CTLs are obtained from the same ethnic group. In addition, by adopting other immunophenotyping readouts such as production of IL-2 from $CD4^+$ T cells, this method could be applied to identification of MHC class II–restricted minor H antigens which have crucial roles in controlling CTL functions upstream. This may open a new field in the study of allo-HSCT since MHC class II–restricted mHags have been technically difficult to identify by conventional methods.

Finally, the discovery of ACC-1^C as a novel minor H antigen indicates that all the mismatched transplants at this locus could be eligible for allo-immune therapies, since we have previously demonstrated that the counter allele also encodes a minor H antigen, ACC-1^Y, which is preferentially expressed and presented on blood components including leukemic cells and may serve as a target of allo-immunity.[7,34] Indeed, CTLs specific for ACC-2, an HLA-B44–restricted minor H antigen restricted by the third exonic SNP on *BCL2A1*,[7] was independently isolated from the peripheral blood of a patient with recurrent leukemia re-entering complete remission after donor lymphocyte infusion.[32] The number of eligible allo-HSCT recipients has now been effectively doubled, accounting for 50% of transplants with HLA-A24 or 20% of all transplantations performed in the Asian population. In conclusion, we have described a simple but powerful method for minor H mapping to efficiently accelerate the discovery of novel minor H antigens that will be needed to contribute to our understanding of the molecular mechanism of human allo-immunity.

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Authorship

Contribution: T.K. performed most immunologic experiments and preparation of pooled DNA and quantative PCR, analyzed data, and wrote the manuscript; Y.N. performed the majority of genetic analyses and analyzed the data; H.T. performed T-cell receptor analysis and designed q-PCR primers and probes; G.Y. contributed to the organization of software for linkage analysis and simulation; S.M. prepared the pooled DNA; M.O., K.M., Y.K, and Y.M. collected clinical data and specimens; T.T. and K.K. contributed to data analysis and interpretation, and to the writing of the article; S.O. and Y.A. supervised the entire project, designed and coordinated most of the experiments in this study, contributed to manuscript preparation, and are senior coauthors.

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References

1. Thomas ED Sr. Stem cell transplantation: past, present and future. Stem Cells. 1994;12:539-544.
2. Childs RW, Barrett J. Nonmyeloablative allogeneic immunotherapy for solid tumors. Annu Rev Med. 2004;55:459-475.
3. Goulmy E. Human minor histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy. Immunol Rev. 1997;157:125-140.
4. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. Nat Rev Cancer. 2004;4:371-380.
5. den Haan JM, Meadows LM, Wang W, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. Science. 1998;279:1054-1057.
6. Pierce RA, Field ED, Mutis T, et al. The HA-2 minor histocompatibility antigen is derived from a diallelic gene encoding a novel human class I myosin protein. J Immunol. 2001;167:3223-3230.
7. Akatsuka Y, Nishida T, Kondo E, et al. Identification of a polymorphic gene, BCL2A1, encoding two novel hematopoietic lineage-specific minor histocompatibility antigens. J Exp Med. 2003;197:1489-1500.
8. Warren EH, Vigneron NJ, Gavin MA, et al. An antigen produced by splicing of noncontiguous peptides in the reverse order. Science. 2006;313:1444-1447.
9. Kawase T, Akatsuka Y, Torikai H, et al. Alternative splicing due to an intronic SNP in HMSD generates a novel minor histocompatibility antigen. Blood. 2007;110:055-1063.
10. van Bergen CA, Kester MG, Jedema I, et al. Multiple myeloma-reactive T cells recognize an activation-induced minor histocompatibility antigen encoded by the ATP-dependent interferon-responsive (ADIR) gene. Blood. 2007;109:4089-4096.
11. Dolstra H, Fredrix H, Maas F, et al. A human minor histocompatibility antigen specific for B cell acute lymphoblastic leukemia. J Exp Med. 1999;189:301-308.
12. de Rijke B, van Horssen-Zoetbrood A, Beekman JM, et al. A frameshift polymorphism in P2X5 elicits an allogeneic cytotoxic T lymphocyte response associated with remission of chronic myeloid leukemia. J Clin Invest. 2005;115:3506-3516.
13. Brickner AG, Evans AM, Mito JK, et al. The PANE1 gene encodes a novel human minor histocompatibility antigen that is selectively expressed in B-lymphoid cells and B-CLL. Blood. 2006;107:3779-3786.
14. Warren EH, Otterud BE, Linterman RW, et al. Feasibility of using genetic linkage analysis to identify the genes encoding T cell-defined minor histocompatibility antigens. Tissue Antigens. 2002;59:293-303.
15. Consortium TIH. The International HapMap Project. Nature. 2003;426:789-796.
16. Consortium TIH. A haplotype map of the human genome. Nature. 2005;437:1299-1320.
17. Risch N, Merikangas K. The future of genetic studies of complex human diseases. Science. 1996;273:1516-1517.
18. Hirschhorn JN, Daly MJ. Genome-wide association studies for common diseases and complex traits. Nat Rev Genet. 2005;6:95-108.
19. Kennedy GC, Matsuzaki H, Dong S, et al. Large-scale genotyping of complex DNA. Nat Biotechnol. 2003;21:1233-1237.
20. Matsuzaki H, Dong S, Loi H, et al. Genotyping over 100 000 SNPs on a pair of oligonucleotide arrays. Nat Methods. 2004;1:109-111.
21. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005;21:263-265.
22. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol. 1994;152:163-175.
23. Kubo RT, Sette A, Grey HM, et al. Definition of specific peptide motifs for four major HLA-A alleles. J Immunol. 1994;152:3913-3924.
24. Dolstra H, de Rijke B, Fredrix H, et al. Bi-directional allelic recognition of the human minor histocompatibility antigen HB-1 by cytotoxic T lymphocytes. Eur J Immunol. 2002;32:2748-2758.
25. Easton DF, Pooley KA, Dunning AM, et al. Genome-wide association study identifies novel breast cancer susceptibility loci. Nature. 2007;447:1087-1093.
26. Gudmundsson J, Sulem P, Manolescu A, et al.

Genome-wide association study identifies a second prostate cancer susceptibility variant at 8q24. Nat Genet. 2007;39:631-637.

27. Zeggini E, Weedon MN, Lindgren CM, et al. Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. Science. 2007;316:1336-1341.
28. Su AI, Cooke MP, Ching KA, et al. Large-scale analysis of the human and mouse transcriptomes. Proc Natl Acad Sci U S A. 2002;99:4465-4470.
29. Nannya Y, Taura K, Kurokawa M, Chiba S, Ogawa S. Evolution of genome-wide power of genetic association studies based on empirical data from the HapMap project. Hum Mol Genet. 2007;16:3494-3505.
30. Spierings E, Brickner AG, Caldwell JA, et al. The minor histocompatibility antigen HA-3 arises from differential proteasome-mediated cleavage of the lymphoid blast crisis (Lbc) oncoprotein. Blood. 2003;102:621-629.
31. Brickner AG, Warren EH, Caldwell JA, et al. The immunogenicity of a new human minor histocompatibility antigen results from differential antigen processing. J Exp Med. 2001;193:195-206.
32. Kloosterboer FM, van Luxemburg-Heijs SA, van Soest RA, et al. Minor histocompatibility antigen-specific T cells with multiple distinct specificities can be isolated by direct cloning of IFNgamma-secreting T cells from patients with relapsed leukemia responding to donor lymphocyte infusion. Leukemia. 2005;19:83-90.
33. Tykodi SS, Warren EH, Thompson JA, et al. Allogeneic hematopoietic cell transplantation for metastatic renal cell carcinoma after nonmyeloablative conditioning: toxicity, clinical response, and immunological response to minor histocompatibility antigens. Clin Cancer Res. 2004;10:7799-7811.
34. Kenny JJ, Knobloch TJ, Augustus M, Carter KC, Rosen CA, Lang JC. GRS, a novel member of the Bcl-2 gene family, is highly expressed in multiple cancer cell lines and in normal leukocytes. Oncogene. 1997;14:997-1001.

