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ORIGINAL REPORT

KIR3DL1/HLA-B Subtypes Govern Acute Myelogenous Leukemia Relapse After Hematopoietic Cell Transplantation

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A B S T R A C T

Purpose

Disease relapse remains a major challenge to successful outcomes in patients who undergo allogeneic hematopoietic cell transplantation (HCT). Donor natural killer (NK) cell alloreactivity in HCT can control leukemic relapse, but capturing alloreactivity in HLA-matched HCT has been elusive. HLA expression on leukemia cells—upregulated in the post-HCT environment—signals for NK cell inhibition via inhibitory killer immunoglobulin-like (KIR) receptors and interrupts their antitumor activity. We hypothesized that varied strengths of inhibition among subtypes of the ubiquitous KIR3DL1 and its cognate ligand, HLA-B, would titrate NK reactivity against acute myelogenous leukemia (AML).

Patients and Methods

By using an algorithm that was based on polymorphism-driven expression levels and specificities, we predicted and tested inhibitory and cytotoxic NK potential on the basis of *KIR3DL1/HLA-B* subtype combinations in vitro and evaluated their impact in 1,328 patients with AML who underwent HCT from 9/10 or 10/10 HLA-matched unrelated donors.

Results

Segregated by *KIR3DL1* subtype, NK cells demonstrated reproducible patterns of strong, weak, or noninhibition by target cells with defined *HLA-B* subtypes, which translated into discrete cytotoxic hierarchies against AML. In patients, *KIR3DL1* and *HLA-B* subtype combinations that were predictive of weak inhibition or noninhibition were associated with significantly lower relapse (hazard ratio [HR], 0.72; $P = .004$) and overall mortality (HR, 0.84; $P = .030$) compared with strong inhibition combinations. The greatest effects were evident in the high-risk group of patients with all KIR ligands (relapse: HR, 0.54; $P < .001$; and mortality: HR, 0.74; $P < .008$). Beneficial effects of weak and noninhibiting *KIR3DL1* and *HLA-B* subtype combinations were separate from and additive to the benefit of donor activating *KIR2DS1*.

Conclusion

Consideration of *KIR3DL1*-mediated inhibition in donor selection for HLA-matched HCT may achieve superior graft versus leukemia effects, lower risk for relapse, and an increase in survival among patients with AML.

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INTRODUCTION

As the only known curative therapy for most persons with acute myelogenous leukemia (AML), allogeneic hematopoietic cell transplantation (HCT) enlists an immune-mediated graft-versus-leukemia alloreactivity that is distinct from graft-versus-host disease (GVHD).[1] Although GVHD can be significantly reduced with greater HLA matching, relapse remains responsible for 46% of deaths beyond 100 days post-transplant, which suggests that immune mediated AML control differs between donors.[2] Understanding variations that govern graft-versus-leukemia reactivity may inform donor selection to improve HCT outcomes.

Natural killer (NK) cells are innate lymphocytes capable of recognizing transformed cells. Killer immunoglobulin-like receptors (KIRs) control NK function and are encoded by the highly polymorphic, multimembered KIR gene family.[3] Interaction between self-specific inhibitory KIR and cognate HLA ligands is fundamental to NK education,[4] where cells that express inhibitory

KIR for self-HLA are licensed and more responsive than their unlicensed counterparts.[4,5] Inflammatory cytokines induced after HCT[6,7] activate unlicensed NK cells, but concurrently prompt HLA upregulation on the tumor, which puts the educated NK population at risk for inhibition.

In patients with AML who undergo HCT, lack of HLA ligand for donor KIR is associated with superior NK reactivity and lower relapse as a result of lack of NK inhibition.[8-11] The missing ligand classification is defined by the patients' unchangeable HLA; therefore, enlisting a similar effect in the 40% of patients who express all KIR ligands has been an elusive goal. We hypothesized that KIR allele variation between donors titrates the strength with which donor NK cells are inhibited by the HLA-laden tumor, which leads to differences in leukemotoxicity. We examined one common KIR (KIR3DL1) and its ligand (HLA-Bw4) for which allele subtype variation influences receptor and ligand expression, binding affinity, education, and inhibition.[12-17] Compared with other KIR ligands, the lack of HLA-Bw4 conveys notably higher protection from leukemic relapse and solid tumor progression, which makes it likely that diversity in KIR3DL1/HLA-B interaction will have a clinical effect.[18-21]

The *KIR3DL1/S1* gene is one of the most polymorphic KIRs[22-24]; subtypes are displayed at high (KIR3DL1-h) or low (KIR3DL1-l) cell-surface densities or retained within the cell (KIR3DL1-n).[25,26] KIR3DS1 receptors are displayed on the cell surface but do not bind HLA-Bw4.[14,27] Dimorphism between isoleucine and threonine at position 80 in HLA-Bw4 (Bw4-80I *v* Bw4-80T) is similarly associated with surface expression on healthy cells.[13] Receptor density is broadly associated with affinity to HLA-Bw4 allomorphs. KIR3DL1-h receptors preferentially bind Bw4-80I in favor of Bw4-80T allotypes, but KIR3DL1-l receptors bind both HLA-Bw4 allomorphs similarly.[13,28] Clinical data, however, suggest that both KIR3DL1-l and -h subtypes are impacted by coinherited HLA-Bw4 subtypes; therefore, affinity alone is unlikely to control receptor-ligand avidity and NK responses. Receptor density, receptor availability, ligand density, and affinity combine to influence NK education and effector function, with impacts on HIV control.[13,29] These findings suggest a complex receptor-ligand interaction that may also impact inhibition and leukemia control.

Allelic combinations of *KIR3DL1-h* and *Bw4-80I* are enriched among patients with AML, which suggests that this is a strongly inhibiting combination that may predispose individuals to developing cancer.[20] Furthermore, in patients with neuroblastoma, *KIR3DL1* and *HLA-B* subtype combinations with predicted weak or no engagement are associated with increased disease-free survival compared with combinations with strong interaction.[19]

We now demonstrate that HLA-Bw4 subtypes differentially inhibit primary NK cells on the basis of the KIR3DL1 subtypes they express. In 1,328 patients with AML who received HLA-compatible allografts, donor-recipient *KIR3DL1/HLA-B* subtype combinations that demonstrate weak or no inhibition in vitro are associated with significantly lower relapse and higher survival compared with strong inhibition combinations. The benefit of weak or no KIR3DL1 inhibition is not driven by other known KIR-mediated benefits, including the activating *KIR2DS1+HLA-C1*[30,31]; when combined, these configurations elicit superior outcomes compared with either one alone. In sum, we identify an influential axis that calibrates NK function against AML, elucidating a novel immunogenetic criterion with which stem cell donors can be chosen for maximum anti-AML activity and improved transplant outcome.

<div style="text-align:center">

PATIENTS AND METHODS

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Clinical Samples and Healthy Donor Peripheral Blood Mononuclear Cells

We evaluated 1,328 patients with AML who received an allograft from a 9/10 or 10/10 HLA-matched unrelated donor. The National Marrow Donor Program facilitated all transplants, and all donor-patient pairs for whom *HLA* typing and donor DNA were available were included in this study (Appendix Table A1, online only). Clinical data, *HLA* genotyping, sequence-based typing for *KIR3DL1* alleles, and genomic DNA were provided by the Center for International Blood and Marrow Transplant Research. Studies were performed in compliance with federal regulations that pertained to the protection of human research participants and were approved by the National Marrow Donor Program institutional review board.

Patients and donors provided informed written consent for research. Healthy anonymous donor peripheral blood mononuclear cells (PBMCs) were collected from buffy coats obtained from the New York Blood Center (New York, NY), as described.[13] Studies were approved by the Memorial Sloan Kettering Cancer Center institutional review board.

Donor KIR and KIR3DL1 Typing

KIR genotyping was performed by using sequence-specific PCR[32,33] or KIR sequence-specific oligonucleotide probes (SSOP) (Thermo Fisher Scientific Life Sciences, Waltham, MA; and One Lambda, Canoga Park, CA). Sequence-based *KIR3DL1* allele typing was available for 299 donors.[34-36] By using multiplex PCR,[37] 1,029 donors were assessed for *KIR3DL1* subtypes. Allele frequencies were similar to previous findings.[38]

KIR3DL1 alleles were classified as *KIR3DS1*, *KIR3DL1-high* (*h*), *KIR3DL1-low* (*l*), or *KIR3DL1-null* (*n*) subtypes on the basis of known polymorphisms and expression (Appendix Tables A1–A3, online only)[37]; Bw6, Bw4-80T and Bw4-80I epitopes were assigned by using the Immuno Polymorphism Database.[39] KIR3DL1 and HLA-B were grouped on the basis of their compound subtypes, as described[19,29] (Appendix Tables A2 and A3).

AML Cell Lines and Primary Blasts

AML blasts were collected from patient peripheral blood and bone marrow. Cell lines were confirmed to be mycoplasma negative, and HLA was determined by sequencing (Histogenetics, Ossining, NY) or KIR ligand (Olerup, West Chester, PA) typing. Cells were maintained in RPMI-1640 that was supplemented with 10% fetal bovine serum. To upregulate HLA expression, cells were cultured for 3 days with 1,000 IU/mL human interferon-γ (Peprotech, Rocky Hill, NJ).

