Human leukocyte antigen-E mismatch is associated with better hematopoietic stem cell transplantation outcome in acute leukemia patients

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ABSTRACT

The immunomodulatory role of human leukocyte antigen (HLA)-E in hematopoietic stem cell transplantation (HSCT) has not been extensively investigated. To this end, we genotyped 509 10/10 HLA unrelated transplant pairs for *HLA-E*, in order to study the effect of HLA-E as a natural killer (NK)-alloreactivity mediator on HSCT outcome in an acute leukemia (AL) setting. Overall survival (OS), disease free survival (DFS), relapse incidence (RI) and non-relapse mortality (NRM) were set as endpoints. Analysis of our data revealed a significant correlation between HLA-E mismatch and improved HSCT outcome, as shown by both univariate (53% *vs.* 38%, *P*=0.002, 5-year OS) and multivariate (hazard ratio (HR)=0.63, confidence interval (CI) 95%=0.48-0.83, *P*=0.001) analyses. Further subgroup analysis demonstrated that the positive effect of HLA-E mismatch was significant and pronounced in advanced disease patients (n=120) (5-year OS: 50% *vs.* 18%, *P*=0.005; HR=0.40, CI 95%=0.22-0.72, *P*=0.002; results from univariate and multivariate analyses, respectively). The study herein is the first to report an association between HLA-E incompatibility and improved post–transplant prognosis in AL patients who have undergone matched unrelated HSCT. Combined NK and T cell HLA-E-mediated mechanisms may account for the better outcomes observed. Notwithstanding the necessity for *in vitro* and confirmational studies, our findings highlight the clinical relevance of HLA-E matching and strongly support prospective HLA-E screening upon donor selection for matched AL unrelated HSCTs.

Introduction

HSCT has long been established as an indispensable life-saving treatment, in particular against acute hematologic malignancies.[1] Despite the significant progress made in the last ten years, transplantation related mortality and graft-*versus*-host disease (GvHD) continue to substantially constrain the curative potential of HSCT, even in an HLA-matched context, underscoring the need to explore the role of other immune system-related genetic factors in HSCT.[2] In this respect, a rather limited number of studies sought to investigate the effect of HLA-E on HSCT outcome, considering the significant immunomodulatory features of this molecule implicated in both innate and adaptive immunity.[3,4] HLA-E, a member of

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the non-classical HLA-Ib family, is ubiquitously expressed on all nucleated cells, but at lower expression levels than the classical HLA-class I molecules.[5] It is rather nonpolymorphic, with basically two functional forms of the protein found worldwide at similar prevalence rates,[6] shares an almost identical structural pattern with its classical HLA-class I counterpart and is viewed as a surrogate marker for HLA-class I expression, as the leader sequences of the latter constitute its main peptide reservoir.[7] Even though this prominent allelic variation derives from a single arginine to a glycine amino acid substitution at position 107 of the heavy chain α2 domain (*HLA-E*01:01* and *HLA-E*01:03*, respectively), the codominance of the two alleles in conjunction with their significantly different expression levels on cell surfaces imply functional differences which are yet to be fully understood.[8-10] As a basic ligand to CD94/NKG2A,[11] a robust inhibitory receptor found on the surface of NK cells and NK-like cytotoxic T lymphocytes (CTLs), the principal role of HLA-E is considered to be the protection of normal cells from aberrant NK killing. However, continuously arising data highlight that HLA-E may hold a much more multifaceted role in immune response by presenting "unconventional" peptides under stress conditions[12,13] and by interacting with HLA-E-restricted $CD8^+$ CTLs and regulatory T cells (Tregs) *via* their αβ T-cell receptors (TCRs) as well as with the activating CD94/NKG2C receptor on the surface of NK-cells and NK-like CTLs.[14,15] Despite the evident role of HLA-E in immune response, no definite conclusions can be drawn from studies published thus far aiming to establish an association between HLA-E and HSCT outcome.[16-24] The aim of the present study was to explore not only the role of *HLA-E* genotype but, primarily, the effect of HLA-E patient-donor compatibility on HSCT outcome, as the weak linkage disequilibrium between *HLA-E* and its classical HLA counterparts leads to a rather high rate of HLA-E mismatches among HLA-A, -B, -C, -DRB1, and HLA-DQB1 allele-matched HSCT pairs.[17,25] HLA-E as an NK-alloreactivity mediator is expected to have a more prominent role in an AL context where the graft-*versus*-leukemia effect (GvL) is of utmost relevance. Hence, we applied a study design including only adult AL patients who had undergone a 10/10 HLA-matched unrelated HSCT in order to evaluate the role of patient/donor *HLA-E* genotypes as well as of HLA-E matching status in HSCT outcome.