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Donor Killer Immunoglobulin-Like Receptor (KIR) Genotype-Patient Cognate KIR Ligand Combination and Antithymocyte Globulin Preadministration Are Critical Factors in Outcome of HLA-C-KIR Ligand-Mismatched T Cell–Replete Unrelated Bone Marrow Transplantation

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ABSTRACT

We previously reported the potent adverse effects of killer immunoglobulin-like receptor (KIR) ligand mismatch (KIR-L-MM) on the outcome of T cell–replete unrelated hematopoietic stem cell transplantation (UR-HSCT) through the Japan Marrow Donor Program. Other UR-HSCT studies have yielded inconsistent results. To address this discrepancy, we evaluated candidate factors contributing to the effects of KIR-L-MM on transplantation outcomes in retrospectively selected hematologic malignancy cases with uniform graft-versus-host disease (GVHD) prophylaxis (n = 1489). KIR-L-MM in the graft-versus-host direction (KIR-L-MM-G) was associated with a higher incidence of acute GVHD (aGVHD; $P < .002$) and a lower overall survival (OS; $P < .0001$) only without the preadministration of antithymocyte globulin (ATG). Furthermore, in KIR-L-MM-G, the donor *KIR2DS2* gene with the patient cognate C1 ligand was associated with a higher incidence of aGVHD ($P = .012$). Multivariate analysis by Cox proportional hazard models suggested that donor *2DS2* and ATG preadministration were critical factors in grade III-IV aGVHD (hazard ratio = 1.96; 95% confidence interval = 1.01-3.80; $P = .045$, and hazard ratio = 0.56; 95% confidence interval = 0.31-0.99; $P = .047$, respectively). These results indicate that the adverse effects of KIR-L-MM-G depend on combination of donor-activating *KIR* genotype-patient cognate KIR ligand type and no ATG preadministration, thereby suggesting the importance of these factors in UR-HSCT and in leukemia treatment using natural killer (NK) cell alloreactivity.

INTRODUCTION

Natural killer (NK) cell alloreactivity plays an important role in hematopoietic stem cell transplantation (HSCT,) and its therapeutic use in leukemia treatment has been considered because of its possible graft-versus-leukemia (GVL) effect [1]. The beneficial effects of NK cell receptor killer immunoglobulin-like receptor (KIR) ligand incompatibility between patient and donor in the HLA-mismatched related hematopoietic stem cell transplantation (R-HSCT) has been reported [2,3]. These effects in unrelated hematopoietic stem cell transplantation (UR-HSCT) have been controversial, however [4]. We recently reported the potent adverse effects of HLA-C-KIR ligand incompatibility

(ligand–ligand analysis) in unrelated T cell–replete HLA-A, -B, and -DR serologically matched bone marrow transplantation without preadministration of antithymocyte globulin (ATG) (n = 1790) through the Japan Marrow Donor Program (JMDP) [5]. Other UR-HSCT studies have documented either adverse or beneficial effects of KIR ligand incompatibility on transplantation outcome [6-14].

Candidate factors possibly accounting for this discrepancy include T cell depletion, *KIR* genotype of patients and donors, sample size, ethnicity, number and source of stem cells, ATG preadministration, graft-versus-host disease (GVHD) prophylaxis, and diseases. Associations between *KIR* genotype and clinical outcome have been reported in both related HLA-identical transplantation [15-22] and unrelated transplantation settings [10,23-27]. However, the contribution of the *KIR* genotype to KIR ligand compatibility has not yet been well defined. Preadministration of ATG in the conditioning regimen both reduces stem cell rejection by host lymphocytes and prevents GVHD by donor-derived lymphocytes, as the drug remains in the patient's blood for several weeks after transplantation and affects residual donor mature lymphocyte activity and reconstitution of the lymphocyte repertoire from donor stem cells [28]. Earlier UR-HSCT studies have demonstrated the need for ATG administration to gain the beneficial effect of NK cell alloreactivity [7], whereas an adverse effect of KIR-L-MM has been reported in both a non-ATG preadministration study [6] and ATG preadministration studies [8,9]; however, a direct comparison of the ATG-administration and ATG-nonadministration groups in a single large cohort has never been performed. Such a study is desirable for a precise evaluation of the effect of ATG on KIR-L-MM.

In this study, patients with hematologic malignancy cases who received uniform GVHD prophylaxis were retrospectively selected from patients undergoing unrelated bone marrow transplantation through the JMDP. All cases were HLA-A, -B, and -DR serologically matched (ie, including HLA-A, -B, and -DR allele-mismatched pairs as well as HLA-Bw4 and HLA-A3 and -A11 KIR ligand matched and HLA-C-KIR ligand matched and mismatched pairs) and mostly uniform with regard to ethnicity and transplantation regimens [29,30]. In these cases, the effects of *KIR* genotype, KIR ligand compatibility, and ATG administration status on transplantation outcomes were analyzed to resolve the discrepant findings regarding the effects of KIR-L-MM.

PATIENTS AND METHODS

Patient and Cohort Selection Criteria

A cohort (n = 1489) was selected from among patients undergoing unrelated bone marrow transplantation between 1993 and 2000 through the JMDP. Characteristics of the patients and donors are summarized in Table 1. A source of hematopoietic stem cells of all transplantations were from T cell–replete and HLA-A, -B, and -DR serologically matched bone marrow. Patients with hematologic malignancies, including 401 cases of acute myelogenous leukemia (AML), 438 cases of acute lymphoblastic leukemia (ALL), 451 cases of chronic myelogenous leukemia (CML), 137 cases of myelodysplastic syndrome (MDS), and 62 cases of malignant lymphoma (non-Hodgkin lymphoma) were analyzed. GVHD prophylaxis other than the combination of cyclosporine and short-term methotexate (the most common treatment reported in the JMDP [68.1%]) was excluded. Ninety-four patients with preadministered ATG were included and analyzed separately or together with the nonadministered cases. Standard risk for relapse was defined as the status of first complete remission (CR) of AML or ALL, first chronic phase (CP) of CML at transplantation, or refractory anemia (RA) in MDS. High risk was defined as a more advanced status than standard risk in AML, ALL, CML, and MDS. All patients were preconditioned with a myeloablative regimen, and 1204 patients received total body irradiation (TBI)-containing regimens, whereas 285 received non–TBI-containing regimens. The final clinical survey of these patients was performed as of

Table 1. *Patient characteristics and matching of HLA allele between patient and donor*

	All patients	C-match	C-mismatch KIR-L-MM-G	C-mismatch KIR-L-M
Analyzed number	1489	1013	81	395
AML	401	286	17	98
ALL	438	306	24	108
CML	451	296	25	130
MDS	137	82	14	41
Malignant lymphoma	62	43	1	18
Patient age	26	27	25	27
Donor age (90 high risk)	34	35	35	34
Sex match	57	56.6	59.3	57.5
TBI	80.9	81	79	80.8
Status of leukemia (% high risk)	55.1	53.5	70	56
HLA-allele mismatch, %				
A	18.5	14.2	28.4	27.6
B	9.1	3.5	25.9	20.1
C	32	0	100	100
DRB1	18.9	15.5	32.1	24.8
DQB1	22	18.7	28.4	29.1
DPB1	71.3	74.8	82.7	76.7
ATG+	94	56	11	27
ATG−	1395	957	70	368
Donor *KIR 2DS2* analyzed	233	83	80	70
Patient–donor *16 KIR* type analyzed	187	70	55	62

June 1, 2005. The mean and range for clinical follow-up were 2914 days and 1639-4597 days, respectively. A part of the subject population (leukemia treated with cyclosporine and short-term methotexate; n = 1210) was overlapped with that reported in our previous study [5]. Written informed consent was obtained from all patients and donors, and the study design was approved by the institutional review boards of the Japanese Red Cross Tokyo Metropolitan Blood Center, the Aichi Cancer Center, and the JMDP.

HLA and KIR Ligand Typing and Compatibility Characterization of Patient–Donor Pairs

HLA-A, -B, -C, -DR, -DQ, and *-DP* alleles of all patients and donors were retrospectively determined by DNA typing as described previously [5]. For analysis of GVHD and leukemia relapse, *HLA* allele mismatch among donor–patient pairs was defined as the patient's alleles not being shared by the donor. KIR ligand specificity of the HLA-C antigen was determined according to the amino acid residues of the HLA-C allele. C1 ligand specificity consists of Asn 80 (Cw1, w3, w7, w8, and others); C2 specificity consists of Lys 80 (Cw2, w4, w5, w6, and others). In the cohort (patients and donors, n = 2978), the numbers of C1C1, C1C2, and C2C2 were 2555 (85.8%), 399 (13.4%), and 24 (0.81%), respectively. HLA-C mismatched pairs (n = 476) were divided into KIR ligand mismatch in the GVH direction (KIR-L-MM-G) (n = 81) and KIR ligand match in the GVH direction (KIR-L-M) (n = 395). KIR-L-MM-G was defined as the donor's KIR ligand for HLA-C not being shared by the patient's ligand. KIR-L-M included ligand match and ligand mismatch in the host-versus-graft (HVG) rejection direction. The combinations of KIR ligands in KIR-L-MM-G were as follows: C1C1 (patient)–C1C2 (donor), 78 (96.2%); C2C2–C1C2, 1 (1.2%); C1C1–C2C2, 2 (2.5%); and C2C2–C1C1, 0.