Fluorescence-Activated Cell Sorting and In Vitro Cytotoxicity

NK and target cells were cocultured (1:1) with anti-CD107a to quantify degranulation. Where specified, AML cell lines were pretreated with 10 μg/mL anti-HLA-B and -C antibody (4E; Memorial Sloan Kettering Cancer Center Monoclonal Antibody and Bioresource core facility) to inhibit KIR engagement. After 6 hour coculture, PBMCs were stained for fluorescence-activated cell sorting by using live/dead fixable stain (Thermo Fisher Scientific Life Sciences) and fluorochrome-tagged antibodies (Appendix Table A4, online only).

To quantify cytotoxicity, target cells were stained by using CFSE (Sigma-Aldrich, St Louis, MO), cocultured with PBMCs (3:1 effector:target) for 48 hours at 37°C, 5% CO_2, and counterstained with 4′,6-diamidino-2-phenylindole

(Sigma-Aldrich). The anti-KIR3DL1/S1 antibody, Z27, was included to block KIR3DL1/HLA-Bw4 interaction.

Statistical Analysis

All models used Cox proportional hazards regression for time-to-event post-HCT outcomes for relapse and death. Probabilities of overall survival and relapse were obtained by using Kaplan-Meier and cumulative incidence estimates, respectively, where death without relapse was regarded as a competing risk for relapse. Multivariate analyses were adjusted for patient age, conditioning regimen, T-cell depletion, graft type, disease status, cytomegalovirus, gender match, and HLA-match. For functional studies, one-way ANOVA using Tukey's post hoc test or Kruskal-Wallis nonparametric assessments with Dunn's correction were used. To assess KIR3DL1-n–positive versus receptor-negative populations, paired Student's t tests compared NK cells derived from the same donor.

Clinical and functional analyses were completed in R and Prism 6 software, respectively, and $P < 0.05$ was considered statistically significant.

RESULTS

HLA-Bw4 Subtypes Hierarchically Inhibit Primary NK Cells

Patients with AML who lack HLA ligands for donor inhibitory KIR have lower relapse and higher survival after HCT compared with patients who exhibit all KIR ligands,[10,11,40] which suggests that HLA expression on the tumor inhibits NK function in vivo. Indeed, we find that total HLA, specifically HLA-Bw4, is expressed on CD33^{+} AML cell blasts and cell lines (Appendix Fig. A1, online
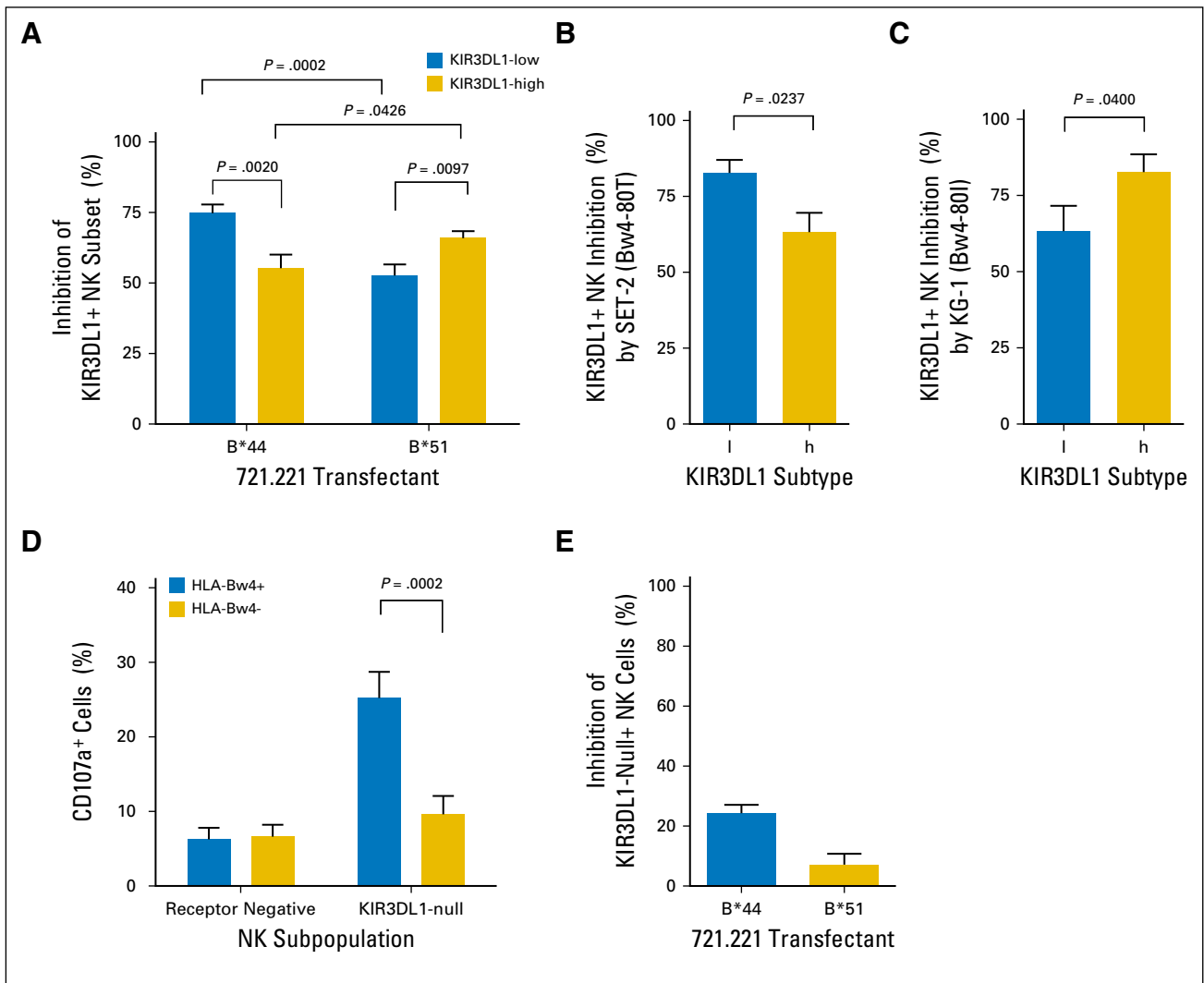