Methods

Patients

509 adult patients diagnosed with AL, receiving their first allogeneic HSCT between 2002 and 2009 were included in the study. All patients were transplanted with 10/10 allele level HLA-A, -B, -C, -DRB1, -DQB1-matched grafts, which were either bone marrow (BM) or peripheral blood stem cells (PBSCs). We included only those patients diagnosed with acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) as well as undefined AL (undifferentiated, biphenotypic or secondary acute). Disease stages were assigned according to a previous report published by the European Society for Blood and Marrow Transplantation (EBMT) study group.[26] Early disease stage included AML, AL, and ALL transplanted in first complete remission, intermediate disease stage was defined as AML and ALL in second complete remission or first relapse as well as AL transplanted in second complete remission. All other disease phases of AML, ALL and AL were characterized as advanced stage. All patients were treated with myeloablative (Mab) or reduced intensity conditioning (RIC).[27,28] Recipient and donor consents for HLA typing and for the analysis of clinical data were obtained in accordance with the Declaration of Helsinki upon initiation of donor search and registration in the EBMT database, respectively. All clinical data were initially recorded in the EBMT ProMISE database and were subsequently provided to us by the German Registry for Stem Cell Transplantation (DRST), which is responsible for the clinical data management of the German patients' subset. The study was approved by the ethical review board of the University of Ulm (project number: 263/09).

HLA-typing

All patients and their respective donors were genotyped at high resolution level for the *HLA*-loci *A, B, C, DRB1* and *DQB1*. *HLA-DPB1* genotyping was performed retrospectively for all study subjects using stored DNA material. Permissiveness of DPB1-mismatches was assessed according to the TCE (T-cell epitope) algorithm.[29] Additional testing for relevant non-expressed alleles was performed according to the National Marrow Donor Program confirmatory typing requirements.[30]

Killer Cell Immunoglobulin-Like Receptors (*KIR*) typing

KIR-typing was performed using the commercially available "KIR Genotyping SSP Kit" from Life Technologies (Carlsbad, CA, USA). Donor *KIR* AA and Bx haplotypes were assigned as previously described.[31]

HLA-E typing

All 509 patient-donor pairs were *HLA-E* high resolution genotyped. *HLA-E* specific primers were designed for complete Exon 2 and 3 sequencing analysis, allowing precise assignment of all known allelic variants. Allelic assignment was based on sequence data retrieved from the immunogenetics (IMGT)/HLA database.

Statistical analysis

The cumulative estimates for the univariate analysis OS and DFS were obtained using the Kaplan-Meier method. For multivariate analyses Cox regression models were implemented. Competing risk analysis was used for the univariate analyses of NRM, RI and chronic (c)GvHD incidence, while competing risk regression models for stratified data were used for multivariate analyses. Acute (a)GvHD and severe infection incidence as well as prevalence of other causes of death are reported descriptively. Center effects were adjusted using a γ frailty term.[32]

Statistical models covered covariates in accordance with the previously published recommendations of the EBMT study group.[28,33] In addition to these, patient and donor cytomegalovirus (CMV) serostatus, treatment with anti-thymocyte globulin (ATG), Karnofsky performance score (KPS) at time of transplantation, donor *KIR* haplotype (AA/Bx),[31] patient C1/C2 KIR ligand status as well as HLA-DPB1 compatibility (based on T-cell epitope algorithm)[29] were also evaluated. Missing data were treated as separate categories in multivariate analyses.[26] A stepwise backward exclusion procedure was used for model selection.[26,28] Statistical significance was set to a P-value≤0.05. All statistical analyses were performed using the open source program for statistical computing "R", version 3.1.0.

More section data available in *Online Supplementary Material*.

Table 1. Cohort characteristics.

Category	Study cohort n(%)	HLA-E-matched n(%)	HLA-E-mismatched n(%)	*P*
Number of patients	509	320	189	
Number of transplantation centers	21	20 (95.2)	20 (95.2)	0.61
Age category				
18-29	97 (19.0)	62 (19.4)	35 (18.5)	
30-39	71 (14.0)	32 (10.0)	39 (20.6)	
40-49	89 (17.5)	62 (19.4)	27 (14.3)	0.24
50-59	129 (25.3)	82 (25.6)	47 (24.9)	
60-69	108 (21.2)	75 (23.4)	33 (17.5)	
70-79	15 (3.0)	7 (2.2)	8 (4.2)	
Diagnosis				
AML	313 (61.5)	196 (61.2)	117 (61.9)	0.89
ALL	132 (25.9)	85 (26.6)	47 (24.9)	
AL	64 (12.6)	39 (12.2)	25 (13.2)	
Disease stage				
Early	237 (46.5)	147 (45.9)	90 (47.6)	
Intermediate	152 (29.9)	92 (28.8)	60 (31.7)	0.46
Advanced	120 (23.6)	81 (25.3)	39 (20.6)	
Conditioning regimen				
Myeloablative	345 (67.8)	215 (67.2)	130 (68.8)	0.78
Reduced intensity	164 (32.2)	105 (32.8)	59 (31.2)	
Karnofsky performance score*				
KPS < 90	98 (30.6)	66 (32.0)	32 (28.1)	0.50
Missing data	189 (37.1)	114 (35.6)	75 (39.7)	
Stem cell source				
BM	32 (6.3)	18 (5.6)	14 (7.4)	0.54
PBSC	477 (93.7)	302 (94.4)	175 (92.6)	
ATG Treatment*				
Yes	252 (63.2)	157 (61.3)	95 (66.4)	0.31
No	147 (37.8)	99 (38.7)	48 (33.6)	
Missing data	110 (21.6)	64 (20.0)	46 (24.3)	
Patient-Donor CMV serostatus combination*				
neg neg	126 (31.6)	81 (32.1)	45 (30.6)	
neg pos	45 (11.3)	31 (12.3)	14 (9.5)	
pos neg	102 (25.5)	61 (24.2)	41(27.9)	0.86
pos pos	126 (31.6)	79 (31.4)	47 (32.0)	
Missing data	110 (21.6)	68 (21.2)	42 (22.2)	
Donor *KIR* Haplotype*				
Haplotype AA	158 (31.3)	95 (30.0)	63 (33.7)	
Haplotype Bx	346 (68.7)	222 (70.0)	124 (66.3)	0.68
Missing data	5 (0.98)	3 (0.94)	2 (1.0)	
Patient C1/C2 KIR ligands				
C1 positive	443 (87.0)	277 (86.6)	166 (87.8)	0.86
C1 negative	66 (13.0)	43 (13.4)	23 (12.2)	
HLA-DPB1 TCE mismatch*				
Permissive	326 (64.3)	208 (65.2)	118 (62.8)	
HvG non-permissive	86 (17.0)	59 (18.5)	27 (14.4)	
GvH non-permissive	95 (18.7)	52 (16.3)	43 (22.8)	0.13
Missing data	2 (0.4)	1 (0.3)	1 (0.5)	

