 (33.3%)		3 (42.8%)		10 (31.3%)		2 (33.3%)	
Patient HLA-A11 present, n (%)	11 (24.4%)	0 (0%)	.02	2 (28.6%)	ns	8 (25%)	ns	1 (20%)	ns
Patient HLA-A11 absent, n (%)	34 (75.6%)	12 (100%)		5 (71.4%)		24 (75%)		5 (80%)	
Donor/recipient KIR gene profile‡			ns		.08		.19		ns
D < R, n (%)	13 (28.8%)	4 (33.3%)		3 (42.8%)		8 (25%)		2 (33.3%)	
D ≥ R, n (%)	16 (35.6%)	4 (33.3%)		0 (0%)		14 (43.7%)		2 (33.3%)	
D ≠ R, n (%)	16 (35.6%)	4 (33.3%)	ne	4 (57.2%)	ne	10 (31.3%)	ne	2 (33.3%)	ne
Patient KIR ligand/donor activatory KIR§									
Patient-C1-absent/donor-KIR2DS2-present, n (%)	9 (20%)	1 (8.3%)	.40	3 (42.8%)	.13	5 (15.6%)	.41	1 (20%)	ns
Other 3 combinations, n (%)	36 (80%)	11 (91.7%)		4 (57.2%)		27 (84.4%)		5 (80%)	
Patient-C2-absent/donor-KIR2DS1-present, n (%)	3 (6.6%)	0 (0%)	ns	0 (0%)	ns	3 (9.4%)	ns	0 (0%)	ns
Other 3 combinations, n (%)	42 (93.4%)	12 (100%)		7 (100%)		29 (90.6%)		6 (100%)	

Table 3. *(Continued)*

No. of Patients (%)	Total no. Patients 45 (100%)	Grade II-IV aGVHD 12 (26.6%)	P	Rejection 7 (15.5%)	P	Thalassemia-Free Survival 32 (71.1%)	P	Transplantation-Related Mortality 6 (13.3%)	P
Patient KIR ligand/donor inhibitory KIR#									
Patient-C1-absent/donor-KIR2DL2-present, n (%)	9 (20%)	1 (8.3%)	.40	3 (42.8%)	.13	5 (15.6%)	.41	1 (20%)	ns
Other 3 combinations, n (%)	36 (80%)	11 (91.7%)		4 (57.2%)		27 (84.4%)		5 (80%)	
Patient-C1-absent/donor-KIR2DL3-present, n (%)	13 (28.8%)	2 (16.7%)	.45	3 (42.8%)	.39	8 (25%)	.47	2 (33.3%)	ns
Other 3 combinations, n (%)	32 (71.2%)	10 (83.3%)		4 (57.2%)		24 (75%)		4 (66.7%)	
Patient-C2-absent/donor-KIR2DL1-present, n (%)	9 (20%)	0 (0%)	.08	3 (42.8%)	.13	6 (18.7%)	ns	0 (0%)	ns
Other 3 combinations, n (%)	36 (80%)	12 (100%)		4 (57.2%)		26 (81.3%)		6 (100%)	
Patient-Bw4-absent/donor 3DL1 present, n (%)	15 (35.7%)	4 (36.6%)	ns	3 (42.8%)	ns	10 (34.5%)	ns	2 (50%)	ns
Patient-Bw4-present/donor 3DL1 present, n (%)	27 (64.3%)	7 (63.4%)		4 (57.2%)		19 (65.5%)		2 (50%)	

CMV indicates cytomegalovirus; C1, HLA-Cw^{Asn80} KIR ligand; C2, HLA-Cw^{Lys80} KIR ligand; HVG, host versus graft; GVH, graft versus host; BU, Busulfan; TT, Thiotepa; CY, Cyclophosphamide; FLU, Fludarabine; ns, not significant; ne, not evaluable; aGVHD, acute graft-versus-host disease.

*Risk Class according to the Pesaro classification [26].

†Classification of the HLA-DPB1 alleles was performed according to the algorithm described by Zino et al [30] and Fleischhauer et al [31].

‡Donor/recipient groups according to Gagne et al [33]: D < R = donor KIR genotype included in the recipient KIR genotype; D ≥ R = recipient KIR genotype identical or included in the donor KIR genotype; D ≠ R = different KIR genotypes in the donor and the recipient.

§Combinations of donor 2DS2 and 2DS1 activatory KIRs and patient C1 and C2 KIR ligands, according to the model proposed by Cook et al [23].