Fig 1. High and low subtypes of KIR3DL1 are differently sensitive to inhibition by Bw4-80I and Bw4-80T. (A) Primary KIR3DL1-low or -high natural killer (NK) cells from healthy Bw4-80T[+] or Bw4-80I[+] donors were challenged with 721.221 cells transfected with HLA-B*44 or HLA-B*51, respectively. Percentage of inhibition of KIR3DL1^{+} NK cells was calculated by comparing degranulation of the same NK cells toward parental 721.221 or 721.221-Bw4^{+} cells. Each bar represents 10 to 15 healthy donors. (B) Degranulation of KIR3DL1-low (I) or KIR3DL1-high (h)-positive NK cells derived from Bw4-80T[+] donors in response to challenge with the Bw4-80T[+] acute myelogenous leukemia (AML) cell line, SET-2. Percentage of inhibition was calculated by comparing NK degranulation in the presence and absence of the KIR3DL1 blocking antibody, DX9. (C) Degranulation of KIR3DL1-low (I) or KIR3DL1-high (h) NK cells derived from Bw4-80I[+] donors in response to challenge with the Bw4-80I+ AML cell line, KG-1. (D) Response of KIR3DL1-null or inhibitory killer immunoglobulin-like receptor–negative cells to HLA-negative 721.221 target cells. NK cells are segregated on the basis of the presence or absence of HLA-Bw4 in the donor. (E) KIR3DL1-null-expressing NK cells from Bw4-80T+ or Bw4-80I+ donors were challenged using 721.221 cells transfected with HLA-B*44 or HLA-B*51, respectively. Percentage of inhibition is calculated by comparing responsiveness against parental 721.221 cells and Bw4-transfected cells. Each bar represents four to six donors and mean ± standard error of the mean.
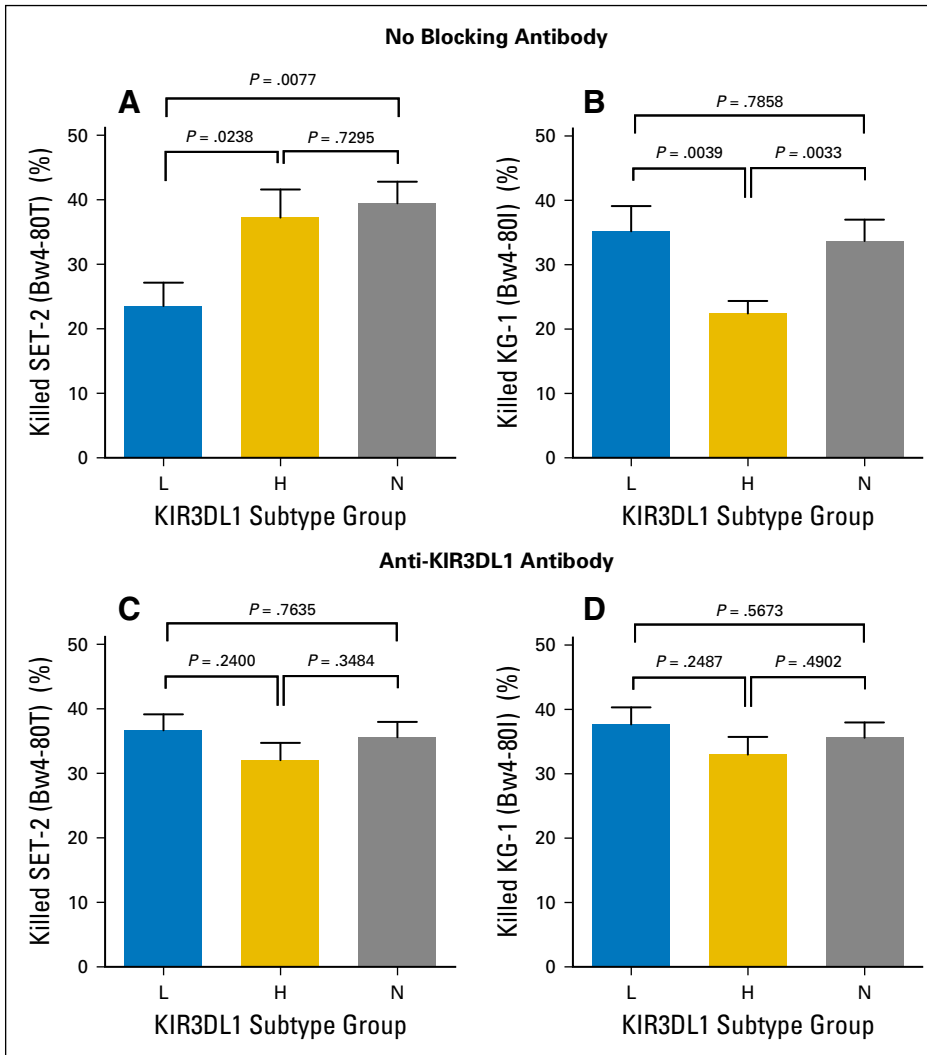
Fig 2. Subtype combinations of KIR3DL1 and HLA-Bw4 predict differential inhibition and killing of leukemia target cells. Peripheral blood mononuclear cells (PBMCs) were cultured with Bw4 subtype-matched target cells for 48 hours and the viability of leukemia cells was measured thereafter. (A) Cytotoxicity of the Bw4-80T+ acute myelogenous leukemia (AML) cell line SET-2 by PBMCs from Bw4-80T+ donors. (B) Cytotoxicity of the Bw4-80I+ AML cell line KG-1 by PBMCs from Bw4-80I+ donors. (C) Cytotoxicity of the Bw4-80T+ AML cell line SET-2 by PBMCs from Bw4-80T+ donors in the presence of Z27 antibody. (D) Cytotoxicity of the Bw4-80I+ AML cell line KG-1 by PBMCs from Bw4-80I+ donors in the presence of Z27 antibody. All bars represent means ± standard error of the mean. Each bar represents a minimum of six independent healthy donors and HLA-C subtype groups are stratified equivalently between groups.

only). Treatment with interferon-γ to mimic inflammation in HCT[6,7,41,42] further upregulates HLA.

In HLA-matched HCT, educated NK cells are at risk of inhibition by HLA expressed on the tumor. To test the hypothesis that NK cells with specific KIR3DL1 subtypes are variably inhibited by HLA-Bw4 subtypes, we evaluated the inhibition of NK cells that were single positive (spNK) for KIR3DL1 by HLA-Bw4–positive target cells. To simulate the HLA-matched HCT setting, we challenged primary NK cells with high or low KIR3DL1 expression from Bw4-80T–positive or Bw4-80I–positive individuals with HLA-Bw4–matched targets. Among *Bw4-80T* donors, KIR3DL1-l–positive spNK cells were more inhibited than KIR3DL1-h–positive spNK cells by the 721.221 transfectant that expressed the *Bw4-80T* allele *HLA-B*44:02* and by the Bw4-80T–positive AML cell line SET-2 (Figs 1A and 1B). The opposite was observed among *Bw4-80I* donors: KIR3DL1-h–positive spNK cells were more inhibited than KIR3DL1-l–positive spNK cells by 721.221 target cells that expressed the Bw4-80I allele HLA-B*51:01 and by the Bw4-80I–positive AML cell line KG-1 (Figs 1A and 1C). Given the higher affinity of KIR3DL1-h for Bw4-80I versus 80T,[13,28] higher inhibition of KIR3DL1-h–positive NK cells by the former was expected and consistent with clinical correlations.[19,21] Higher inhibition of KIR3DL1-l–positive NK cells by Bw4-80T relative to -80I,

however, was not expected on the basis of binding affinity,[28] but had been supported by previous clinical observations.[19,29]

KIR3DL1-Null–Positive NK Cells Are Cytotoxic, Yet Insensitive to Inhibition

KIR3DL1-n receptor is retained intracellularly and does not signal for inhibition.[22] We optimized staining for intracellular KIR3DL1 to investigate whether KIR3DL1-n would educate NK cells, following a recent finding that NK cells are educated by cell-intrinsic HLA[43,44] (Appendix Fig A2, online only). KIR3DL1-n–positive spNK cells from HLA-Bw4–positive donors, but not HLA-Bw4–negative donors, were highly responsive to 721.221 targets (Fig 1D), but insensitive to inhibition by HLA-Bw4–positive 721.221 target cells (Fig 1E), which indicated that they are educated for effector response, though refractory to inhibition.

KIR3DL1 and HLA-Bw4 Subtype Combinations Predict Differential Leukemotoxicity

We next investigated how differences in the inhibition of KIR3DL1-expressing NK cells impacted AML killing. We assigned diploid haplotypes to KIR3DL1 subgroups KIR3DL1-L

Table 1. Impact of *KIR3DL1/HLA-B* Subtype Combinations

Subtype Combination	No.	HR	95% CI	P
Relapse				
Strong inhibitory pairs*	334	1		
Noninhibiting pairs†	632	0.75	0.57 to 0.99	.039
Weak inhibitory pairs‡	362	0.73	0.56 to 0.96	.022
Strong inhibitory pairs*	334	1		
Noninhibiting† and weak inhibitory‡ pairs	994	0.72	0.58 to 0.90	.004
Survival				
Strong inhibitory pairs*	334	1		
Noninhibiting pairs†	632	0.88	0.73 to 1.06	.181
Weak inhibitory pairs‡	362	0.83	0.69 to 0.99	.045
Strong inhibitory pairs*	334	1		
Noninhibiting† and weak inhibitory‡ pairs	994	0.84	0.72 to 0.98	.030
Adjustment for KIR2DS1 effect§				
Relapse				
Strong inhibitory pairs*	334	1		
Noninhibiting pairs†	632	0.77	0.59 to 1.00	.056
Weak inhibitory pairs‡	362	0.74	0.56 to 0.96	.026
Strong inhibitory pairs*	334	1		
Noninhibiting† and weak inhibitory‡ pairs	994	0.74	0.59 to 0.93	.009
Survival				
Strong inhibitory pairs*	334	1		
Noninhibiting pairs†	632	0.89	0.74 to 1.07	.22
Weak inhibitory pairs‡	362	0.83	0.69 to 1.00	.048
Strong inhibitory pairs*	334	1		
Noninhibiting† and weak inhibitory‡ pairs	994	0.86	0.73 to 1.00	.049
Adjustment for Cen-BB effect¶				
Relapse				
Strong inhibitory pairs*	334	1		
Noninhibiting pairs†	632	0.75	0.57 to 0.99	.039
Weak inhibitory pairs‡	362	0.73	0.56 to 0.96	.024
Strong inhibitory pairs*	334	1		
Noninhibiting† and weak inhibitory‡ pairs	994	0.73	0.58 to 0.91	.005
Survival				
Strong inhibitory pairs*	334	1		
Noninhibiting pairs†	632	0.88	0.74 to 1.06	.181
Weak inhibitory pairs‡	362	0.83	0.69 to 1.00	.048
Strong inhibitory pairs*	334	1		
Noninhibiting† and weak inhibitory‡ pairs	994	0.85	0.73 to 0.99	.034

NOTE. In addition to the indicated adjustments (*KIR2DS1, Cen-BB*), all models were adjusted for donor age, treatment regimen, T-cell depletion, graft type, disease status, HLA match, cytomegalovirus, and gender match.
Abbreviation: HR, hazard ratio.
*Donors with KIR3DL1-L + Bw4-80T or KIR3DL1-H + Bw4-80I.
†Donors with any KIR3DL1 + Bw6/Bw6 or with KIR3DL1-N + Bw4-80I or KIR3DL1-N + Bw4-80T.
‡Donors with KIR3DL1-H + Bw4-80T or KIR3DL1-L + Bw4-80I.
§KIR2DS1 effect was defined by donors who exhibited *KIR2DS1* and *HLA-C1* versus all others.
¶*Cen-BB* in donors was defined as KIR2DL2 positive and/or KIR2DS2 positive and KIR2DL3 negative.

or KIR3DL1-H (Appendix Table A2).[45] A third subgroup represented donors who exclusively exhibited KIR3DL1-n and/or KIR3DS1 subtypes (KIR3DL1-N). PBMCs from individuals who represented each subgroup were coincubated with HLA-Bw4 subtype-matched AML target cells.