KIR Genotyping and Profile Analysis

KIR genotyping was performed using genomic DNA from patient and donor, and the presence of the 16 *KIR* genes (*2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, 2DP1,* and *3DP1*) was determined by the polymerase chain reaction sequence-specific primer (PCR-SSP) method [31] with minor modifications [32]. Pairs of all of KIR-L-MM-G (n = 81) cases and also KIR-L-M from HLA-C mismatch cases were selected, and HLA-C–matched cases were randomly selected as controls for the comparison. From the 260 pairs analyzed, all 16 *KIR* types of both patients and donors were successfully obtained in only 187 pairs, because of either insufficient quantity or quality of DNA. These data were used for evaluating *KIR* gene frequency and performing statistical analyses (Table 1). For the KIR-L-MM-G donor *2DS2* analysis, 46 cases, in which donor *2DS2* status was obtained, were added (for a total of 233 cases). KIR haplotype A is defined as carrying a single activating *KIR* gene, *2DS4*; KIR haplotype B has additional activating *KIR* genes [33].

Definition of Transplantation-Related Events

The occurrence of acute GVHD (aGVHD) was evaluated according to grading criteria in patients who survived for more than 8 days after transplantation, as described previously [30].

Statistical Analysis

Statistical analysis was performed as described previously [5]. All analyses were conducted using STATA version 8.2 (STATA Corp, College Station, TX). Overall survival (OS) rate was assessed using the Kaplan-Meier product limit method. Cumulative incidence of aGVHD and leukemia relapse were assessed as described previously [5] to eliminate the effects of competing risks. The competing events regarding aGVHD and relapse were defined as death without aGVHD and death in remission (treatment related mortality), respectively. For each endpoint, a log-rank test was applied to assess the impact of the factor of interest. Multivariate analysis by Cox proportional hazard models was applied to assess the impact of KIR ligand compatibility, donor *KIR* genotype, and ATG administration along with potential confounders. Confounders considered were HLA-A, -B, -DR, -DQ, and -DP matching (GVH direction), sex (donor–patient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard and high, leukemia only analyzed), number of cells transplanted (linear), and preconditioning (TBI vs non-TBI). The number of nucleated cells before the manipulation of bone marrow was replaced with the number of cells transplanted. P values $< .05$ were considered statistically significant. Adjustment of P values for multiple comparison was done because of an a priori hypothesis that activating KIR would interact with the cognate ligand and transduce a stimulatory signal only when the inhibitory signal was inactive.

RESULTS

Adverse Effects of KIR Ligand Incompatibility

We first confirmed the effects of KIR-L-MM in the newly selected cohort in this study (Table 1). The cumulative incidence of aGVHD and OS are shown in Figure 1. KIR-L-MM-G showed a significantly higher incidence of grade III-IV aGVHD (41.1%; 95% confidence interval [CI] = 29.5%-51.9%) compared with KIR-L-M in HLA-C–mismatched patients (29.7%; 95% CI = 25.2%-34.3%; P = .032). A similar trend was seen in grade II-IV aGVHD (data not shown). In addition, in

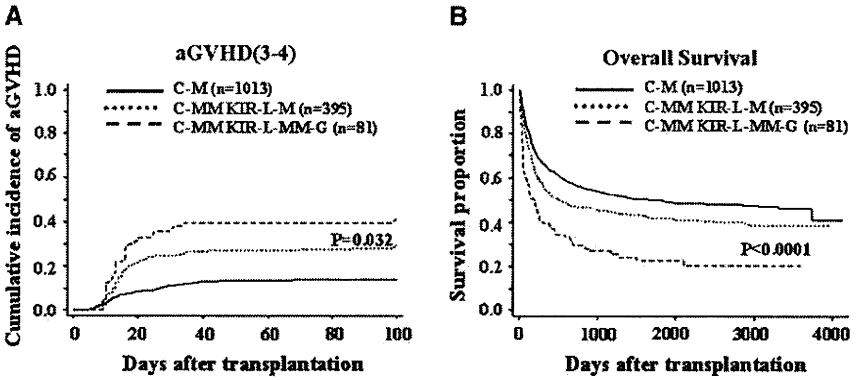

Figure 1. Effects of KIR ligand mismatch on transplantation outcome. Cumulative incidence of acute GVHD (grade III-IV) (A) and overall survival (B) by matching of KIR ligands in the GVHD direction. The directions of HLA-C mismatching were GVH and/or HVG. All patients were analyzed. The solid line represents HLA-C match (CM), the thin dotted line represents HLA-C mismatch KIR ligand match in the GVHD direction (C-MM KIR-L-M), and the thick dotted line represents HLA-C mismatch KIR ligand mismatch in the GVH direction (C-MM KIR-L-MM-G). The log-rank test was applied between CMM KIR-L-MM-G and CMM KIR-L-M.

Table 2. *Multivariate analysis of the effects of KIR ligand matching, donor KIR genotype, and ATG preadministration*

			aGVHD 3-4		aGVHD 2-4		Relapse		OS	
Group	Confounders	Subject number	HR (95%CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value
A	HLA-C (HCX) matched	1013	1.00(reference)		1.00(reference)		1.00(reference)		1.00(reference)	
	HLA-C-MM (HCX) and KIR-L-MM-G	81	3.08(2.05-4.62)	<.001	1.76(1.28-2.43)	.001	1.27(0.73-2.22)	.403	1.93(1.47-2.53)	<.001
	HLA-C-MM (HCX) and KIR-L-M	395	2.00(1.54-2.61)	<.001	1.47(1.23-1.77)	<.001	0.58(0.41-0.81)	.001	1.17(0.99-1.37)	.065
	ATG (yes vs no)	94 vs 1395	0.56(0.31-0.99)	.047	0.63(0.43-0.93)	.019	1.01(0.60-1.71)	.957	1.23(0.92-1.65)	.158
B	KIR-L (MM-G vs M)	80 vs 70	1.36(0.76-2.44)	.304	1.32(0.81-2.15)	.258	2.16(0.89-5.24)	.087	1.60(1.05-2.44)	.027
	Donor KIR genotype (2DS2 + vs −)	28 vs 122	1.96(1.01-3.80)	.045	1.62(0.92-2.85)	.095	0.78(0.24-2.47)	.666	1.04(0.62-1.74)	.889

Adjusted for HLA-A, -B, -DR –DQ, DP(GVH direction), age, donor age, donor-recipient sex pattern, disease, TBI, and risk.
Group A: all pairs, n=1489; group B: HLA-C-mismatched and donor *2DS2*-typed n=150.

HLA-C–mismatched patients, KIR-L-MM-G had a lower 5-year OS rate (23.2%; 95% CI = 14.6%-32.9%) than KIR-L-M (41.8%; 95% CI = 36.9%-46.7%; $P < .0001$). Multivariate analysis (Table 2, group A [n = 1489]) also demonstrated the strong adverse effects of KIR-L-MM-G in HLA-C mismatch on aGVHD (grade III-IV GVHD: hazard rate [HR] = 3.08, $P < .001$; grade II-IV GVHD: HR = 1.76, $P = .001$) and on OS (HR = 1.93; $P < .001$), but not on relapse (HR = 1.27; $P = .40$). Allele mismatches of *HLA-A*, *-B*, *-DR*, *-DQ*, and *-DP* loci of the patient and donor were considered confounders in the analysis; consequently, the observed KIR-L-MM-G effects in HLA-C mismatch were adjusted for other HLA disparities. These adverse effects of KIR-L-MM-G on aGVHD and OS were consistent with those found in our previous study [5]. Consequently, we further analyzed the factors responsible for the effects of KIR-L-MM-G on transplantation outcome using this cohort.

KIR Genotypes and Profiles of Patients and Donors

The selected patients and donors were analyzed using the PCR-SSP method for genotyping 16 different *KIR* genes. Data for 187 pairs were obtained, including 55 cases of KIR-L-MM-G and 62 cases of KIR-L-M in HLA-C mismatch and 70 cases of HLA-C match (Table 1). Table 3 shows the frequency of each *KIR* gene and the KIR profiles of patients and donors, demonstrating no significant differences between the patients and donors. The frequency of each *KIR* was similar to that of the healthy Japanese population [32-34]. Nearly half of the patients had only haplotype A.