#Combinations of donor 2DL2, 2DL3, 2DL1, and 3DL1 inhibitory KIRs with patient C1, C2, and Bw ligands, according to the model proposed by Hsu et al [24].

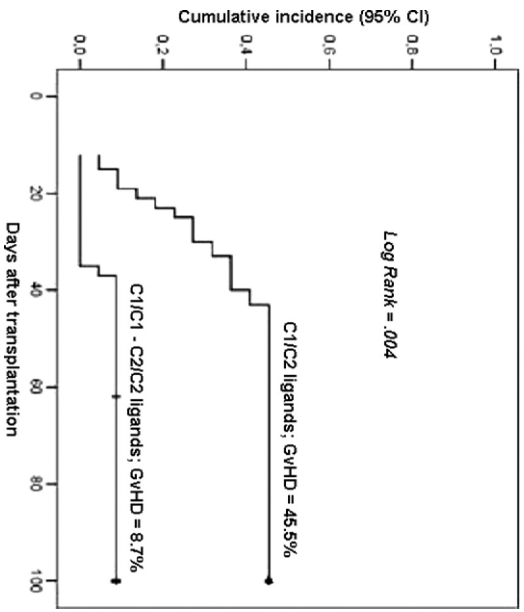

Figure 2. Influence of heterozygosity for the HLA-Cw ligand groups 1 and 2 (C1/C2) on the incidence of grade II-IV aGVHD in 45 thalassemia patients transplanted from an unrelated donor. (C1/C2 patients = 22, events = 10; C1/C1 patients = 9, events = 0; C2/C2 patients = 14, events = 2).

tion (33%). Comparisons with the other 3 combinations (patient-C1-absent/donor-KIR2DL2-absent, patient-C1-present/donor-KIR2DL2-present, and patient-C1-present/donor-KIR2DL2-absent) did not yield any significant differences for aGVHD and rejection. A mild increase of rejection was observed in patients when comparing the 9 pairs of the patient-C1-absent/donor-KIR2DL2-present group with the 13 pairs of the patient-C1-present/donor-KIR2DL2-present group

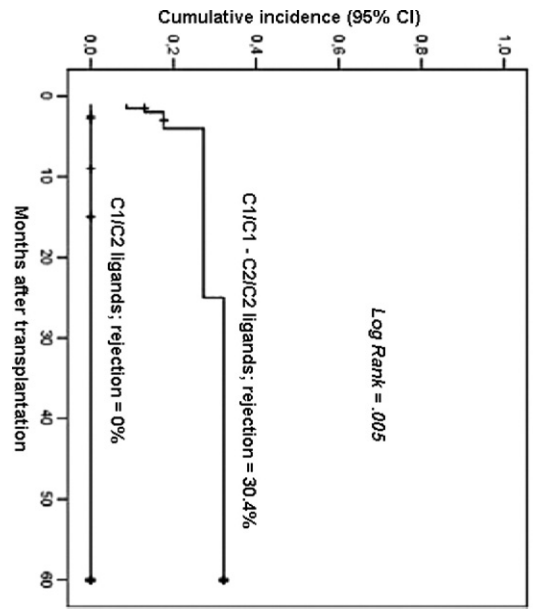

Figure 3. Influence of homozygosity for the HLA-Cw ligand groups (C1/C1 or C2/C2) on the cumulative incidence of rejection in 45 thalassemia patients transplanted from an unrelated donor. (C1/C1 patients = 9, events = 3; C2/C2 patients = 14, events = 4; C1/C2 patients = 22, events = 0).

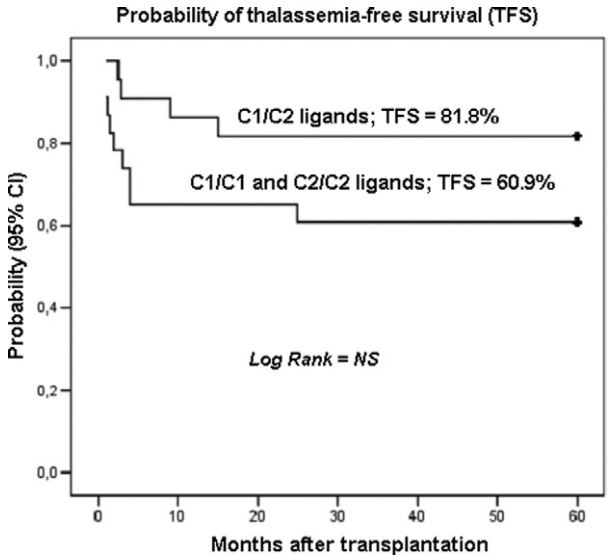

Figure 4. Comparison of the probabilities of thalassemia-free survival (TFS) between 22 C1/C2 heterozygotes (events = 18) and 23 C1/C1 or C2/C2 homozygotes (C1/C1 patients = 9, events 6; C2/C2 patients = 14, events = 8).