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Recipient-donor sex match				
Male-male	228 (44.8)	143 (44.7)	85 (45.0)	
Male-female	46 (9.0)	26 (8.1)	20 (10.6)	
Female-male	168 (33.0)	108 (33.8)	60 (31.7)	0.80
Female-female	67 (13.2)	43 (13.4)	24 (12.7)	
Year of transplantation				
2002-2005	127 (25.0)	77 (24.0)	50 (26.5)	0.62
2006-2009	382 (75.0)	243 (76.0)	139 (73.5)	

*In compliance with the EBMT statistical guidelines, percentages for variables with missing data are presented with reference to the known data cases. AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; AL: acute leukemia not specified as AML or ALL (undifferentiated, biphenotypic or secondary acute); KPS: Karnofsky performance score; BM: bone marrow; PBSC: peripheral blood stem cells; ATG: anti-thymocyte globulin; CMV: cytomegalovirus; pos: positive; neg: negative. KIR: killer cell immunoglobulin-like receptor; HLA: human leukocyte antigen; TCE: T-cell epitope; HvG: Host *vs.* Graft; GvH: Graft *vs.* Host.

Table 2. Human leukocyte antigen (*HLA*)-*E* genotyping results.

HLA-E allele frequencies (n,%)				*HLA-E* genotypes (n,%)			
*HLA-E**	01:01	01:03	01:07	01:01	01:01, 01:03	01:03	01:01, 01:07
Patients	567 (55.7)	451 (44.3)	–	157 (30.8)	253 (49.7)	99 (19.5)	–
Donors	572 (56.0)	445 (43.8)	1 (0.2)	151 (29.7)	269 (52.8)	88 (17.3)	1 (0.2)

Results

Patient characteristics

Patient cohort characteristics regarding HSCT outcome predictors and in relation to HLA-E matching status between patient and donor are summarized in Table 1. For the 509 patients included in the study, median post-transplant follow-up time was almost 5 years (4.97 years), while median patient age was 49 years (range: 18-74 years). Interestingly, 37.1% of the cases were HLA-E-mismatched, and as the *P*-values in Table 1 suggest, there was no biased distribution of HLA-E-matched and mismatched cases with regard to other parameters predictive for the outcome of HSCT which we evaluated.

HLA-E genotyping results

A summary of the *HLA-E* genotyping results is displayed in Table 2. The *HLA-E* allele frequencies found were in accordance with those previously reported for Caucasian populations,[6,17,25] confirming the codominant prevalence of the two basic allelic forms of *HLA-E*. No differences were identified regarding the distribution of the *HLA-E* allelic variants between patients and donors.

*HLA-E**01:03, 01:03 patient genotype is not associated with better HSCT outcome

Our results do not confirm the findings of previously published studies regarding the positive impact of patient *HLA-E**01:03, 01:03 genotype on HSCT outcome. On the contrary, *HLA-E**01:03, 01:03 patients in our cohort had worse OS, DFS and NRM rates compared to the patients carrying the two other genotypes as shown in the multivariate analysis (OS: HR=1.45, CI 95%=1.00-2.10, *P*=0.05; DFS: HR=1.47, CI 95%=1.04-2.07, *P*=0.03; NRM: HR=1.74, CI 95%=1.09-2.78, *P*=0.02). Of note, this finding did not reach statistical significance in any of the univariate models (*data not shown*).