Among *Bw4-80T* individuals, KIR3DL1-H–positive PBMCs killed the Bw4-80T–positive AML target more efficiently than did KIR3DL1-L–positive PBMCs (Fig 2A). In contrast, among *Bw4-80I* individuals, KIR3DL1-L–positive PBMCs killed Bw4-80I–positive AML targets more efficiently than did KIR3DL1-H–positive PBMCs (Fig 2B). Antibody blockade of KIR3DL1 equalized target cell lysis between groups, which indicated that the differences in cytotoxicity could be explicitly attributed to differential inhibition of the KIR3DL1–positive

cell population (Figs 2C and 2D). KIR3DL1-N PBMCs exhibited high cytotoxicity against both cell lines, unchanged by the addition of anti-KIR3DL1/S1, which reflected their simultaneous education and insensitivity to inhibition. NK cells that were heterozygous for KIR3DL1-l+h exhibited greater killing of Bw4-80I–positive targets than Bw4-80T–positive targets, which supported their inclusion in the KIR3DL1-L group[19,29] (Appendix Fig A3, online only).

Strong Inhibitory Subtypes of KIR3DL1 and HLA-B Are Associated With Increased AML Relapse and Mortality

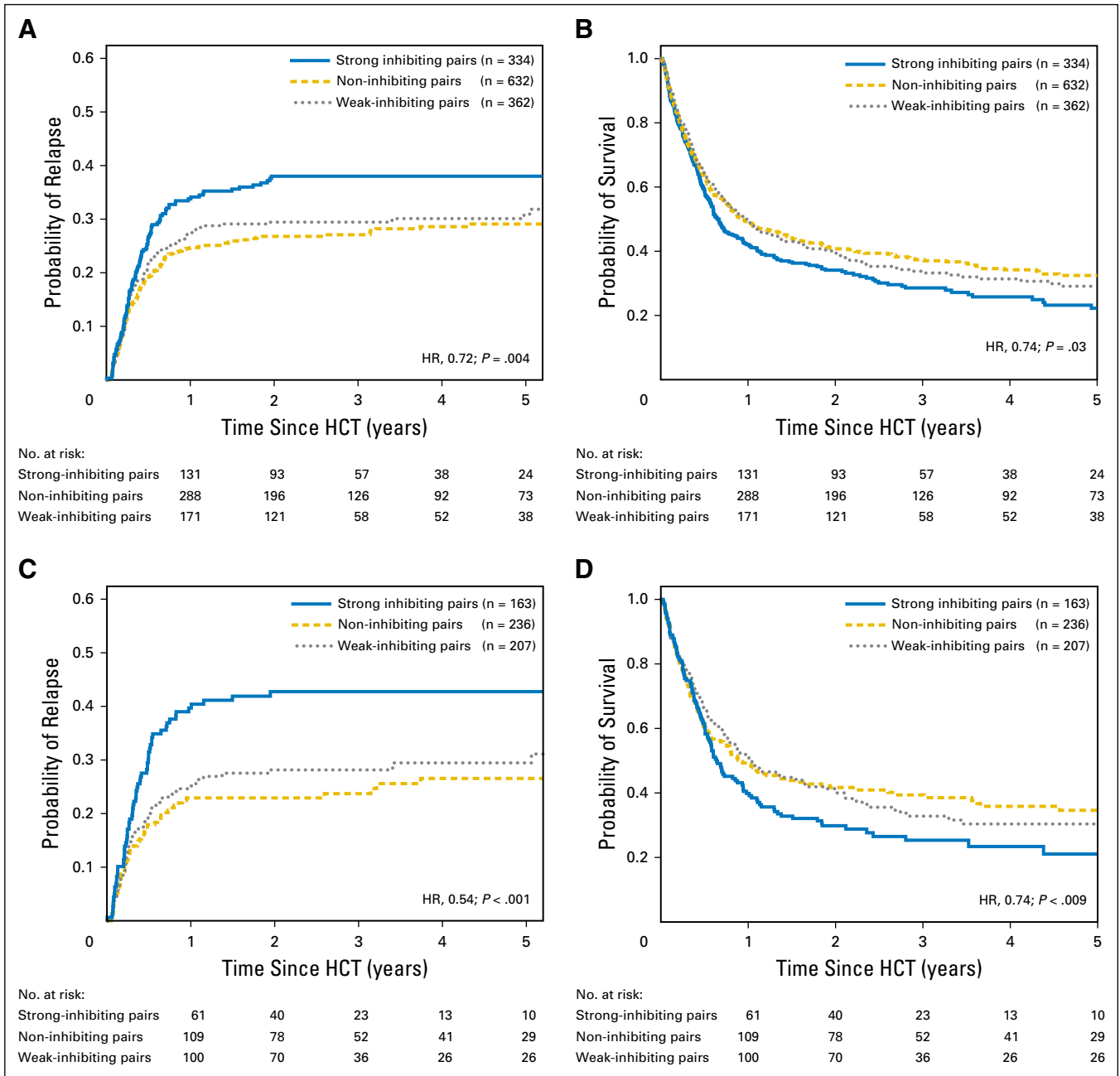To determine whether the hierarchy of inhibitory sensitivities established by KIR3DL1 and HLA-Bw4 subtypes affect AML

Fig 3. KIR3DL1 and HLA-B subtype combinations predict outcomes post–hematopoietic cell transplantation (HCT). (A and B) Patients with acute myelogenous leukemia (AML; N = 1,328) who underwent HCT were segregated according to donor *KIR3DL1* and *HLA-B* subtypes into strong inhibiting (blue lines), noninhibiting (dashed yellow lines), or weak inhibiting (dotted gray lines) subtype combinations. The number of donor-patient pairs per group is shown. (A) Cumulative incidence curves for relapse and (B) Kaplan-Meier plot for survival among all donor-patient pairs. (C and D) Patients with AML (n = 606) that exhibited HLA-C1 and -C2 who underwent HLA-matched HCT were segregated according to donor *KIR3DL1 and HLA-B* subtypes into strongly-interacting (blue lines), non-inhibiting (dashed yellow lines), or weakly interacting (dotted gray lines) subtype combinations. The number of donor-patient pairs per group is shown. (C) Cumulative incidence curves for relapse and (D) Kaplan-Meier plot for survival among donor-patient pairs exhibiting HLA-C1 and -C2. The indicated hazard ratios (HRs) and P values compare strong inhibiting pairs with weak and non-inhibiting pairs combined and reflect adjustment for patient's age, conditioning regimen, T-cell depletion, graft type, disease status, cytomegalovirus match, and gender match. All curve comparisons were completed using Cox proportional hazards regression analysis for the time-to-event post-HCT outcomes.

control, we retrospectively evaluated donor *KIR3DL1* and *HLA-B* subtypes for 1,328 patients with AML who received an unrelated HLA-compatible HCT. Neither donor-recipient HLA-B epitope, Bw4 subtype, nor *KIR3DL1* subtype alone was associated with overall mortality or relapse (data not shown). In contrast, any

impact of *KIR3DL1* on outcomes, particularly relapse, was dependent on the HLA-Bw4 subtype (test of interaction: $P = .06$).

On the basis of their relative inhibitory and cytotoxic strengths against targets in vitro, *KIR3DL1-H*+Bw4-80I and *KIR3DL1-L*+Bw4-80T were considered collectively to be strong
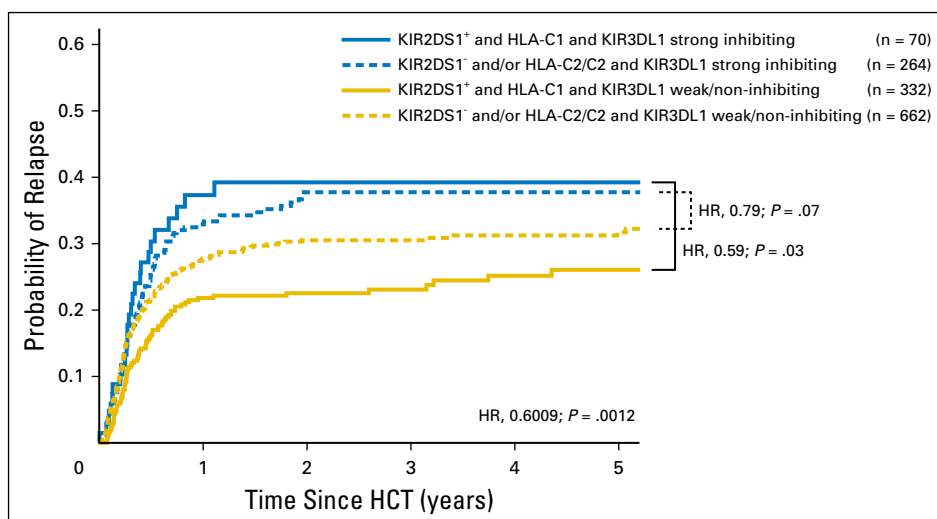
Fig 4. Independent and additive benefits of weak or noninhibiting KIR3DL1 and HLA-B pairs or KIR2DS1 and HLA-C1. Patients with acute myelogenous leukemia (N = 1,328) who underwent hematopoietic cell transplantation (HCT) from an unrelated donor were segregated on the basis of the presence/absence of *KIR3DL1* and *KIR2DS1*-mediated benefits. A cumulative incidence curve for relapse is shown. Patients with beneficial KIR2DS1 + HLA-C1 are shown as solid lines; patients who lacked KIR2DS1 and/or HLA-C1 are shown in dashed lines. Patients with strong inhibiting partnerships of KIR3DL1 and HLA-B are shown as blue lines; patients with weak inhibiting or noninhibiting partnerships of KIR3DL1 and HLA-B are shown as yellow lines. Hazard ratios (HRs) compare the indicated groups and patients with neither KIR2DS1/KIR3DL1 benefit to those with both.