(3 of 9 versus 0 of 13; RR =3.16; 95% CI = 1.63-6.13; P = .054). No significant differences were found in comparisons between donor/recipient pairs of the patient-C1-absent/donor-KIR2DL3-present group (13/45) and those of the other groups: patient-C1-absent/donor-KIR2DL3-absent, patient-C1-present/donor-KIR2DL3-present, and patient-C1-present/donor-KIR2DL3-absent. The comparison between the patient-C2-absent/donor-KIR2DL1-present group (9 of 45) and the other 3 groups (patient-C2-absent/donor-KIR2DL1-absent, patient-C2-present/donor-KIR2DL1-present, and patient-C2-present/donor-KIR2DL1-absent), although not statistically significant, revealed a decreased risk for aGVHD (0 of 9 versus 12 of 36) (RR = 0.10; 95% CI = 0-1.92; P = .086). No significant differences were observed for the rates of rejection.

Furthermore, no significant differences were found for aGVHD and rejection when the 15 pairs of the patient-Bw4-absent/donor-KIR3DL1-present group were compared with the 27 pairs of the patient-Bw4-present/donor-KIR3DL1-present group (Table 3).

DISCUSSION

Unrelated donor BMT in thalassemia patients represents an ideal model for studying the impact of immunogenetic factors on transplantation outcome. Different to patients with hematologic malignancies, thalassemia patients have comparable clinical characteristics, a competent immune system, and receive homogeneous conditioning regimens and GVHD prophylaxis. Moreover, the investigation of pathophysiologic mechanisms implicated in GVHD, and particularly rejection, is not hampered by the presence of neoplastic cell clones and previous chemotherapy treatment.

So far, only 1 report has analyzed the effect of KIR-KIR ligand interaction on the occurrence of GVHD and rejection in thalassemia [40]. That study included patients transplanted from an HLA identical sibling, whereas the present study was performed on the largest cohort of thalassemia patients transplanted from an unrelated donor.

The KIR gene frequencies observed in our donor/recipient pairs are similar to those previously reported in other Caucasoid populations. No significant differences in the KIR gene profile were observed between patients with aGVHD and their donors.

Incomplete elimination of recipient immunocompetent cells is a factor that plays a major role in primary or secondary graft failure, although the mechanisms underlying graft rejection remain to be completely elucidated. It is now accepted that rejection of donor hematopoietic cells is not only mediated by T cell-specific immune response, but also by NK cells [41]. In our study, the patients who rejected the allograft had 1 or 2 additional activatory KIRs compared to their donors, even if the difference did not reach statistical significance. When the donor/recipient pairs were stratified according to their KIR gene profiles as proposed by Gagne et al [33], there was a tendency toward rejection when the donor KIR genotype was included in the recipient KIR genotype.

In previous studies, Ruggeri et al [9,18] showed the impact of HLA-C differences as a cause of NK alloreactivity and, more recently, Cook et al [23] dem-

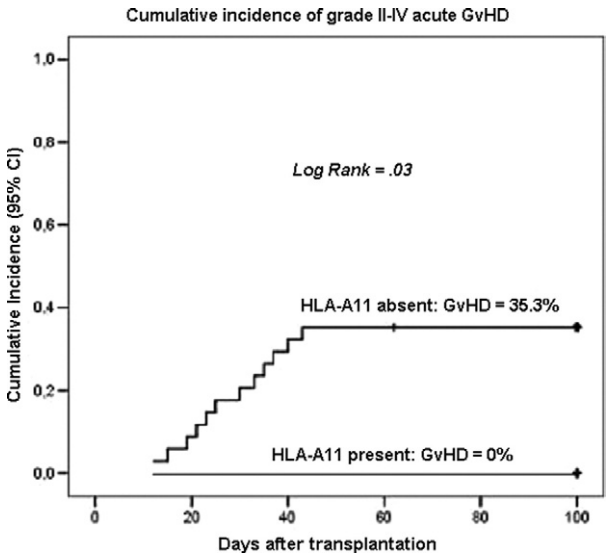

Figure 5. Impact of the presence of the HLA-A11 KIR antigen on the incidence of grade II-IV aGVHD in 45 thalassemia patients transplanted from an unrelated donor. (HLA-A11 positive patients = 11, events = 0; HLA-A11 negative patients = 34, events 12).