HLA-E incompatibility significantly improves OS, DFS and NRM

Analysis of OS, DFS and NRM with respect to HLA-E matching status between patients and donors revealed a significant favorable effect of HLA-E mismatch on these endpoints. As shown in Figure 1, patients transplanted with HLA-E-mismatched donors exhibit a significantly improved 5-year OS (53% *vs.* 38%, *P*=0.002), 5-year DFS (45% *vs.* 32%, *P*=0.007) and a significantly lower 5-year NRM (26% *vs.* 37%, *P*=0.006) when compared to cases receiving an HLA-E compatible graft. Multivariate analyses confirmed the above findings as the beneficial effect of HLA-E mismatch was statistically significant for all of the above HSCT outcome endpoints (OS: HR=0.63, CI 95%=0.48-0.83, *P*=0.001; DFS: HR=0.71, CI 95%=0.55-0.92, *P*=0.008; NRM: HR=0.63, CI 95%=0.43-0.91, *P*=0.015). Since better OS appeared to stem from lower NRM rates in the HLA-E-mismatched patient subgroup, we separately analyzed the prevalence rates of aGvHD and severe infection along with an overall cause of death analysis. Although Grade III-IV aGvHD rates were similar in the two groups (~10%), the death rate of 9% from GvHD in the HLA-E-matched group was substantially higher than the 5.8% found among HLA-E-mismatched patients. Furthermore, severe infection was reported in 17.2% of HLA-E-matched patients *vs.* 9.5% of HLA-E-mismatched patients. Accordingly, infection-related mortality was higher in the HLA-E-matched group (10.9% *vs.* 7.9%).

It should be noted that data on both aGvHD and cGvHD were incomplete for 9% (46/509) and 43% (217/509) of cases, respectively. No cause of death data were available for 2.1% of patients (11/509). With regard to cGvHD, presuming that missing values were most likely randomly distributed among HLA-E-matched and mismatched cases within our cohort, we decided to include this parameter in the statistical analysis. The analysis of

Table 3. Univariate and multivariate analyses as to the effect of human leukocyte antigen (HLA)-E mismatch on HSCT outcome.

Endpoints	Univariate Analysis			Multivariate Analysis		
Overall Survival	HLA-E-matched	HLA-E-mismatched	*P*	HR	CI 95%	*P*
1 year	0.59 (0.53-0.65)	0.67 (0.61-0.75)				
3 year	0.42 (0.36-0.49)	0.57 (0.50-0.65)	*0.002*	0.63	0.48-0.83	*0.001*
5 year	0.38 (0.32-0.44)	0.53 (0.46-0.62)				
Disease free survival						
1 year	0.51 (0.46-0.58)	0.59 (0.52-0.66)	*0.007*	0.71	0.55-0.92	*0.008*
3 year	0.36 (0.31-0.43)	0.51 (0.44-0.59)				
5 year	0.32 (0.27-0.39)	0.45 (0.38-0.53)				
Non-relapse mortality						
1 year	0.27 (0.22-0.32)	0.19 (0.14-0.26)	*0.006*	0.63	0.43-0.91	*0.015*
3 year	0.36 (0.30-0.41)	0.22 (0.16-0.29)				
5 year	0.37 (0.31-0.43)	0.26 (0.19-0.33)				
Relapse incidence						
1 year	0.25 (0.20-0.31)	0.25 (0.19-0.32)	0.84	1.02	0.73-1.43	0.90
3 year	0.32 (0.27-0.38)	0.31 (0.24-0.38)				
5 year	0.35 (0.29-0.41)	0.34 (0.26-0.41)				
cGvHD incidence						
6 months	0.34 (0.27-0.42)	0.24 (0.17-0.32)	0.102	0.70	0.47-1.04	0.074
12 months	0.39 (0.31-0.46)	0.28 (0.21-0.37)				
24 months	0.39 (0.32-0.47)	0.32 (0.24-0.40)				

Number of patients included in the analyses, n=509. Omitted observations due to missing data for overall survival (OS)=6, disease free survival (DFS)=4, non-relapse mortality=6, relapse incidence= 20 and cGvHD incidence=217. Statistical significance is marked in italics. Hazard ratio (HR) values for survival endpoints (Overall survival and Disease free survival) refer to the risk of death and/or relapse as measured in the analyses for these endpoints. cGvHD: chronic graft-*versus*-host disease.

the cumulative probability of cGVHD revealed a tendency toward association between HLA-E mismatch and less cGvHD. However, given the admittedly high number of missing data, these results should be interpreted with caution.

All results for both univariate and multivariate analyses are summarized in Table 3. After stepwise backward exclusion procedure used for model selection, patient age, disease stage, diagnosis, CMV serostatus compatibility, ATG treatment and patient HLA-E haplotype were integrated as significant clinical predictors in our multivariate analyses.