Donor *KIR2DS2* Exacerbated aGVHD in KIR-L-MM-G

To statistically evaluate the possible involvement of *KIR* genotype in the adverse effects of KIR-L-MM-G, we investigated the particular combinatory

Table 3. *KIR genotype analysis of patient and donor of the cohort (n = 374)*

Haplotype	Profile	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DL2	3DL3	3DS1	2DP1	3DP1	Patient Number	Patient Frequency	Donor Number	Donor Frequency	KIR number** Inhibitory	KIR number** Activating	KIR number** Total
A	#1	+	−	+	+	−	−	−	−	+	−	+	+	+	−	+	+	95	0.51	92	0.49	6	1	7
B	#2	+	−	+	+	−	+	−	−	+	+	+	+	+	+	+	+	24	0.13	35	0.19	7	4	11
B	#3	+	+	+	+	−	−	+	−	+	+	+	+	+	−	+	+	13	0.07	9	0.05	7	2	9
B	#4	+	−	+	+	+	−	+	−	+	−	+	+	+	+	+	+	12	0.06	4	0.02	7	4	11
B	#5	+	−	+	+	+	−	−	−	+	−	+	+	+	+	+	+	8	0.04	8	0.04	6	3	9
B	#6	+	−	+	+	−	−	−	−	−	+	+	+	+	−	+	+	6	0.03	5	0.03	6	0	6
B	#7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	6	0.03	5	0.03	8	6	14
B	#8	+	−	+	+	+	+	−	−	+	+	+	+	+	+	+	+	4	0.02	3	0.02	7	3	10
B	#9	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	4	0.02	3	0.02	8	5	13
B	#10	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	2	0.01	2	0.01	7	5	12
	Others*																	13	0.07	21	0.11			
Frequency	Patient	0.99	0.16	1.00	1.00	0.38	0.37	0.17	0.17	0.88	0.25	0.94	1.00	1.00	0.35	1.00	1.00							
	Donor	0.99	0.14	0.98	1.00	0.41	0.39	0.16	0.14	0.87	0.32	0.93	1.00	0.99	0.37	0.99	1.00							
	Total	0.99	0.15	0.99	1.00	0.39	0.38	0.16	0.15	0.87	0.28	0.93	1.00	1.00	0.36	0.99	1.00							

*Combined profiles < 1% frequency; ** Not include pseudo-gene.

effects of donor *KIR* genotype and patient cognate KIR ligand type (receptor-ligand analysis). For the inhibitory KIR, we attempted to examine the combination of a particular ligand in the patient and absence of the cognate inhibitory *KIR* gene in the donor. But with regard to HLA-C-KIR ligand specificity, almost all individuals were positive for both C1 and C2 inhibitory KIRs (*2DL2* and/or *2DL3*, and *2DL1*, respectively; Table 3); therefore, there was no mismatch between patient KIR ligand and donor inhibitory *KIR* genotype combination or vice versa. In contrast, activating *KIR* genotypes were quite variable among individuals, and mismatch (ie, reactive) combinations of activating KIR with its presumed ligand (*2DS1* with C2 and *2DS2* with C1, respectively) were present.

For activating KIR, the combination of a particular ligand in the patient and presence of the cognate-activating KIR but absence of the corresponding inhibitory KIR in the donor was selected and analyzed. This choice was based on dominance of the inhibitory signal over the cognate-activating signal [35,36]. The corresponding activating donor *KIR* genotypes to patients C1C1 and C2C2 in KIR-L-MM-G were *2DS2* and *2DS1*, respectively. However, the frequency of C2C2 in the JMDP cases was too low (only 1 case in this study) to permit statistical evaluation. As shown in Figure 2, donor *2DS2*-positive cases in KIR-L-MM-G had a significantly higher incidence of aGVHD (grade III-IV GVHD, 70.9% [95% CI = 40.0%-87.9%]; grade II-IV GVHD, 78.6% [95% CI = 47.2%-92.5%]) compared with the donor *2DS2*-negative cases (grade III-IV GVHD, 33.6% [95% CI = 22.0%-45.7%]; grade II-IV GVHD, 54.4% [95% CI = 40.8%-66.1%]; $P = .012$ and .029, respectively). This was not true for KIR-L-M cases, however. These results suggest that the adverse effects of KIR-L-MM-G depend on combinations of the donor-activating *KIR* genotype and cognate patient ligand C1.

To explore the possibility of the neighboring activating *KIR* loci being the primary factor in outcomes because of possible linkage disequilibrium, we next investigated the associations between other *KIR* genotypes and transplantation outcomes. No other activating *KIR*, but inhibitory *2DL2* (located adjacent to and tightly linked with *2DS2*) showed a significant association with the incidence of aGVHD (data not shown). No significant associations between donor *2DS2* with relapse or OS in KIR-L-MM-G could be observed (Figures 2C and D, respectively).

Multivariate analysis (Table 2; group B [n = 150]) demonstrated that the donor *2DS2* was a possible risk factor for grade III-IV aGVHD in HLA-C–mismatched cases (HR = 1.96; $P = .045$). The same trend was observed for grade II-IV GVHD (HR = 1.62; $P = .095$). We also tested the currently proposed model for the *KIR* genotype effects on HSCT outcomes (donor *KIR* gene numbers [10,16,18,26,37], comparison of

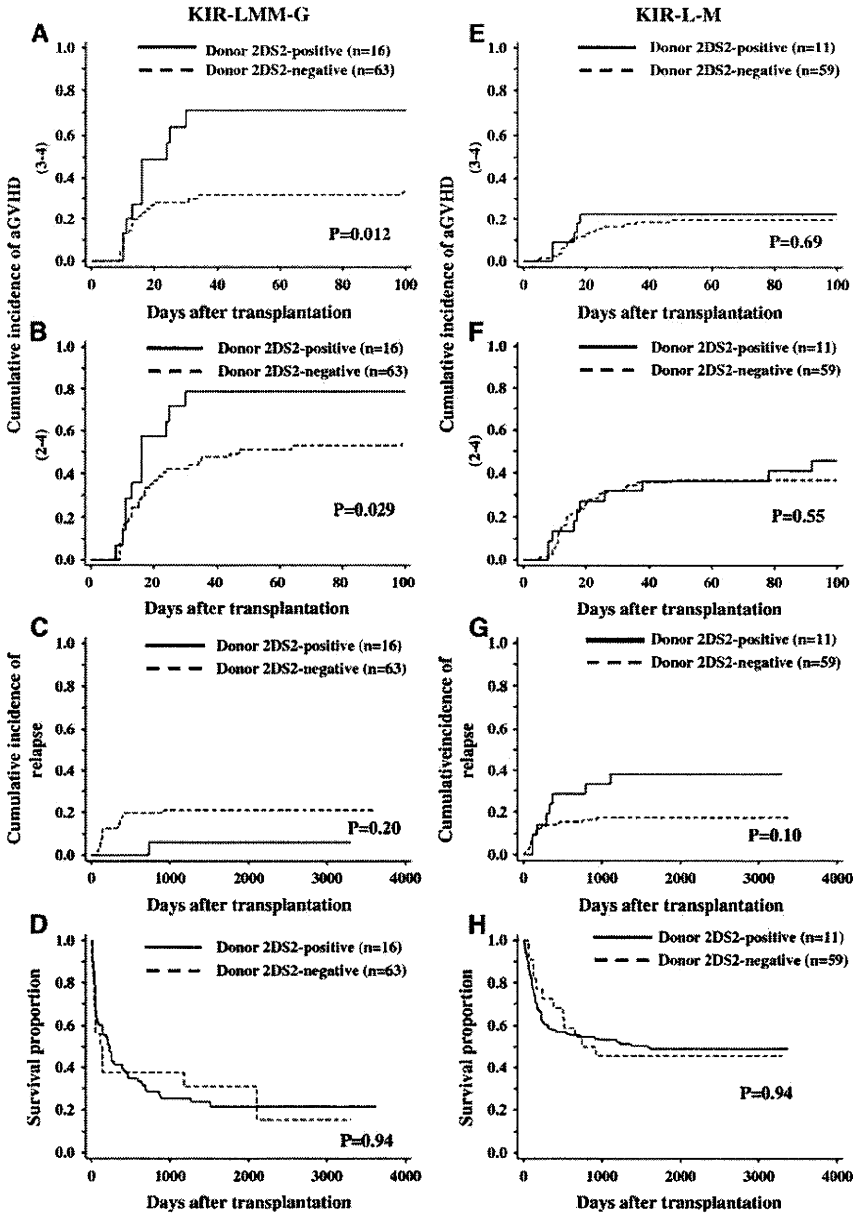

Figure 2. Effects of donor *KIR2DS2* in KIR ligand mismatch on transplantation outcome: Cumulative incidence of aGVHD, relapse, and overall survival with presence or absence of donor *KIR2DS2* gene in HLA-C–mismatched patients. Grade III-IV GVHD (A and E), grade 2-4 GVHD (B and F), relapse (C and G), and overall survival (D and H) with KIR-L-MM-G (A-D) or KIR-L-M (E-H) cases were analyzed. The solid line represents donor *KIR2DS2*-positive; the dotted line, donor *KIR2DS2*-negative.