inhibiting pairs; reciprocal combinations were considered to be weak inhibiting pairs. A third noninhibiting classification was composed of donors with *Bw6* and/or *KIR3DL1-N*. In a multivariable analysis, weak inhibiting pairs demonstrated significantly lower relapse (hazard ratio [HR], 0.73; $P = .019$) and mortality (HR, 0.83; $P = .041$) compared with strong inhibiting pairs; noninhibiting pairs were similarly beneficial (relapse: HR, 0.72; $P = .008$; and mortality: HR, 0.87; $P = .064$; Table 1). Combined, donors with weak or noninhibiting pairs were associated with superior outcomes compared with strong inhibiting pairs (relapse: HR, 0.72; $P = .004$; Fig 3A; mortality: HR, 0.84; $P = .03$; Fig 3B).

Removal of the 55 *KIR3DS1* homozygous donors did not alter our conclusions; therefore, the benefit of noninhibiting pairs was not a result of enrichment for *KIR3DS1* or other activating receptors in positive linkage disequilibrium (Table 1). There was no impact of Bw4 epitopes encoded by HLA-A alleles. Previous studies have demonstrated an association between lower relapse and donors with the partial KIR haplotype that contains the *KIR2DS2* and *KIR2DL2* genes, *cenB*, where homozygosity (*cenBB*) confers particular protection.[30] Correcting for *cenBB* did not alter *KIR3DL1/Bw4* effects (Table 1).

Weak or Noninhibiting KIR3DL1/HLA-B Subtype Combinations Are Most Protective in HLA-C1/C2 HCT

Among HCT recipients, 40% exhibit *HLA-Bw4/C1/C2*, or all KIR ligands, a configuration that is associated with higher relapse and mortality compared with patients who lack at least one KIR ligand.[30] Segregating HCT pairs according to *HLA-C* KIR ligands, we found that the protective effects of weak or noninhibiting versus strong inhibiting combinations for relapse (HR, 0.54; $P < .001$) and mortality (HR, 0.74; $P = .009$) were most evident in the high-risk HLA-C1/C2 transplant pairs (Figs 3C and 3D).

Benefits of KIR2DS1 and KIR3DL1/HLA-B Are Distinct

In our present cohort of 1,328 donor-patient pairs, 1,220 were assessed in our previous study of KIR2DS1, where we

described a benefit of donor *KIR2DS1+HLA-C1*.[31] This finding was unchanged within the larger cohort (relapse: HR, 0.79; $P = .04$; and mortality: HR, 0.89; $P = .12$). The protection associated with weak or noninhibiting *KIR3DL1/HLA-B* subtype combinations was not altered by correcting for *KIR2DS1/HLA-C1* (Table 1).

Donors who exhibited the combined benefits of weak or noninhibiting *KIR3DL1/HLA-B* with *KIR2DS1/HLA-C1* conveyed the lowest relapse and highest survival to patients. Strong inhibiting *KIR3DL1/HLA-Bw4* partnerships exhibited the highest relapse and mortality, which could not be improved by combination with *KIR2DS1+HLA-C1* (Appendix Table A5, online only, and Fig 4). Therefore, although the benefits of KIR3DL1/HLA-B and KIR2DS1/HLA-C1 are separate, the former exhibits primacy in HCT outcomes.

KIR3DL1 Subtypes: A Novel Donor Selection Criterion

To examine the association of increased NK inhibition with risk of failure, we estimated relative hazards for AML relapse and survival among all *KIR3DL1/HLA-B* subtype combinations in HLA-matched HCT, clustering groups on the basis of in vitro assessments of education and inhibitory sensitivity (Figs 5A and 5B). *Bw6* donor-patient pairs experienced intermediate protection, which reflected the known missing ligand benefit, but the lowest relapse and mortality was observed among *KIR3DL1-N+HLA-Bw4*, where cells were educated but refractory to inhibition. KIR3DL1-Bw4 partnerships that were predictive of weak inhibition (*Bw4-80T +KIR3DL1-H* or *Bw4-80I+KIR3DL1-L*) were associated with intermediate relapse and mortality, which implied a benefit of education and a weak sensitivity to inhibition. Strong inhibiting partnerships (*Bw4-80I+KIR3DL1-H* and *Bw4-80T +KIR3DL1-L*) were associated with the highest relapse and mortality, which implied that a strong inhibitory signal overrides the benefit of NK education.

We compared outcomes among HLA-B patient groups to understand whether donor selection on the basis of *KIR3DL1* subtypes may be an effective intervention to improve AML
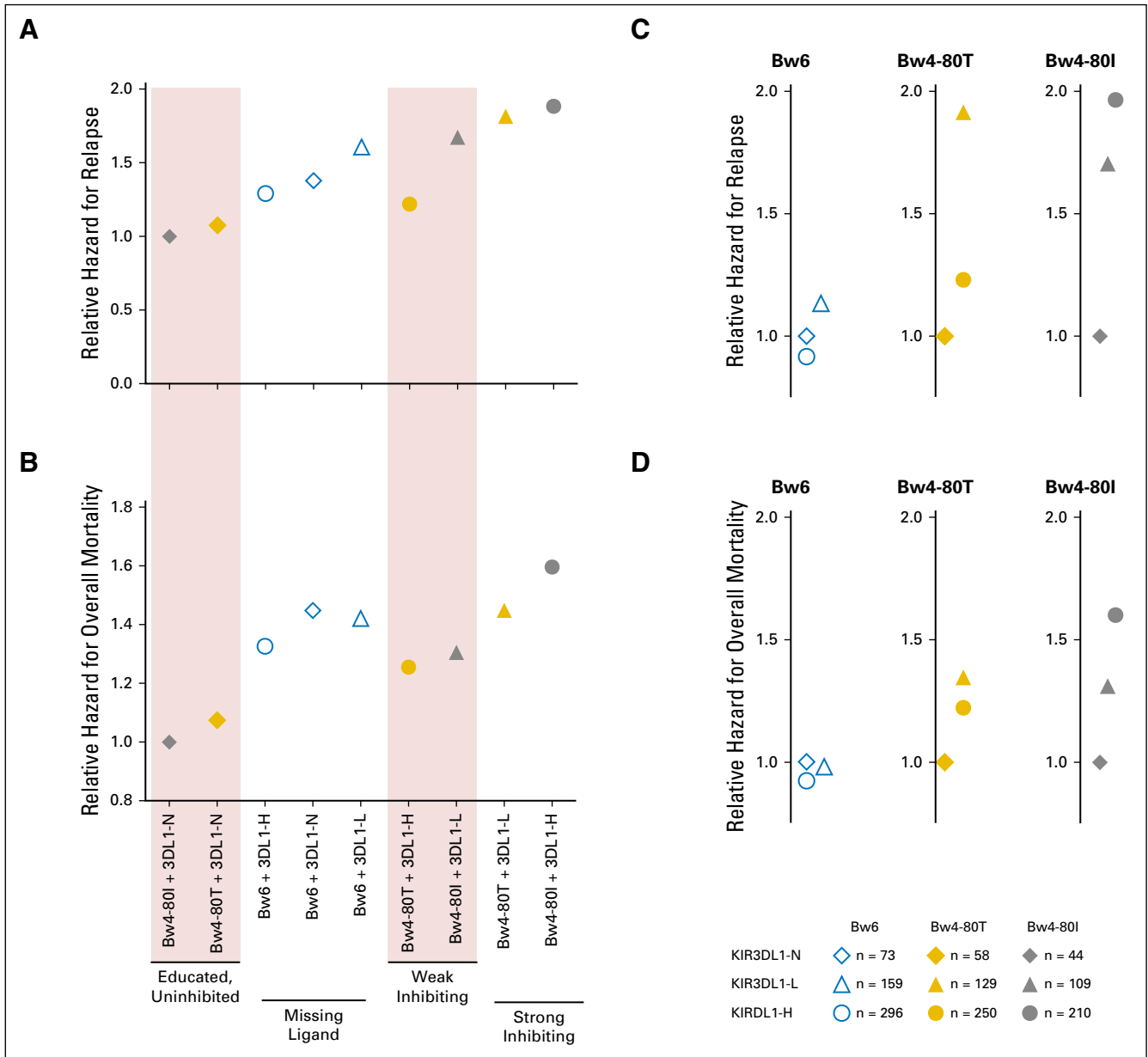
Fig 5. *KIR3DL1* and *HLA-B* subtype combinations predict a spectrum of post-transplant outcomes. Relative hazards were calculated by Cox proportional hazards regression analysis to compare the impacts of donor and recipient HLA-B. (A) Overall relapse and (B) mortality among hematopoietic cell transplantation (HCT) pairs with specific donor *KIR3DL1* and *HLA-B* subtype combinations are shown. (C) Relapse and (D) mortality segregated by recipient *HLA-B* subtype (*Bw6*, *Bw4-80T*, or *Bw4-80I*) stratified by donor *KIR3DL1* subtypes (*KIR3DL1-N*, *-L*, or *-H*) are shown. Diamonds (◊) represent *KIR3DL1-N* donors, triangles (△) represent *KIR3DL1-L* donors, and circles (○) represent *KIR3DL1-H* donors. Open blue, yellow, and gray symbols represent donor-recipients who encode *Bw6*, *Bw4-80T*, or *Bw4-80I*, respectively, and the numbers of donor-patient pairs in each compound subgroup are shown. Relative hazards reflect adjustment for patient's age, conditioning regimen, T-cell depletion, graft type, disease status, and cytomegalovirus and gender match. The legend indicates the number of patients present in each subgroup assessed.