Advanced disease acute leukemia patients benefit the most from *HLA-E*-mismatched unrelated 10/10 HLA matched HSCT

Exploratory controls for potential interactions between HLA-E matching status and other clinical predictors revealed an association between the "HLA-E mismatch effect" and advanced disease stage. For this reason we extended our analysis by dividing patients into an advanced (n=120) and a non-advanced disease (n=389) group, with the latter including patients in early or intermediate disease stage. Both univariate and multivariate analyses for OS, DFS and NRM revealed a much stronger effect of HLA-E mismatch in the advanced disease group compared to the early/intermediate stage patients. The 5-year survival rates were markedly improved in advanced disease patients who received HLA-E disparate grafts (OS: 50% *vs.* 18%, *P*=0.005; DFS: 40% *vs.* 12%, *P*=0.002), as likewise depicted by the Kaplan-Meier curves in Figure 2. NRM was also notably lower among these patients (32% *vs.* 55%, *P*=0.038, Figure 2). Multivariate analyses confirmed the above findings for all three endpoints in advanced disease patients (OS: HR=0.40, CI 95%=0.22-0.72, *P*=0.002; DFS: HR=0.42, CI 95%=0.25-0.72, *P*=0.001; NRM: HR=0.44, CI 95%=0.20-0.95, *P*=0.036). Additionally, HLA-E mismatch in advanced disease patients was associated with markedly higher rates of none or mild (grade 0-I) aGvHD (66.7% vs. 56.8%) and lower rates of grade II-IV aGvHD (7.7% *vs.* 12.3%). Moreover, 14.8% of HLA-E-matched patients died due to severe GvHD compared to only 2.6% of HLA-E-mismatched cases. No significant differences were observed on account of severe infection prevalence between the two groups (21.0% of HLA-E-matched *vs.* 17.9% of HLA-E-mismatched cases). Interestingly, infection-related mortality was higher in the HLA-E-mismatched group (17.9% *vs.* 12.3%). Possible subjectivity involved in the reporting of only one cause of death in the case of concomitant fatal conditions may account for this discordance. It should be underscored that no aGvHD data were available in 17.5% (21/120) of cases, while cause of death data were incomplete for 2.5% (3/120) of advanced disease patients. The effect of HLA-E mismatch in non-advanced disease patients, albeit noticeable, did not reach statistical significance for any of the endpoints in either univariate or multivariate analyses. No significant differences were identified in this subset of patients with respect to aGvHD rates and GvHD-related death. However, there was a marked difference observed regarding severe infection prevalence with 15.9% in HLA-E-matched cases *vs.* 7.3% in HLA-E-mismatched ones, likewise regarding infection-related mortality rates (10.5% *vs.* 5.3% in HLA-E-matched and

mismatched cases, respectively). Cause of death data were missing for 2% (8/389) of early/intermediate disease patients. All results for both univariate and multivariate analyses and for both patient subgroups are listed in Tables 4 and 5, respectively.

HLA-E mismatch has no effect on relapse incidence rates

No differences in RI were observed with respect to HLA-E matching status. Moreover, advanced as well as non-advanced disease patients exhibited similar relapse rates regardless of HLA-E matching status to their donor. The results for RI are summarized in Tables 3-5.

Discussion

The immunomodulatory role of HLA-E and its implication in both innate and adaptive immunity has long been investigated and established.[4] Its impact, however, on HSCT remains markedly elusive, as there are only a relatively few number of studies with small and heterogeneous cohorts to be found in the literature;[3] most of which have aimed at establishing a correlation between certain patient *HLA-E* genotypes and HSCT outcome. The study herein is, to our knowledge, the first to report a favorable effect of HLA-E incompatibility in an AL-matched unrelated HSCT setting. Our data suggest significantly improved