KIR genotype and profile between patient and donor [receptor–receptor analysis] [17,19,25], compatibility score [24], ligand homozygosity in patients [21,27,38,39], and "missing ligand" effect [3,11,13,40,41]), and found no significant associations in this cohort (data not shown).

ATG Preadministration Ameliorates the Adverse Effects of KIR-L-MM-G on aGVHD and OS

In our previous study [5], the incidence of aGVHD was high in KIR-L-MM-G, where all cases did not involve ATG administration in the conditioning regimen, which is common in the JMDP cases. In the present study, we included rare ATG-administered cases (n = 94) in the analysis and evaluated the effects of ATG administration on KIR-L-MM-G. We found no significant differences in most of the parameters between the ATG-administered and non–ATG-administered groups, except for patient average age (18 years vs 27 years). Multivariate analysis (Table 2; group A [n = 1489]) indicated that ATG administration was a risk-reducing factor for severe aGVHD (grade III-IV

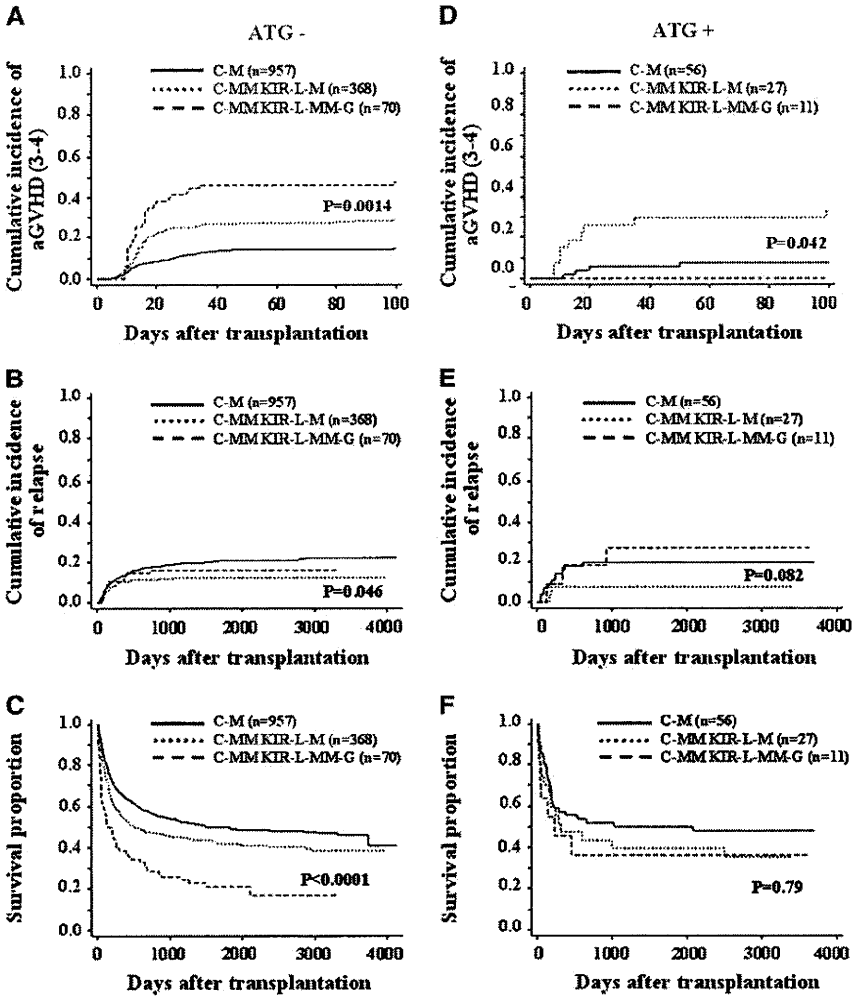

Figure 3. Effects of ATG preadministration in KIR ligand mismatch on transplantation outcome: Cumulative incidence of aGVHD, relapse, and overall survival of patients not receiving ATG (A-C) and those receiving ATG (D-F). The solid line represents HLA-C match (C-M), the thick dotted line represents HLA-C mismatch KIR ligand match in the GVHD direction (C-MM KIR-L-M), and the thin dotted line represents HLA-C mismatch KIR ligand mismatch in the GVH direction (C-MM KIR-L-MM-G). The log-rank test was applied between CMM KIR-L-MM-G and CMM KIR-L-M.

GVHD: HR = 0.56; P = .047; grade II-IV GVHD: HR = 0.63, P = .019), whereas no significant effects on relapse or OS could be seen.

The cumulative incidence of aGVHD was assessed separately in the non–ATG-administered and ATG-administered groups (Figures 3A and 3D, respectively). In the non–ATG-administered group, the incidence of grade III-IV GVHD was significantly higher in KIR-L-MM-G than in KIR-L-M (47.7% [95% CI = 35.2%-59.2%] vs 29.4% [95% CI=24.8%-34.1%]; P = .0014), as found in our previous study [5]. In contrast, no grade III-IV aGVHD was observed in KIR-L-MM-G cases in the ATG-administered group (2 cases of grade 2, 2 cases of grade 1, and 7 cases of grade 0), and the preventive effects of KIR-L-MM-G on severe aGVHD were significant (P = .042) although only a small number were analyzed (n = 38). We analyzed the effects of *2DS2* in the non–ATG-administered cases. In KIR-L-MM-G, the incidence of grade III-IV aGVHD was significantly higher in the donor *2DS2*-positive cases (n = 15) than in the donor *2DS2*-negative cases (n = 54) (76.4% [95% CI = 43.5-91.7%] vs 40.1% [95% CI = 26.5%-53.2%]; P = .048), suggesting that the adverse effects of donor *2DS2* are independent of ATG administration. In ATG-administered cases, no grade III-IV aGVHD was observed in donor *2DS2*-negative KIR-L-MM-G (n = 15); in 1 donor *2DS2*-positive KIR-L-MM-G case, the patient failed engraftment but showed no aGVHD, and died on day 35. Therefore, we could not statistically evaluate the effect of ATG on the *2DS2*-positive cases.

As shown in Figure 3B, in non–ATG-administered cases, the cumulative incidence of relapse was higher in KIR-L-MM-G than in KIR-L-M (16.1% [95% CI = 8.6%-25.8%] vs 11.9% [95% CI = 8.9%-15.3%];

P = .046), which was seen mainly in ALL (data not shown), as was found in our previous study [5]. In contrast, no significant increase in relapse was obtained in ATG-administered cases (P = .082) (Figure 3E). As in our previous study [5], in non–ATG-administered cases, overall survival rate was significantly lower in KIR-L-MM-G than in KIR-L-M (21.0% [95% CI = 12.2%-31.3%] vs 42.0% [95% CI =36.8%-47.0%]; P < .0001) (Figure 3C). On the other hand, in ATG-administered cases, no significant difference was observed between KIR-L-MM-G and KIR-L-M (36.4% [95% CI = 11.2%-62.7%] vs 39.5% [95% CI = 21.2%-57.3%]; P = .79) (Figure 3F), suggesting that ATG preadministration in the conditioning regimen abolished the adverse effect of KIR-L-MM-G on survival.

DISCUSSION

In the present study, we identified donor *KIR* genotype–patient KIR ligand combination and no ATG preadministration as critical factors for the adverse effects of KIR-L-MM-G on transplantation outcomes in the JMDP. The cases analyzed in this study were all HLA-A, -B, and -DR serologically matched; thus, we were able to evaluate the HLA-C ligand compatibility effects, because the HLA-Bw4 and HLA-A3 and -A11 KIR ligands were all matched. Other groups included mostly Bw4 ligand mismatch cases in KIR-L-MM-G analysis [2,6,7,10-12,14,21,42]. The Bw4 (patient) *-3DL1* (or *-3DS1*) (donor) combinatory effect also may affect transplantation outcome.