onstrated the influence of HLA-Cw KIR ligands as risk factors, also in the absence of an HLA-mismatch with the donor. In fact, patients with myeloid disease transplanted from an HLA-identical sibling had a better outcome when donor and recipient pairs were C1/C2 heterozygotes or C1/C1 homozygotes. The same effect was not observed in transplants for lymphoid diseases. The same authors report a significant reduction of disease-free survival (DFS) when the donor carried the activatory KIR2DS2 gene and the recipient lacked the respective C1 KIR ligand (patient-C1-absent/donor-KIR2DS2-present) in comparison with other combinations (patient-C1-present/donor-KIR2DS2-present and patient-C1-present/donor-KIR2DS2-absent). A similar analysis performed for KIR2DS1 and the presence or absence of the KIR ligand C2 in the recipient did not reveal any additional effect for overall survival or aGVHD. In our donor/recipient pairs, analysis of the 2DS1 and 2DS2 activatory KIRs and their ligands did not have any significant effect on the incidence of aGVHD, rejection, OS, and/or thalassemia-free survival (Tables 2 and 3).

Hsu et al [24] tested the "missing ligand hypothesis" by investigating the effect of the presence/absence of HLA ligands for donor 2DL1, 2DL2, 2DL3, and 3DL1 inhibitory KIRs on the outcome of HLA identical sibling transplantation. They demonstrated that absence in the recipient of the HLA Class I ligand for the 2DL1, 2DL2, 2DL3, and 3DL1 inhibitory KIRs was an independent predictive factor significantly associated with a better transplantation outcome. In particular, patients lacking the HLA-Cw and HLA-Bw4 ligands for donor inhibitory KIRs displayed a better DFS and OS than patients exhibiting all HLA class I ligands for donor inhibitory KIRs. When we applied the same algorithm to our patient sampling, we observed a slight increase in the risk of rejection in the C1-absent/donor-KIR2DL2-present group, compared to the C1-present/donor-KIR2DL2-present group. Analogously, when comparing the C2-absent/donor-KIR2DL1-present combination with those of the other 3 groups (patient-C2-absent/donor-KIR2DL1-absent, patient-C2-present/donor-KIR2DL1-present and patient-C2-present/KIR2DL1-absent), we observed a lower risk for aGVHD, but again, the difference did not reach statistical significance.

In our cohort of patients, the most significant data emerge from the analysis of the HLA C1 and C2 KIR ligands. In fact, the probability of rejection was significantly higher ($P = .009$) when donor and recipient pairs were homozygotes for the ligands of the C1 and C2 groups (Figure 3). A possible explanation of this finding is that the presence of a single subset of HLA-Cw ligands for activatory KIRs in C1 or C2 donor/recipient homozygotes could determine an insufficiency of donor NK cell activation, thus precluding the complete eradication of recipient hematopoietic and immune cells. Conversely, it is not clear why C1/C2 heterozygotes had a significantly increased risk of experiencing GVHD ($P = .007$) (Figure 2). Moreover, C1/C2 heterozygotes had a better, although not statistically significant, thalassemia-free survival than C1/C1 or C2/C2 homozygotes (Figure 4).

The presence of the HLA-A11 antigen in donor/recipient pairs was associated with a protective effect against GVHD ($P = .02$) (Figure 5 and Table 3). It has been postulated that HLA-A11 and HLA-A3 antigens are ligands of KIR3DL2, although direct evidence is lacking [42,43]. Indeed, it would seem that recognition of HLA-A3 and HLA-A11 by KIR3DL2 is strongly peptide-specific [15]. However, the functional role of this interaction remains to be established.

In conclusion, our data suggest that the status of donor-recipient HLA class I ligands, and not the KIR receptor genotype, is predictive for the outcome of unrelated donor HSCT in patients with β-thalassemia. Evaluation of the presence of HLA Class I ligands, particularly those of the C1 and C2 groups, and the HLA-A11 antigen may facilitate the identification of patients at high risk of developing either aGVHD or rejection, and thus help the clinician modulate the intensity of conditioning regimen and/or immunosuppressive therapy accordingly.

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