control (Figs 5C and 5D). Predictably, there were no distinct advantages among donor KIR3DL1 subtypes in *Bw6*$^+$ donor-recipient pairs. For *Bw4-80T*$^+$ recipients, the greatest protection from relapse occurred if donors exhibited *KIR3DL1-H* (HR, 0.65; *P* = .031) or *KIR3DL1-N* (HR, 0.52; *P* = .058) compared with donors with *KIR3DL1-L*; overall mortality followed the same trend. For *Bw4-80I*$^+$ recipients, *KIR3DL1-N* donors were most protective for relapse (HR, 0.52; *P* = .055) and mortality (HR, 0.64; *P* = .054) compared with *KIR3DL1-H* donors.

DISCUSSION

Disease relapse remains the leading cause of death after allogeneic HCT.[2] It is increasingly evident that NK cells—educated by *HLA* and *KIR*—impact HCT outcomes for AML.[46] We now demonstrate that primary KIR3DL1-positive NK cells exhibit a hierarchy of inhibition by HLA-Bw4 subtypes that limits their capacity for lysing leukemia. Retrospective analysis of patients with AML who underwent HLA-matched HCT revealed that strong inhibiting

KIR3DL1/HLA-Bw4 combinations predict for higher relapse and mortality, whereas weak or /noninhibiting combinations are protective. The ideal NK effector against AML is educated for reactivity but is insensitive to inhibition by the patient's HLA, a combination found with *KIR3DL1-N+HLA-Bw4*.

The observation that Bw4-80I allotypes are stronger inhibitors of KIR3DL1-h–positive NK cells is consistent with their known binding preference[13,28]; however, for KIR3DL1-l subtypes, specificity between the two HLA-Bw4 subtypes is less dichotomous. By comparing inhibition of primary KIR3DL1-l–positive NK cells by different allotypes, we found that KIR3DL1-l–positive NK cells are more inhibited by Bw4-80T–positive targets than Bw4-80I–positive targets, in contrast to a recent report that found no discernible difference.[16,28] The discrepant results likely underscore the importance of considering the educating HLA of the NK cell in inhibition assays; to approximate an autologous or HLA-matched setting, both the NK and target cell should express the same KIR ligand. The mechanisms that underlie the association between receptor expression and differences in inhibitory sensitivity to HLA-Bw4 subtypes are not fully known, and amino acid residues that are important for receptor-ligand affinity do not dictate expression.[17] Assignment of strong versus weak inhibition on the basis of receptor expression phenotype and HLA-Bw4 dimorphism may be an oversimplification of a more nuanced system. Nevertheless, the associations of subtype combinations with inhibitory dichotomy, AML cytotoxicity, and relapse protection are consistent.

Proinflammatory cytokines that are present during immune reconstitution lower the threshold for NK activation but upregulate HLA on leukemia cells.[6,7,41,42] The protection from relapse that is conveyed by weak or noninhibiting *KIR3DL1/HLA-B* subtypes reflects a relative insensitivity to HLA-Bw4 that favors cytotoxicity over inhibition. In direct contrast to a suggestion that *KIR3DL1-n* donors should be avoided,[47] our results demonstrate a distinct advantage of *KIR3DL1-N+HLA-Bw4*: Intracellular receptor sequestration separates NK education from inhibitory susceptibility.

The benefit of *KIR3DL1/HLA-B* subtype combinations on HCT outcomes persists even after correcting for *KIR2DS1* with *HLA-C1*.[31] Combined, *KIR3DL1* and *KIR2DS1* exhibit the added benefit of minimizing inhibition while maximizing activation. Together, these studies indicate that consideration of *HLA* and *KIR* allele typing in donor selection to enable the antileukemic benefits of NK alloreactivity in HCT is warranted. In the frequent case in which a patient has more than one HLA-equivalent donor available, priority should be given to minimize KIR3DL1 inhibition over KIR2DS1 benefit.

In a pilot study, we performed *KIR3DL1* subtyping for 941 *HLA*-matched donors who were screened for 252 patients, 115 of whom completed HCT.[48] Weak or noninhibiting *KIR3DL1* subtype donors were identified for 93% of 211 patients who had more than one donor available. Of importance, the random 27% risk of a donor exhibiting high inhibition was reduced to 4% upon evaluation of up to three additional donors. Patients with weak or noninhibiting KIR3DL1/HLA-B partnerships experienced higher 2-year disease-free survival after HCT compared with those with strong inhibiting donors (64% *v* 39%; *P* = .05). Whether screening for KIR3DL1 subtypes among HLA-matched HCT donors will effect superior outcomes, especially in the high-risk HCT patients who encode all three KIR ligands, forms the basis of a larger, prospective clinical trial (NCT02450708).

The hallmark complications of HCT are GVHD, infection, and relapse. Whereas advances in allograft manipulation, HLA genetics, and antimicrobial therapies have improved the prevention of GVHD and the treatment of infection, disease relapse remains high. KIR and HLA titrate NK inhibition in a predictable, subtype-specific manner, which translates to hierarchical leukemia control. Therefore, refining donor selection algorithms to include KIR3DL1/HLA-B subtype analysis to avoid strong inhibition donors may reduce relapse and improve survival.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at jco.org.

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■ ■ ■

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

KIR3DL1/HLA-B Subtypes Govern Acute Myelogenous Leukemia Relapse After Hematopoietic Cell Transplantation

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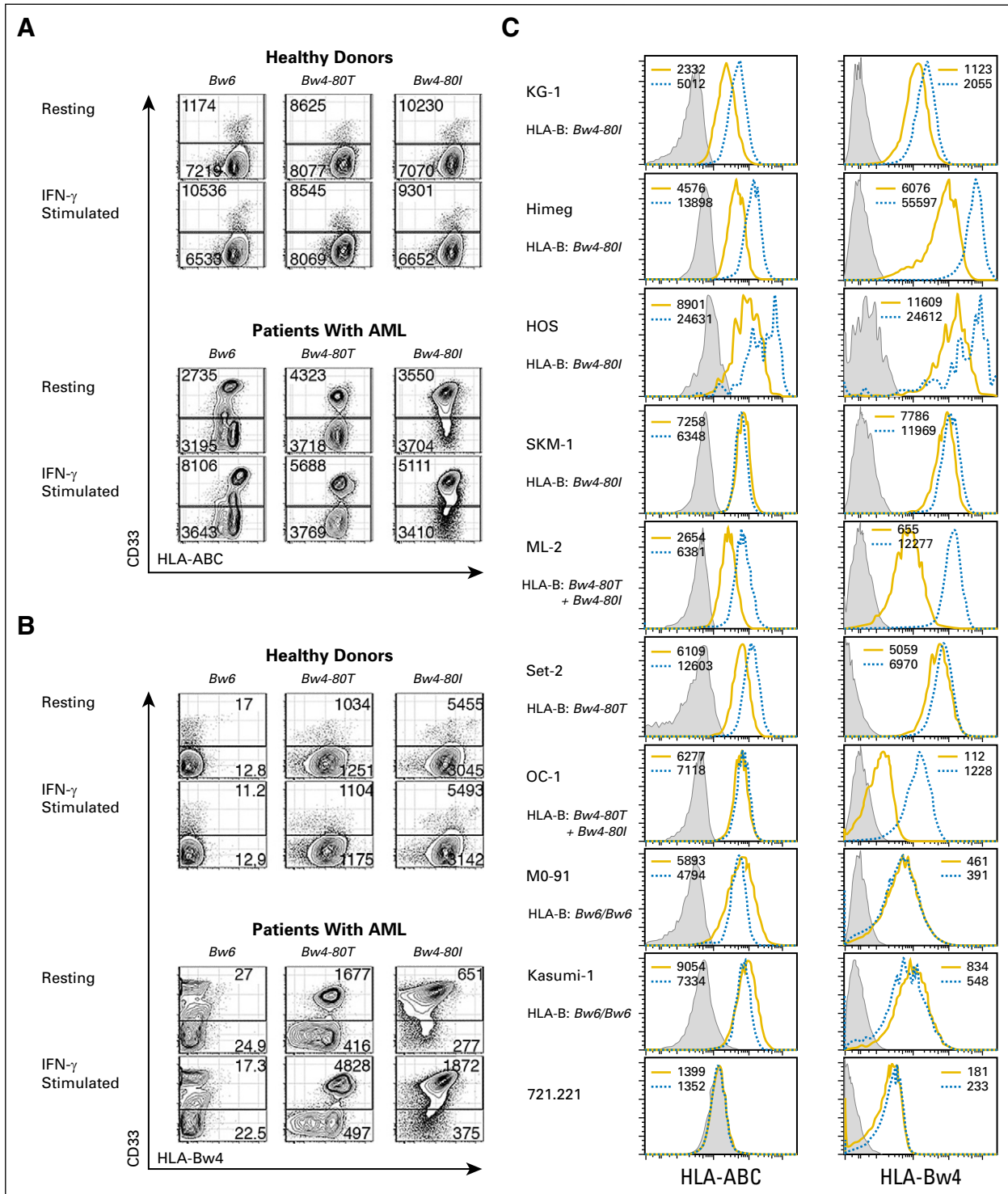
Acknowledgment