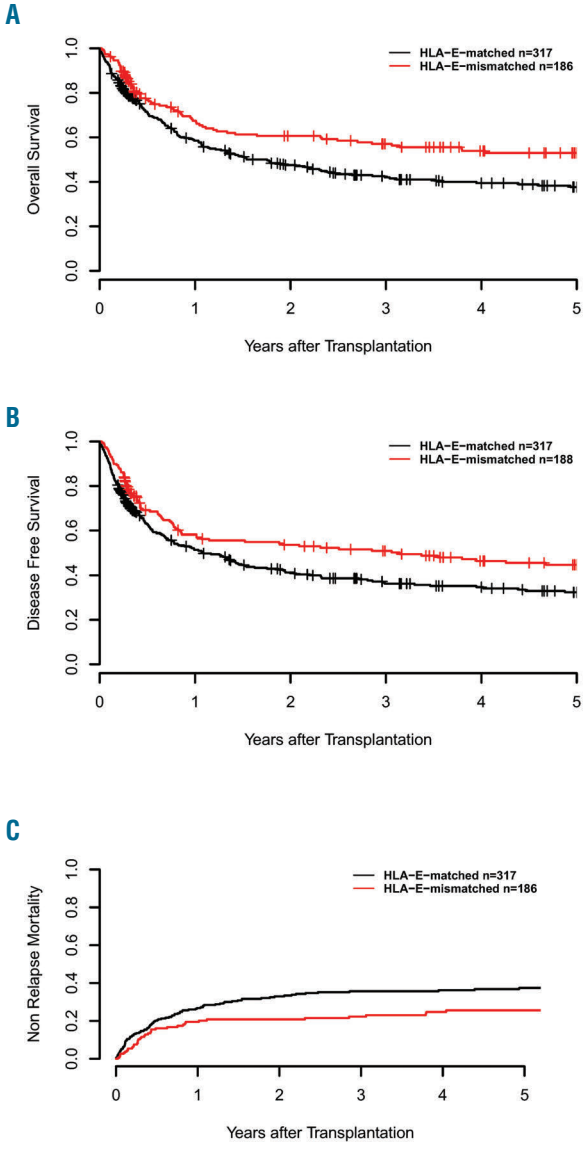

Figure 1. Hematopoietic stem cell transplantation outcome with respect to human leukocyte antigen (HLA)-E matching status in acute leukemia patients, n=509. (A) Overall survival (*P*=0.002); (B) Disease free survival (*P*=0.007) and (C) Non-relapse mortality (*P*=0.006) curves, respectively, of patients transplanted with HLA-E-matched donors (black line) *versus* patients transplanted with HLA-E-mismatched donors (red line).

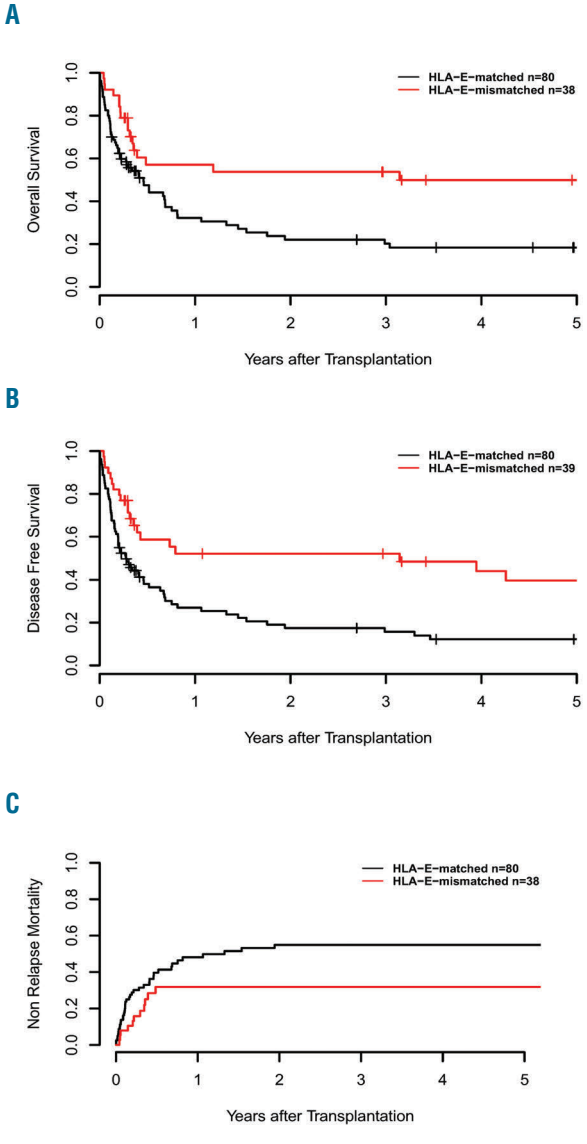

Figure 2. Hematopoietic stem cell transplantation outcome with respect to human leukocyte antigen (HLA)-E matching status in advanced disease patients, n=120. (A) Overall survival (*P*=0.005); (B) Disease free survival, (*P*=0.002) and (C) Non-relapse mortality (*P*=0.038) curves, respectively, of patients transplanted with HLA-E-matched donors (black line) *versus* patients transplanted with HLA-E-mismatched donors (red line).

overall and disease free survival rates as well as lower NRM in adult AL patients transplanted with 10/10 HLA-matched unrelated donors when grafts received were HLA-E disparate. No effect was found in relation to relapse incidence. Other confounding factors putatively responsible for this observation were excluded, as HLA-E-matched and mismatched pairs had no significant differences from one another with respect to other known HSCT outcome predictors[33] (Table 1). In previous studies which investigated the role of HLA-E compatibility in HSCT outcome, Fürst *et al.* did not observe any association between HLA-E mismatch and HSCT outcome, while the results of Harkensee *et al.* suggested a negative impact of HLA-E incompatibility on survival.[20,23] These two studies, however, were designed on a different basis, hence the results are not comparable. The cohort of Fürst *et al.*, apart from its significantly smaller size (n=116), was heterogeneous in terms of diagnoses, which for reasons that will be analyzed subsequently, may be of fundamental importance. The Harkensee *et al.* study rationale was performed in an HLA-mismatched setting and its primary goal was to establish associations between various non-HLA genetic factors and HSCT outcome for HLA disparate transplant pairs. Previous studies[16-19,21,22] reported lower transplantation related mortality, less severe bacterial infection rates as well as lower relapse and severe GvHD incidences in patients with the *HLA-E**01:03 genotype. We could not confirm these associations. In our multivariate models, where patient *HLA-E* genotype was a significant covariate, patient *HLA-E**01:03 homozygosity was, in fact, correlated with inferior outcome. However, it must be acknowledged that any comparison between these studies and ours is not applicable, as some of them included HSCT from related or HLA-E-matched donors,[16,18,22] and cohorts in all of them were not only significantly smaller in size but also heterogeneous with regard to diagnoses.[16-19,21,22]