In the KIR-L-MM-G combination, the patient lacks the donor's KIR ligand. In this situation, donor NK cells may react with the patient cells according to the "missing self" model [43]. Previous KIR ligand compatibility data, together with the present data, confirm that the KIR-L-MM-G has potent adverse effects on UR-HSCT. In most KIR-L-MM-G cases in the JMDP, the donor and patient ligand types are C1C2 and C1C1, respectively, suggesting that C1C2 donor NK cells (and/or some T cells) respond to C1C1 patient cells. In this case, donor NK cells lack the inhibitory KIR for C1 (2DL2 and 2DL3) in terms of genotype or phenotype, or both. As shown in the present results, almost all JMDP donors examined posessed an inhibitory *KIR* gene for C1 (*2DL3*). The subpopulation of donor NK cells thus appears to lack cell surface expression of the C1-inhibitory KIR molecule, despite the presence of the genes. This is explained by the "at least one inhibitory receptor expression" model [44], in which each NK cell must express 1 inhibitory receptor for the self–major histocompatibility complex (MHC) class I to avoid autoreactivity, but expression of other receptors is "stochastic." Consequently, NK cell subpopulations lacking the C1-inhibitory *KIR* (*2DL2* and *2DL3*) but having the C2-inhibitory *KIR* (*2DL1*) would react with C1C1 (C2-lacking) patient cells. Therefore, the donor inhibitory KIR repertoire at the expression level, not at the genomic level, appears to influence outcome in the JMDP. The importance of the inhibitory KIR expression repertoire and functional analysis of donor NK cells has been discussed previously [3,45].

With a lack of inhibitory KIR signals, NK cells respond to target cells through activation signals from activating receptors. 2DS1 and 2DS2 are assigned to recognize C2 and C1, respectively, but other activating KIR ligand specificities (2DS3-5 and 3DS1) are unidentified [35]. Therefore, we were able to evaluate only these 2 KIRs for combinatory effects with their ligands. As described in Results, a higher incidence of severe aGVHD was observed in the *2DS2*-positive donors in the KIR-L-MM-G cases, but not in the KIR-L-M cases. This suggests that *2DS2*-positive lymphocytes (NK cells and/or some T cells) react with cognate ligand (C1)-positive cells and exacerbate aGVHD. Recently, La Nasa et al.[27] reported that the patient KIR ligand homozygosity, but not donor KIR genotype, is predictive for the outcome of HLA-matched UR-HCT in patients with beta-thalassemia. Their cases were all KIR ligand-matched transplantation and the donor-activating *KIR*–patient cognate ligand combination had no significant effect on the outcomes. Their results are consistent with our findings indicating that the donor *2DS2*–patient C1 combination of ligand-matched pairs has no effect on any outcomes (Figure 2E-H). This is in accordance with the notion that an activating *KIR* works only when the patient has the cognate ligand and that the donor inhibitory KIR does not function (Fig. 4). Chewning et al [36] reported that *KIR 2DS1*-positive NK cells recognized C2-expressing target cells and showed alloreactivity in vitro supporting the concept of this model.

Although adverse impacts of donor *2DS2* on transplantation outcome have been documented previously [15,18,23], the present study is the first report on the adverse effects of the *2DS2*–cognate ligand C1 combination on aGVHD incidence. Because we had an a priori hypothesis, we did not apply adjustment of P-value in our analysis; however, our results must be interpreted with caution. KIR-L-MM-G is infrequent in the JMDP (only 81 of 1489 cases in the present study), and the frequency of *2DS2* is low in Japan [32,33], and confirmation in other independent cohorts from different populations will support our findings. Combinatory effects of *2DS2* and cognate ligand C1 also have been reported in disease susceptibility studies, including studies of type I diabetes mellitus [46], ulcerative colitis [47], rheumatoid vasculitis [48], and tuberculosis [49]. Furthermore, extensive genetic analysis of *KIR* and HLA genotypes of various ethnic populations have demonstrated a strong negative correlation of activating *KIR* and its putative ligand

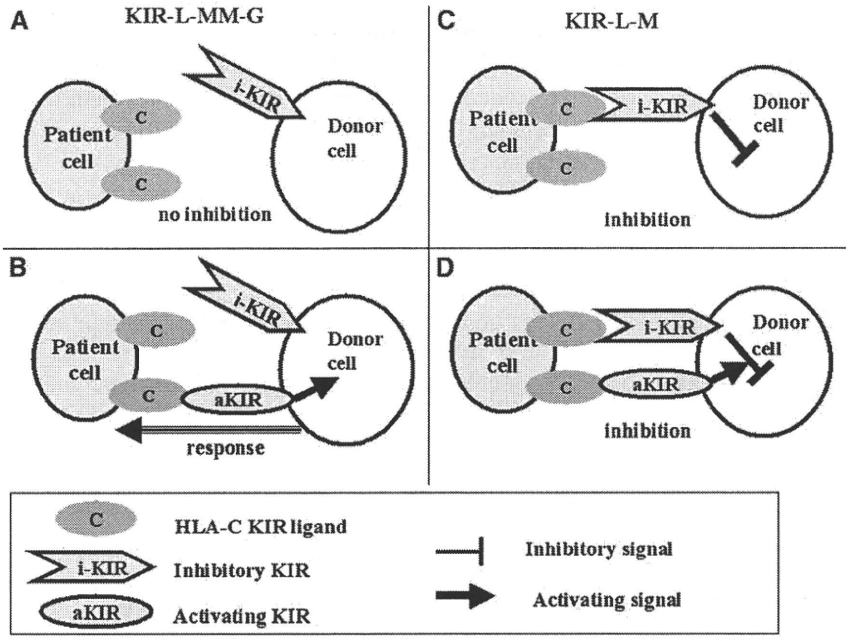

Figure 4. Model of interaction between activating KIR and cognate KIR ligand. Donor-activating KIR transduces an activating signal on recognition of the cognate KIR ligand of the patient cell in KIR-L-MM-G case (B). The activating signal is canceled by an inhibitory signal from inhibitory KIR, which recognizes the KIR ligand of the patient cell in KIR-L-M case (D).

combination including *2DS2*–C1, suggesting coevolution of the activating receptor–ligand loci [50]. Taken together with our data, these clinical and population genetic studies suggest a direct receptor–ligand interaction between 2DS2 and C1; however, binding studies using soluble 2DS2 molecules have shown no or a very weak binding to C1 molecules or C1-transfected cells, challenging the notion of C1 as a 2DS2 ligand [51-53]. Recombinant 2DS1 also showed very low or no affinity to C2 [54]. This disparity may be linked to differences in the nature of ligand binding between inhibiting and activating receptors. One possible factor is class I-binding peptides. The peptide-dependent binding with class I-binding receptors is recognized in most of the inhibitory receptors [53,55-58] and also has been suggested in activating KIR [53,58,59]. The peptide repertoire that allows strong KIR binding might be more restricted in activating KIR cases than in inhibitory ones. Alternatively, activating KIR–ligand binding may be somehow strengthened under stress conditions, such as transplantation or viral infection. Epstein Barr virus–transformed C1-positive cells were found to be stained slightly by recombinant 2DS2 tetramers [53]. A mutation study found that only 1 amino acid substitution in 2DS2 increased its level of binding to C1 to that of inhibitory 2DL2, suggesting that a very fine conformational microstructure change controls KIR binding specificity [60].

Inhibitory *2DL2* also showed a significant association with the incidence of severe aGVHD. This may be secondary to the *2DS2*–C1 association [50]; alternatively, donor 2DL2-positive NK cells might have a different effect than 2DL3-positive NK cells on acute GVHD incidence, because the binding affinity to C1 is higher in 2DL2 than in 2DL3 [60]. Other groups have analyzed activating *KIR* gene number and outcome and have reported both beneficial and adverse associations [10,16,26,37]. We did not find such quantitative *KIR* loci effects in this JMDP cohort (data not shown); *KIR* genotype variation among various ethnic groups may be responsible for these differences.

Preadministration of ATG to a patient is also a critical factor in attenuating the adverse effects of KIR-L-MM-G on transplantation outcome. Our findings demonstrate that KIR-L-MM-G had potent adverse effects (higher aGVHD incidence and lower OS) without ATG administration, and that ATG administration in the conditioning regimen ameliorated most of these adverse effects. Although the average patient age in the ATG-administered group was about 10 years younger than that in the non–ATG-administered group in this study, multivariate analysis including age as a confounder also identified the ATG effect as an independent factor for incidence of aGVHD (see Table 2). To the best of our knowledge, this is the first direct comparison UR-HSCT study on the effects of ATG preadministration under the same transplantation regimen with similar genetic backgrounds. Because far fewer ATG-administrated cases than

non–ATG-administered cases are included in the JMDP (an imbalance that could bias statistical results), further evaluation of large numbers of ATG-preadministered cases in different ethnic populations are needed.