Appendix

Fig A1. HLA class I is expressed on acute myelogenous leukemia (AML) blasts and cell lines and upregulated in response to interferon-gamma (IFN-γ). (A and B) Peripheral blood mononuclear cells (PBMCs) from healthy donors or primary AML blasts from 12 patients were stained for CD33 and assessed for (A) total HLA class I expression or (B) for Bw4 expression at rest and after stimulation with IFN-γ. Three patients with AML and three HLA-B–matched healthy donors matched for HLA-B subtypes are shown and are representative of three to four samples per HLA-B subtype analyzed. Values indicate the mean fluorescence intensities of HLA-ABC or Bw4 staining among CD33+ and CD33− populations. (C) Nine cell lines of differing HLA-B epitope backgrounds are stained for total HLA class I expression and Bw4 at rest (yellow histograms) and after stimulation with IFN-γ (blue dashed histograms). Control (unstained) cells are shown as filled light gray histograms. The B lymphoid cell line, 721.221, which does not express HLA, is shown for comparison. The cell lines ML-2, OC-1, MO-91, and Kasumi-1 exhibit HLA-A epitopes that contain Bw4 motifs. Data represent two independent trials and numbers indicate mean fluorescent intensities.
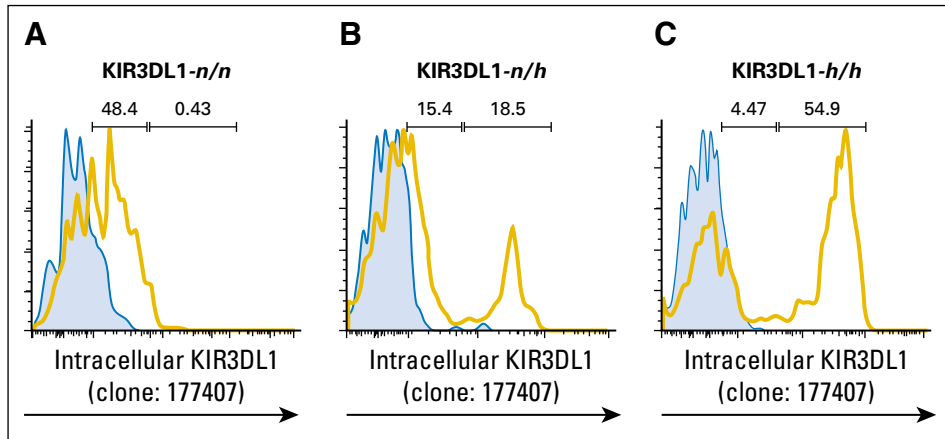
Fig A2. Optimization of staining for intracellular KIR3DL1-n. To enable assessment of intracellular KIR3DL1-n, cells were permeabilized and stained with anti-KIR3DL1 clone 177407. (A–C) Staining was optimized on natural killer (NK) cells from (A) KIR3DL1-n homozygous donors and verified on NK cells from donors exhibiting (B) KIR3DL1-n + KIR3DL1-h or (C) homozygous for KIR3DL1-h. Blue histograms indicate FMO control staining and yellow histograms indicate NK cells stained with anti-KIR3DL1 clone 177407.
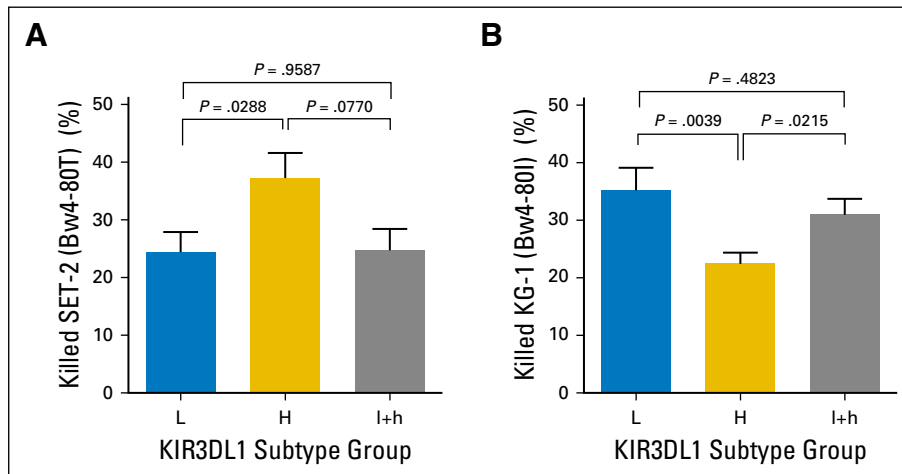
Fig A3. Donors who were heterozygous for KIR3DL1-l+h exhibit cytotoxicity most similar to KIR3DL1-L. (A) Cytotoxicity of the Bw4-80T+ acute myelogenous leukemia (AML) cell line SET-2 by peripheral blood mononuclear cells (PBMCs) from Bw4-80T+ healthy donors coexpressing KIR3DL1-l and KIR3DL1-h (l+h) or exhibiting only one of either KIR3DL1-h or KIR3DL1-l. (B) Cytotoxicity of the Bw4-80I+ AML cell line KG-1 by PBMCs from healthy Bw4-80I+ donors coexpressing KIR3DL1-l and KIR3DL1-h (l+h) or exhibiting only one of either KIR3DL1-h (H) or KIR3DL1-l (L). Bars represent means ± standard error of the mean and a minimum of seven independent donors and three independent trials. Means are compared by one-way ANOVA using Tukey's post hoc test.

Table A1. Donor, Recipient, and Transplant Characteristics According to Disease and *KIR3DL1/HLA-B* Subtypes

Characteristic	Noninhibiting (n = 632), No. (%)	Weak Inhibiting (n = 362), No. (%)	Strong Inhibiting (n = 334), No. (%)
Patient age at transplant, years			
0-20	113 (17.88)	63 (17.40)	49 (14.67)
20-30	87 (13.77)	61 (16.85)	57 (17.07)
30-40	112 (17.72)	41 (11.33)	40 (11.98)
40-50	144 (22.8)	81 (22.38)	82 (24.55)
50-60	123 (19.5)	84 (23.20)	62 (18.56)
60-70	50 (7.91)	29 (8.01)	44 (13.17)
>70	3 (0.47)	3 (0.83)	0 (0)
Donor age, years			
18-19	7 (1.107)	4 (1.104)	3 (0.898)
20-39	413 (65.34)	244 (67.40)	228 (68.26)
≥40	212 (33.54)	114 (31.49)	103 (30.83)
Year of transplantation			
1989-1994	14 (2.215)	16 (4.419)	8 (2.395)
1995-2000	159 (25.15)	93 (25.69)	80 (23.95)
2001-2005	211 (33.38)	117 (32.32)	123 (36.82)
2006-2008	248 (39.24)	136 (37.56)	123 (36.82)
Patient-donor sex			
Male-male	230 (36.39)	131 (36.18)	116 (34.73)
Male-female	96 (15.18)	65 (17.95)	56 (16.76)
Female-male	186 (29.43)	98 (27.07)	91 (27.24)
Female-female	119 (18.82)	68 (18.78)	71 (21.25)
Unknown	1 (0.158)		
Disease stage*			
Early (low risk)	239 (37.81)	121 (33.42)	107 (32.03)
Intermediate (intermediate risk)	182 (28.79)	98 (27.07)	101 (30.23)
High (high risk)	207 (32.75)	143 (39.50)	122 (36.52)
Unknown	4 (0.632)	0	4 (1.197)
Patient-donor serologic status for cytomegalovirus			
Negative-negative	191 (30.22)	106 (29.28)	107 (32.03)
Negative-positive	65 (10.28)	43 (11.87)	42 (12.57)
Positive-negative	240 (37.97)	112 (30.93)	104 (31.13)
Positive-positive	114 (18.03)	80 (22.09)	70 (20.95)
Unknown	22 (3.48)	21 (5.80)	11 (3.29)
Transplant type			
Myeloablative	540 (85.44)	304 (83.97)	279 (83.53)
Reduced-intensity/nonmyeloablative	81 (12.81)	55 (15.19)	50 (14.97)
Unknown	11 (1.74)	3 (0.83)	5 (1.50)
TBI			
No TBI	268 (42.41)	173 (47.79)	151 (45.21)
TBI	356 (56.33)	189 (52.21)	180 (53.89)
Unknown	8 (1.27)	0 (0.00)	3 (0.90)
Source of cells			
Bone marrow	349 (55.22)	197 (54.41)	176 (52.69)
Peripheral blood stem cells	283 (44.77)	165 (45.58)	158 (47.30)
GVHD prophylaxis			
Cyclosporine with or without other agents	261 (41.29)	139 (38.39)	127 (38.02)
Tacrolimus with or without other agents	257 (40.66)	153 (42.26)	138 (41.31)
T-cell depletion	50 (7.91)	31 (8.56)	31 (9.28)
Other combinations	64 (10.12)	39 (10.77)	38 (11.37)
Patient race or ethnic group†			
African American	7 (1.11)	9 (2.49)	15 (4.49)
Asian/Pacific Islander	4 (0.63)	2 (0.55)	7 (2.10)
White	588 (93.03)	330 (91.16)	292 (87.42)
Hispanic	27 (4.28)	18 (4.97)	12 (3.59)
Native American	1 (0.158)	0	0
Other	1 (0.16)	1 (0.28)	2 (0.60)
Unknown	4 (0.63)	2 (0.55)	6 (1.80)
(continued on following page)			