According to our findings, HLA-E mismatch appears to confer its beneficial effect through dampening of NRM. On account of this, two very interesting observations are of note. First, that HLA-E mismatch seems to differentially impact patients according to their disease stage, and secondly, that a putatively combined mechanism may account for the overall beneficial effect, as the lower NRM rates in advanced disease patients appear to be prevalently related with lower GvHD rates, whereas in early/intermediate disease patients there is better control of infection. As far as the first observation is concerned, our results clearly suggest a much stronger impact of HLA-E mismatch on advanced disease patients' outcome (Tables 4, 5). In fact, the results within this subgroup of patients drive the findings in the entire study cohort since they clearly reach significance, while the effect of HLA-E mismatch in the larger group of early/intermediate disease patients, although visible, does not reach statistical significance. This is most likely due to the different "baseline" prognostic odds of the two subgroups.[34]

It is well known that HLA-E is an important modulator of NK-cytotoxicity, as it constitutes the main ligand to the CD94/NKG2A/C group of NK receptors.[11] According to the murine model proposed by Olson *et al.*, early post-transplant NK alloreactivity could be associated with better OS rates due to lower GvHD incidence and NRM.[35] The fact that CD94/NKG2A/C receptors are the first to appear on freshly reconstituted NK cells immediately following HSCT, strengthens the assumption that this "HLA-

Table 4. Univariate analysis of advanced *vs.* non-advanced patients with respect to human leukocyte antigen (HLA)-E mismatch.

Endpoints	Advanced disease patients n=120*			Early/intermediate disease patients n=389†		
Overall Survival	HLA-E-matched	HLA-E-mismatched	*P*	HLA-E-matched	HLA-E-mismatched	*P*
1 year	0.32(0.23-0.46)	0.57(0.43-0.76)	*0.005*	0.68(0.62-0.74)	0.72(0.65-0.80)	0.071
3 year	0.20(0.12-0.33)	0.54(0.39-0.74)		0.50(0.43-0.57)	0.60(0.52-0.69)	
5 year	0.18(0.11-0.31)	0.50(0.35-0.71)		0.45(0.38-0.53)	0.56(0.48-0.65)	
Disease free survival						
1 year	0.27(0.18-0.40)	0.52(0.38-0.72)	*0.002*	0.60(0.53-0.67)	0.60(0.52-0.69)	
3 year	0.16(0.09-0.28)	0.52(0.38-0.72)		0.43(0.37-0.51)	0.51(0.43-0.60)	0.26
5 year	0.12(0.06-0.24)	0.40(0.25-0.62)		0.39(0.33-0.47)	0.46(0.38-0.56)	
Non-relapse mortality						
1 year	0.48(0.36-0.59)	0.32(0.17-0.48)	*0.038*	0.20(0.15-0.26)	0.16(0.11-0.23)	0.083
3 year	0.55(0.42-0.66)	0.32(0.17-0.48)		0.26(0.20-0.32)	0.18(0.12-0.25)	
5 year	0.55(0.42-0.66)	0.32(0.17-0.48)		0.29(0.23-0.36)	0.20(0.14-0.27)	
Relapse incidence						
1 year	0.31(0.20-0.43)	0.24(0.10-0.41)	0.60	0.24(0.18-0.30)	0.25(0.18-0.33)	0.86
3 year	0.33(0.22-0.45)	0.24(0.10-0.41)		0.29(0.23-0.35)	0.30(0.23-0.28)	
5 year	0.37(0.25-0.49)	0.35(0.16-0.54)		0.32(0.26-0.39)	0.32(0.24-0.40)	
cGvHD incidence						
6 months	0.50(0.30-0.67)	0.13(0.03-0.30)	*0.009*	0.31(0.24-0.39)	0.27(0.19-0.36)	0.60
12 months	0.50(0.30-0.67)	0.13(0.03-0.30)		0.36(0.29-0.44)	0.32(0.23-0.41)	
24 months	0.50(0.30-0.67)	0.13(0.03-0.30)		0.37(0.29-0.45)	0.36(0.27-0.46)	

*Advanced disease patients, n=120. Omitted observations due to missing data for overall survival=2, disease free survival=1, non-relapse mortality=2, relapse incidence= 14 and cGvHD incidence=69. †Early/intermediate disease patients, n=389. Omitted observations due to missing data for overall survival=4, disease free survival=3, non-relapse mortality=4, relapse incidence= 6 and cGvHD incidence=148. Statistical significance is marked in italics. cGvHD: chronic graft-*versus*-host disease.

Table 5. Multivariate analysis of advanced *vs.* non-advanced patients with respect to HLA-E mismatch.