Administration of ATG extensively depletes patient and donor T cells, thus strongly inhibiting the responses of alloreactive T cells. Because the JMDP cases are all unmanipulated T cell–replete marrow, donor alloreactive T cell response may be very strong, which would obscure some of the NK cell beneficial effects [61]. In KIR-L-MM-G without ATG preadministration, alloreactive NK cells were activated by 2DS2–C1 interaction without inhibitory KIR signals and may have augmented alloreactive donor T cell responses, resulting in increased aGVHD incidence and mortality. Alternatively, KIR-positive T cells may have been responsible for inducing aGVHD. In contrast, with ATG preadministration, donor T cells are largely depleted, and the beneficial effects of NK cell alloreactivity on aGVHD incidence may become prominent. Too few ATG-treated cases were analyzed (n = 11) to allow confirmation of the preventive effects of KIR-L-MM-G on acute GVHD, but the results are consistent with those for the HLA haplo-mismatched, ATG-preadministered R-HSCT [2]. In mouse GVHD models, alloreactive NK cells prevented donor alloreactive T cell stimulation and suppressed aGHVD by lysing donor antigen-presenting cells [2]. These mechanisms might explain the preventive effects of KIR-L-MM-G on the incidence of aGVHD. NK cell reconstitution after transplantation might be influenced by ATG treatment as well as by KIR ligand and KIR genotype variability [39,62,63]. Our data suggest that the KIR-L-MM-G combination must be avoided in JMDP transplantation unless ATG is used in the conditioning regimen.

Another possible factor is mismatch combination dissimilarity resulting from genetic variability in *HLA* and *KIR* in populations with different ethnic backgrounds. There are allele frequency differences in *HLA-C* among human populations in terms of the *HLA-C* KIR ligand [50]. Because the C1 ligand type is dominant in the Japanese population (allele frequency 0.92), KIR-L-MM-G is relatively rare (5%) compared with the incidence in White populations. Furthermore, in the KIR-L-MM-G, the C1C1 (patient)–C1C2 (donor) combination is common (95%) [5]. Therefore, we could focus on the KIR ligand incompatibility and the *2DS2* effects on the C1-homozygous patients in this study. In contrast, the White population more frequently exhibits the C2 type [50]. Consequently, the KIR-L-MM-G frequency is higher in Caucasian than Japanese and might include C2C2 (patient)–C1C2 (donor), C2C2–C1C1, and C1C1–C2C2 combinations, in addition to the C1C1–C1C2 combination. Therefore, not only the C1C1 (patient)–2DS2 (donor) combination, but also the C2C2 (patient)–2DS1 (donor) combination, might contribute considerably to the effects of KIR-L-MM-G in White [36]. The inhibitory capacity of C1 is reportedly weaker than that of C2 [64], and the binding strength of inhibitory KIR to the ligand HLA-C is different as well (2DL1 > 2DL2 > 2DL3) [60]. There may be more variability in inhibitory pathways in White populations; indeed, several groups have reported that the transplantation outcomes vary between C1-homozyous and C2-homozygous patients [15,19,38,39]. *KIR* genotype also shows ethnic variability [33,50]; Japanese have a markedly high frequency of the A haplotype and a very low frequency of *2DS2* (16% in the JMDP, compared with a frequency of > 40% in most Caucasian and African populations). One potential factor not examined in the present study is *KIR* allelic polymorphism. Yawata et al. [34] have shown that allelic polymorphism modulates the level and frequency of KIR3D expression, as well as its inhibitory capacity. These allelic differences might influence outcomes even though HLA-A- and B-KIR ligand specificities were the same in donors and recipients in the present study.

Here we found that the combination of donor-activating *KIR* genotype–patient cognate KIR ligand type and ATG administration in the conditioning regimen were critical factors in the adverse effects of KIR-L-MM-G on transplantation outcome. Alloreactivity of NK cells may be either beneficial or adverse depending on the above factors. However, other important parameters also may contribute to transplantation outcome. Further large-scale international collaborative studies, including a variety of ethnic populations and statistical comparisons under uniform regimens, are needed to gain further insight into the effects of NK cell alloreactivity on transplantation and to guide the development of cell therapy using alloreactive NK cells for leukemia and other diseases.

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REFERENCES

1. Ruggeri L, Aversa F, Martelli MF, et al. Allogeneic hematopoietic transplantation and natural killer cell recognition of missing self. *Immunol Rev.* 2006;214:202-218.
2. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science.* 2002;295:2097-2100.
3. Leung W, Iyengar R, Turner V, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol.* 2004;172:644-650.
4. Witt CS, Christiansen FT. The relevance of natural killer cell human leucocyte antigen epitopes and killer cell immunoglobulin-like receptors in bone marrow transplantation. *Vox Sang.* 2006;90:10-20.
5. Morishima Y, Yabe T, Matsuo K, et al. Effects of HLA allele and killer immunoglobulin-like receptor ligand matching on clinical outcome in leukemia patients undergoing transplantation with T-cell–replete marrow from an unrelated donor. *Biol Blood Marrow Transplant.* 2007;13:315-328.
6. Davies SM, Ruggieri L, DeFor T, et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. *Blood.* 2002;100:3825-3827.
7. Giebel S, Locatelli F, Lamparelli T, et al. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. *Blood.* 2003;102:814-819.
8. Bornhauser M, Schwerdtfeger R, Martin H, et al. Role of KIR ligand incompatibility in hematopoietic stem cell transplantation using unrelated donors. *Blood.* 2004;103:2860-2862.
9. Schaffer M, Malmberg KJ, Ringden O, et al. Increased infection-related mortality in KIR ligand–mismatched unrelated allogeneic hematopoietic stem-cell transplantation. *Transplantation.* 2004;78:1081-1085.
10. De Santis D, Bishara A, Witt CS, et al. Natural killer cell HLA-C epitopes and killer cell immunoglobulin-like receptors both influence outcome of mismatched unrelated donor bone marrow transplants. *Tissue Antigens.* 2005;65:519-528.
11. Hsu KC, Gooley T, Malkki M, et al. KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. *Biol Blood Marrow Transplant.* 2006;12:828-836.
12. Farag SS, Bacigalupo A, Eapen M, et al. The effect of KIR ligand incompatibility on the outcome of unrelated donor transplantation: a report from the Center for International Blood and Marrow Transplant Research, the European Blood and Marrow Transplant Registry, and the Dutch Registry. *Biol Blood Marrow Transplant.* 2006;12:876-884.
13. Sun JY, Dagis A, Gaidulis L, et al. Detrimental effect of natural killer cell alloreactivity in T cell–replete hematopoietic cell transplantation (HCT) for leukemia patients. *Biol Blood Marrow Transplant.* 2007;13:197-205.
14. Sivula J, Volin L, Porkka K, et al. Killer-cell immunoglobulin-like receptor ligand compatibility in the outcome of Finnish unrelated donor hematopoietic stem cell transplantation. *Transpl Immunol.* 2007;18:62-66.