Table A1. Donor, Recipient, and Transplant Characteristics According to Disease and *KIR3DL1/HLA-B* Subtypes (continued)

Characteristic	Noninhibiting (n = 632), No. (%)	Weak Inhibiting (n = 362), No. (%)	Strong Inhibiting (n = 334), No. (%)
Donor race or ethnic group†			
African American	8 (1.27)	9 (2.49)	12 (3.59)
Asian/Pacific Islander	5 (0.79)	3 (0.83)	6 (1.80)
White	556 (87.97)	306 (84.53)	281 (84.13)
Hispanic	23 (3.64)	18 (4.97)	18 (5.39)
Native American	6 (0.95)	4 (1.10)	1 (0.30)
Other	19 (3.01)	17 (4.70)	9 (2.69)
Unknown	15 (2.37)	5 (1.38)	7 (2.10)
HLA match status‡			
HLA 10/10	352 (55.69)	194 (53.59)	170 (50.89)
HLA 9/10	280 (44.30)	168 (46.40)	164 (49.10)
Cytogenetic risk			
Good	54 (8.54)	28 (7.73)	17 (5.09)
Intermediate	208 (32.91)	128 (35.36)	119 (35.63)
Poor	37 (5.85)	20 (5.52)	22 (6.59)
No abnormalities	180 (28.48)	94 (25.97)	100 (29.94)
Unknown	153 (24.21)	92 (25.41)	92 (22.75)
cGVHD			
No (0)	381 (60.28)	208 (57.46)	212 (63.47)
Yes (1)	241 (38.13)	147 (40.61)	327 (34.43)
Unknown	10 (1.58)	7 (1.93)	7 (2.10)
aGVHD (2-4)			
No (0)	308 (48.73)	172 (47.51)	173 (51.80)
Yes (1)	321 (50.79)	185 (51.10)	156 (46.71)
Unknown	3 (0.47)	5 (1.38)	5 (1.50)
aGVHD (3-4)			
No (0)	488 (77.22)	285 (78.73)	265 (79.34)
Yes (1)	141 (22.31)	72 (19.89)	62 (18.56)
Unknown	3 (0.47)	5 (1.38)	7 (2.10)

Abbreviations: aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; TBI, total body irradiation.
*Low risk indicates first complete remission, intermediate risk second or greater complete remission, and high risk primary induction failure or relapse.
†Race and ethnic groups were self-reported.
‡HLA donor-recipient matches at *HLA-A, -B, -C, -DRB1,* and *-DQB1.*

Table A2. Donor *KIR3DL1* and *HLA-B* Subtype Distributions

	Bw6	Bw4-80T		Bw4-80I			
Subtype	Bw6 Bw6	80T Bw6	80T/80T	80I/Bw6	80I/80T	80I/80I	Total
3DL1-N	73	53	5	34	7	3	175
3DL1-L	158	110	19	79	20	11	397
3DL1-H	299	217	35	138	44	23	756
Total	530	380	59	251	71	37	1,328

	Bw6	Bw4-80T	Bw4-80I	Total
3DL1-N				
n/n	23	13	11	47
n/s	27	27	19	73
s/s	23	18	14	55
3DL1-L				
l/n	24	22	12	58
l/l	20	12	18	50
l/h	81	71	54	206
l/s	33	24	26	83
3DL1-H				
h/n	81	69	48	198
h/h	120	110	99	329
h/s	98	73	58	229
Total	530	439	359	1,328

Table A3. Alleles Comprising KIR3DL1 Subtype Groups and Their Differential Primer Binding Sites

Subtype	High-Frequency Alleles (> 2%)	Low-Frequency Alleles (< 2%)	Exon 3 (D0 domain) 193	Exon 3 (D0 domain) 202	Exon 4 (D1 domain) 607	Exon 7 (transmembrane domain) 1021/22
KIR3DL1-n	*004	*019, *021, *036, *037, *039, *040, *056, *063, *072	C**G**GAA	C**G**GAA	C**T**CCT	A**TG**TT
KIR3DS1*	*013	*010, *011, *012, *014, *045, *046, *047, *048, *049N, *050, *055, *058	C**A**GAA	C**A**GAA	C**C**CCT	A**CA**TT
KIR3DL1-l	*005	*041, *044, *053	C**A**GAA	C**A**GAA	C**T**CCT	A**CA**TT
	*007	*032, *033, *068	C**A**GAA	C**A**GAA	C**C**CCT	A**CG**TT
KIR3DL1-h	*001	*016, *026, *027, *043, *052, *059, *060, *061, *064, *065, *067, *075	C**A**GAA	C**A**GAA	C**C**CCT	A**CA**TT
	*002, *015, *008	*006, *009, *017, *018, *020, *022, *023, *024N, *025, *028, *029, *030, *031, *034, *035, *038, *042, *051, *054, *057, *062, *066, *074, *076, *077	C**A**GAA	C**G**TTCC	C**C**CCT	A**CA**TT

NOTE. The polymorphic sites that differentiate allele subtypes are shown in bold and column labels indicate the polymorphic site in the mature coding sequence. Banded rows indicate alleles identified by medium resolution PCR-SSP.[7]
*KIR3DS1 alleles are differentiated from KIR3DL1*002 group high alleles by product size; intron 3 in KIR3DS1 alleles is 200-bp longer than that of KIR3DL1.

Table A4. Antibody Clones and Sources Used for Flow Cytometry

Target	Clone	Source
CD3	OKT-3	BioLegend, San Diego, CA
CD56	N901	Beckman Coulter, Brea, CA
KIR3DL1 (binds KIR3DL1-l and KIR3DL1-h)	DX9	BioLegend, San Diego, CA
NKG2A	Z199	Beckman Coulter, Brea, CA
KIR2DL1/S1	EB6B	Beckman Coulter, Brea, CA
KIR2DL2/L3/S2	GL183	Beckman Coulter, Brea, CA
KIR3DL1 (binds all KIR3DL1)	177407	Beckman Coulter, Brea, CA
CD33	AC104.3E3	Miltenyi Biotec, Auburn, CA
HLA-A, -B, -C	G46-2.6	BD Biosciences, San Jose, CA
HLA-Bw4	REA 274	Miltenyi Biotec, Auburn, CA
CD107a	H4A3	BD Biosciences, San Jose, CA

Table A5. Combined Benefits Mediated by KIR3DL1 and KIR2DS1

KIR3DL1	KIR2DS1	Relapse HR	Relapse 95% CI	Relapse P	Survival HR	Survival 95% CI	Survival P
Strong inhibiting partnership	KIR2DS1- and/or HLA-C2/C2	1			1		
Strong inhibiting partnership	KIR2DS1+ and HLA-C1+	1.0118	0.64 to 1.60	.9649	0.9323	0.67 to 1.30	.6795
Weak inhibiting/noninhibiting partnership	KIR2DS1- and/or HLA-C2/C2	0.7895	0.61 to 1.02	.0592	0.8632	0.73 to 1.03	.0982
Weak inhibiting/noninhibiting partnership	KIR2DS1+ and HLA-C1+	0.6002	0.44 to 0.82	.0012	0.7741	0.64 to 0.95	.0136

NOTE. Analyses were adjusted for age, treatment regimen, T-cell depletion, graft type, disease status, cytomegalovirus match, sex match, and HLA match.
Abbreviation: HR, hazard ratio.