Endpoints	Advanced disease patients n=120*			Early/intermediate disease patients n=389†		
	HR	CI 95%	*P*	HR	CI 95%	*P*
Overall Survival	0.40	0.22-0.72	*0.002*	0.75	0.55-1.04	0.088
Disease free survival	0.42	0.25-0.72	*0.001*	0.85	0.63-1.15	0.29
Non-relapse mortality	0.44	0.20-0.95	*0.036*	0.72	0.46-1.12	0.14
Relapse incidence	1.10	0.50-2.43	*0.81*	1.05	0.72-1.55	0.80
cGvHD incidence	0.18	0.05-0.65	*0.008*	0.86	0.56-1.31	0.48

*Advanced disease patients, n=120. Omitted observations due to missing data for overall survival=2, disease free survival=1, non-relapse mortality=2, relapse incidence= 14 and cGvHD incidence=69. †Early/intermediate disease patients, n=389. Omitted observations due to missing data for overall survival=4, disease free survival=3, non-relapse mortality=4, relapse incidence= 6 and cGvHD incidence=148. Hazard ratio (HR) values for survival endpoints (Overall survival and Disease free survival) refer to the risk of death and/or relapse as measured in the analyses for these endpoints. cGvHD: chronic graft-*versus*-host disease.

E effect", at least as far as the "dampening" of GvHD is concerned, could be NK-mediated.[36,37]

Numerous studies have highlighted the prominent effect of peptide specificity in peptide/HLA-E (pHLA-E) complexes as to the affinity and intensity of HLA-E interactions with its corresponding NK receptors, namely the inhibitory CD94/NKG2A and the activating CD94/NKG2C.[9-10,38-43] The impact of HLA-E polymorphism, with respect to the NK "licensing" process, has not yet been investigated and as such remains elusive. Given the apparent ability of CD94/NKG2 receptors to discriminate different pHLA-E constellations through differential binding affinity, however, it is plausible to assume that during their "licensing" phase NK cells may be educated and tuned according to "self" pHLA-E patterns. Moreover, it has been shown that under abnormal conditions (e.g., infection, stress or tumorigenesis) HLA-E molecules are able to present "unconventional" peptides, generating pHLA-E complexes that go unnoticed by the dominant inhibitory CD94/NKG2A receptor, while on certain occasions they instigate activating signals through the CD94/NKG2C receptor.[12] This in turn may lead to exacerbated NK activation. According to our hypothesis model, in an advanced-stage AL setting, aggravated stress conditions, heavier leukemia-cell burden and further alterations due to advanced leukemogenesis[44] may lead to an enhanced NK-mediated attenuation of T cell alloreactivity.[45] This, in succession, could explain the significantly lower GvHD related mortality observed in advanced disease patients.

As previously mentioned, cause of death analysis in advanced and non-advanced disease patients revealed two potential mechanisms implicated - at a different degree according to disease stage - in a significant reduction of NRM rates. The decrease of GvHD-related death in advanced disease patients, as discussed above, may be NK-mediated. The reduction of fatal infection-related death in non-advanced disease patients, on the other hand, is more likely to be T cell-mediated, as it has been reported that HLA-E-restricted αβ T cells may play a significant role in the control of viral as well as bacterial infections (CMV, Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), *M.tuberculosis, S. typhi* etc.).[14] Given the role of HLA-E allelic variation in the specificity of HLA-E bound peptides, the ability of HLA-E to bind pathogen-derived peptides[13] and the importance of peptide specificity in TCR recognition of pHLA-E complexes,[14] it is plausible to presume that in an HLA-E-mismatched context, the chances of pathogen-specific HLA-E-restricted T cells to encounter the right pHLA-E constellation may be significantly higher due to a theoretically extended pHLA-E repertoire on account of HLA-E disparity. In an infection setting, "unconventional" pHLA-E complexes can be presented by both donor antigen presenting cells (APCs) and patient infected cells, hence pathogen-specific donor HLA-E-restricted T cells are more likely to encounter an immune-response-instigating pHLA-E pattern.[14,43] These two independent mechanisms probably act synergistically but to a different degree according to disease stage. The differences observed in the two subgroups may be the result of NK interference in the T cell-mediated infection control potential in advanced disease patients on the one hand, and the less intense NK activation in early/intermediate disease patients due to lighter disease burden on the other.

Significant limitations of our study are the incompleteness of the data regarding significant clinical parameters, such as aGvHD, cGvHD, type of infection and CMV reactivation, which would allow for a much more thorough and precise understanding of the way in which HLA-E mismatch exerts its beneficial effect on NRM and OS. Despite these drawbacks, however, the size and homogeneity of our cohort with respect to diagnosis, type of donor and HLA compatibility, certainly justify further investigation with larger confirmatory cohorts and functional *in vitro* studies. Considering that AL patients constitute the majority of all HSC-transplanted patients, and that even 10/10 HLA-matched unrelated transplant pairs have about 30-40% chance to be HLA-E disparate, our data support future integration of HLA-E compatibility as an additional clinical predictor, which ought to be considered upon selection of an optimal donor in an AL setting. Even though our findings, from a statistical point of view, did not confirm the effect of HLA-E mismatch in "early/intermediate disease" patients, we suspect, on account of our hypothesis model, that all AL patients, albeit to a different degree, could benefit from HLA-E disparate grafts. Future larger independent cohort studies, such as that of our ongoing CIBMTR IB16-01 project with more than 1500 AL patients enrolled, which may or may not confirm these results, will undoubtedly show the way.

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