15. Cook MA, Milligan DW, Fegan CD, et al. The impact of donor KIR and patient HLA-C genotypes on outcome following HLA-identical sibling hematopoietic stem cell transplantation for myeloid leukemia. *Blood.* 2004;103:1521-1526.
16. Cook M, Briggs D, Craddock C, et al. Donor KIR genotype has a major influence on the rate of cytomegalovirus reactivation following T-cell–replete stem cell transplantation. *Blood.* 2006;107: 1230-1232.
17. Chen C, Busson M, Rocha V, et al. Activating KIR genes are associated with CMV reactivation and survival after non–T-cell–depleted HLA-identical sibling bone marrow transplantation for malignant disorders. *Bone Marrow Transplant.* 2006; 38:437-444.
18. Verheyden S, Schots R, Duquet W, et al. A defined donor-activating natural killer cell receptor genotype protects against leukemic relapse after related HLA-identical hematopoietic stem cell transplantation. *Leukemia.* 2005;19:1446-1451.
19. McQueen KL, Dorighi KM, Guethlein LA, et al. Donor–recipient combinations of group A and B KIR haplotypes and HLA class I ligand affect the outcome of HLA-matched, sibling donor hematopoietic cell transplantation. *Hum Immunol.* 2007;68: 309-323.
20. Clausen J, Wolf D, Petzer AL, et al. Impact of natural killer cell dose and donor killer-cell immunoglobulin-like receptor (KIR) genotype on outcome following human leucocyte antigen-identical haematopoietic stem cell transplantation. *Clin Exp Immunol.* 2007;148:520-528.
21. Sobecks RM, Ball EJ, Maciejewski JP, et al. Survival of AML patients receiving HLA-matched sibling donor allogeneic bone marrow transplantation correlates with HLA-Cw ligand groups for killer immunoglobulin-like receptors. *Bone Marrow Transplant.* 2007;39:417-424.
22. Zhao XY, Huang XJ, Liu KY, et al. Prognosis after unmanipulated HLA-haploidentical blood and marrow transplantation is correlated to the numbers of KIR ligands in recipients. *Eur J Haematol.* 2007;78:338-346.
23. Giebel S, Nowak I, Wojnar J, et al. Impact of activating killer immunoglobulin-like receptor genotype on outcome of unrelated donor-hematopoietic cell transplantation. *Transplant Proc.* 2006;38:287-291.
24. Sun JY, Gaidulis L, Dagis A, et al. Killer Ig-like receptor (KIR) compatibility plays a role in the prevalence of acute GVHD in unrelated hematopoietic cell transplants for AML. *Bone Marrow Transplant.* 2005;36:525-530.
25. Gagne K, Brizard G, Gueglio B, et al. Relevance of KIR gene polymorphisms in bone marrow transplantation outcome. *Hum Immunol.* 2002;63:271-280.
26. Kroger N, Binder T, Zabelina T, et al. Low number of donor activating killer immunoglobulin-like receptors (KIR) genes but not KIR-ligand mismatch prevents relapse and improves disease-free survival in leukemia patients after in vivo T-cell–depleted unrelated stem cell transplantation. *Transplantation.* 2006;82:1024-1030.
27. La Nasa G, Littera R, Locatelli F, et al. 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. *Biol Blood Marrow Transplant.* 2007; in press.
28. Bacigalupo A. Antilymphocyte/thymocyte globulin for graft-versus-host disease prophylaxis: efficacy and side effects. *Bone Marrow Transplant.* 2005;35:225-231.
29. Sasazuki T, Juji T, Morishima Y, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. Japan Marrow Donor Program. *N Engl J Med.* 1998;339:1177-1185.
30. Morishima Y, Sasazuki T, Inoko H, et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A–, HLA-B–, and HLA-DR–matched unrelated donors. *Blood.* 2002;99:4200-4206.
31. Gomez-Lozano N, Vilches C. Genotyping of human killer-cell immunoglobulin-like receptor genes by polymerase chain reaction with sequence-specific primers: an update. *Tissue Antigens.* 2002;59:184-193.
32. Miyashita R, Tsuchiya N, Yabe T, et al. Association of killer cell immunoglobulin-like receptor genotypes with microscopic polyangiitis. *Arthritis Rheum.* 2006;54:992-997.
33. Yawata M, Yawata N, McQueen KL, et al. Predominance of group A KIR haplotypes in Japanese associated with diverse NK cell repertoires of KIR expression. *Immunogenetics.* 2002; 54:543-550.
34. Yawata M, Yawata N, Draghi M, et al. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. *J Exp Med.* 2006;203:633-645.
35. Moretta A, Sivori S, Ponte M, et al. Stimulatory receptors in NK and T cells. *Curr Top Microbiol Immunol.* 1998;230:15-23.
36. Chewning JH, Gudme CN, Hsu KC, et al. KIR2DS1-positive NK cells mediate alloresponse against the C2 HLA-KIR ligand group in vitro. *J Immunol.* 2007;179:854-868.
37. Bishara A, De Santis D, Witt CC, et al. The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. *Tissue Antigens.* 2004;63:204-211.
38. Giebel S, Locatelli F, Wojnar J, et al. Homozygosity for human leucocyte antigen-C ligands of KIR2DL1 is associated with increased risk of relapse after human leucocyte antigen C–matched unrelated donor haematopoietic stem cell transplantation. *Br J Haematol.* 2005;131:483-486.
39. Fischer JC, Ottinger H, Ferencik S, et al. Relevance of C1 and C2 epitopes for hemopoietic stem cell transplantation: role for sequential acquisition of HLA-C–specific inhibitory killer Ig-like receptor. *J Immunol.* 2007;178:3918-3923.
40. Hsu KC, Keever-Taylor CA, Wilton A, et al. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood.* 2005;105:4878-4884.
41. Miller JS, Cooley S, Parham P, et al. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood.* 2007;109:5058-5061.
42. Beelen DW, Ottinger HD, Ferencik S, et al. Genotypic inhibitory killer immunoglobulin-like receptor ligand incompatibility enhances the long-term antileukemic effect of unmodified allogeneic hematopoietic stem cell transplantation in patients with myeloid leukemias. *Blood.* 2005;105:2594-2600.
43. Ljunggren HG, Karre K. In search of the "missing self": MHC molecules and NK cell recognition. *Immunol Today.* 1990;11: 237-244.
44. Valiante NM, Uhrberg M, Shilling HG, et al. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity.* 1997;7:739-751.

45. Han M, Fallena M, Guo Y, et al. Natural killer cell cross-match: functional analysis of inhibitory killer immunoglobulin-like receptors and their HLA ligands. *Hum Immunol.* 2007;68:507-513.
46. van der Slik AR, Koeleman BP, Verduijn W, et al. KIR in type 1 diabetes: disparate distribution of activating and inhibitory natural killer cell receptors in patients versus HLA-matched control subjects. *Diabetes.* 2003;52:2639-2642.
47. Jones DC, Edgar RS, Ahmad T, et al. Killer Ig-like receptor (KIR) genotype and HLA ligand combinations in ulcerative colitis susceptibility. *Genes Immun.* 2006;7:576-582.
48. Yen JH, Moore BE, Nakajima T, et al. Major histocompatibility complex class I–recognizing receptors are disease risk genes in rheumatoid arthritis. *J Exp Med.* 2001;193:1159-1167.
49. Mendez A, Granda H, Meenagh A, et al. Study of KIR genes in tuberculosis patients. *Tissue Antigens.* 2006;68:386-389.
50. Single RM, Martin MP, Gao X, et al. Global diversity and evidence for coevolution of KIR and HLA. *Nat Genet.* 2007;39: 1114-1119.
51. Vales-Gomez M, Erskine RA, Deacon MP, et al. The role of zinc in the binding of killer cell Ig-like receptors to class I MHC proteins. *Proc Natl Acad Sci U S A.* 2001;98:1734-1739.
52. Saulquin X, Gastinel LN, Vivier E. Crystal structure of the human natural killer cell– activating receptor KIR2DS2 (CD158j). *J Exp Med.* 2003;197:933-938.
53. Stewart CA, Laugier-Anfossi F, Vely F, et al. Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. *Proc Natl Acad Sci U S A.* 2005;102: 13224-13229.
54. Biassoni R, Pessino A, Malaspina A, et al. Role of amino acid position 70 in the binding affinity of p50.1 and p58.1 receptors for HLA-Cw4 molecules. *Eur J Immunol.* 1997;27:3095-3099.
55. Maenaka K, Juji T, Nakayama T, et al. Killer cell immunoglobulin receptors and T cell receptors bind peptide major histocompatibility complex class I with distinct thermodynamic and kinetic properties. *J Biol Chem.* 1999;274:28329-28334.
56. Thananchai H, Gillespie G, Martin MP, et al. Cutting edge: allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. *J Immunol.* 2007;178:33-37.
57. Hansasuta P, Dong T, Thananchai H, et al. Recognition of HLA-A3 and HLA-A11 by *KIR3DL2* is peptide-specific. *Eur J Immunol.* 2004;34:1673-1679.
58. Vales-Gomez M, Reyburn HT, Erskine RA, et al. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. *EMBO J.* 1999;18:4250-4260.
59. Carr WH, Rosen DB, Arase H, et al. Cutting edge: KIR3DS1, a gene implicated in resistance to progression to AIDS, encodes a *DAP12*-associated receptor expressed on NK cells that triggers NK cell activation. *J Immunol.* 2007;178:647-651.
60. Winter CC, Gumperz JE, Parham P, et al. Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J Immunol.* 1998;161: 571-577.
61. Lowe EJ, Turner V, Handgretinger R, et al. T-cell alloreactivity dominates natural killer cell alloreactivity in minimally T-cell–depleted HLA-nonidentical paediatric bone marrow transplantation. *Br J Haematol.* 2003;123:323-326.
62. Savani BN, Mielke S, Adams S, et al. Rapid natural killer cell recovery determines outcome after T-cell–depleted HLA-identical stem cell transplantation in patients with myeloid leukemias but not with acute lymphoblastic leukemia. *Leukemia.* 2007;21: 2145-2152.
63. Zhao XY, Huang XJ, Liu KY, et al. Reconstitution of natural killer cell receptor repertoires after unmanipulated HLA-mismatched/haploidentical blood and marrow transplantation: analyses of CD94:NKG2A and killer immunoglobulin-like receptor expression and their associations with clinical outcome. *Biol Blood Marrow Transplant.* 2007;13:734-744.
64. Parham P. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol.* 2005;5:201-214.