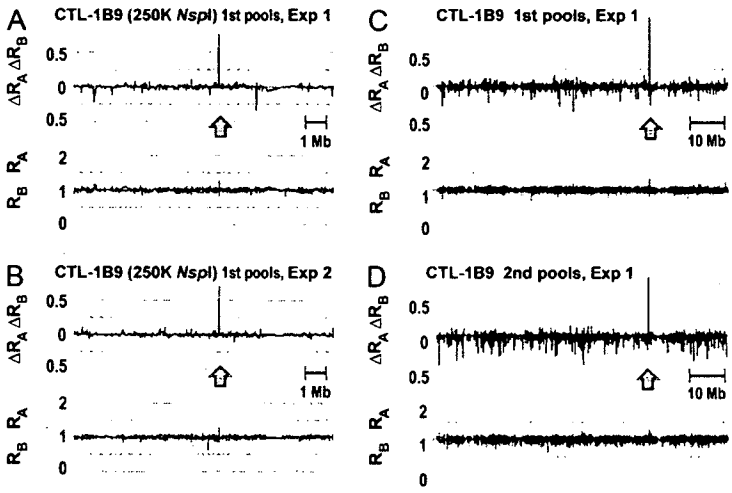

Figure 4. Reproducible detection of association with the immunophenotypes determined by CTL-1B9 at the *BCL2A1* locus. The maximum test statistic value was observed at a single SNP (rs1879894) within 15q25.1 in duplicate experiments for the first pools consisting of 57 CTX$^+$ and 38 CTX$^-$ B-LCLs (A-C). The peak association at the same SNP was reproduced in the experiments with the second pools consisting of 75 CTX$^+$ and 34 CTX$^-$ LCLs (D). Test statistic values ($\Delta R_A \Delta R_B$) are plotted by blue lines together with their R_A (red) and R_B (green) values. The expected $\Delta R_A \Delta R_B$ values multiplied by r^2 correlation coefficients for the adjacent SNPs within 500 kb from the SNP rs1879894 are overlaid by red lines (A,B).

application of WGASs to transplantation immunology, which provides a simple but robust method to fine-map the genetic loci of minor H antigens whose expression is readily determined by standard immunophenotyping with CTL clones established from patients who have undergone transplantation.

The current WGA/CTL method has several desirable features that should contribute to the acceleration of minor H locus mapping. In comparing the method to those of linkage analysis and other nongenetic approaches, including direct peptide sequencing of chemically purified minor H antigens[5,6,10,13] and conventional

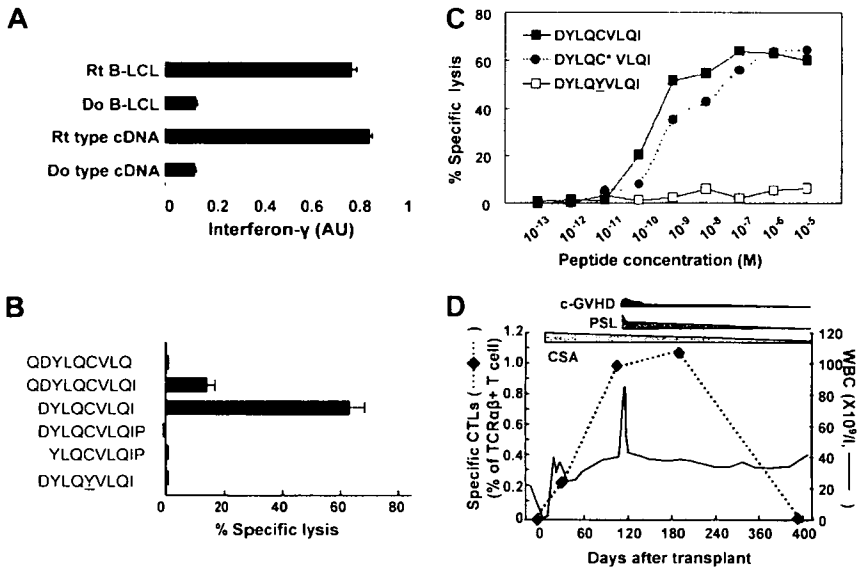

Figure 5. Identification of the CTL-1B9 minimal minor H epitope. (A) Interferon-γ production from CTL-1B9 against HLA-A*2402–transduced 293T cells transfected with plasmid encoding full-length *BCL2A1* cDNA cloned from either the recipient (Rt) from whom CTL-1B9 was isolated or his donor (Do). Rt B-LCL and Do B-LCL were used as positive and negative controls, respectively. Secreted interferon-γ was measured by ELISA and is expressed in arbitrary units (AUs) corresponding to optical density at 630 nm. Results are typical of 2 experiments and data are the mean plus or minus SD of triplicates. (B) A peptide reconstitution assay was conducted to determine the minimal epitope for CTL-1B9. Nonameric peptide (DYLQCVLQI), 2 nonameric peptides shifted by one amino acid to N- or C-terminus, N- and C-terminal extended decameric peptides, and its allelic counterpart (DYLQYVLQI) were synthesized and tested by adding to antigen-negative donor B-LCL at 10 nM in a standard ^{51}Cr release assay. Results are typical of 2 experiments and data are the mean plus or minus SD of triplicates. (C) Titration of the candidate minor H peptide by epitope reconstitution assay. Chromium-labeled donor B-LCLs were distributed to wells of 96-well round-bottomed plates, pulsed with serial dilutions of the indicated peptides for 30 minutes at room temperature, and then used as targets for CTL-1B9 in a standard ^{51}Cr release assay. A cysteinylated peptide (indicated by an asterisk) was included as an alternative form of the potential epitope. Results are typical of 2 experiments. (D) Tracking of ACC-1^C–specific T cells in the recipient's peripheral blood. In order to longitudinally analyze the kinetics of the ACC-1^C–specific CTLs in peripheral blood from the patient from whom CTL-1B9 was established, a real-time quantitative PCR was conducted. Complementary DNAs of peripheral blood mononuclear cells from the donor and patient before and after HSCT were prepared from the patient. Real-time PCR analysis was performed using a TaqMan assay as described previously.[9] The primers and fluorogenic probe sequences spanning the CTL-1B9 complementarity-determining region 3 (CDR3) were used to detect T cells carrying the CDR3 sequences identical to that of CTL-1B9. The primers and fluorogenic probe sequences spanning constant region of TCR beta chain (TCRBC) mRNA were used as internal control. Samples were quantified with the comparative CT method. The delta CT value was determined by subtracting the average CT value for TCRBC from the average CTL-1B9 CDR3 CT value. The standard curve for the proportion of CTL-1B9 among TCR$\alpha\beta^+$ T cells was composed by plotting mean delta CT values for each ratio, and the percentages of T cells carrying the CDR3 sequence identical to CTL-1B9 were calculated by using this standard curve. During this period, quiescent chronic GVHD, which required steroid treatment, developed; however, involvement of immune reaction to ACC-1^C minor H antigen was unlikely since its frequency increased even after resolution of most chronic GVHD symptoms. c-GVHD, chronic GVHD; CSA, cyclosporine A; PSL, prednisolone; WBC, white blood cell count.

Table 2. Correlation of *BCL2A1* sequence polymorphisms with susceptibility to CTL-1B9

	HLA-A*2402–positive B-LCLs								
	Rt	Do	UR1	UR2	UR3	UR4	UR5	UR6	UR7
Cytolysis by CTL-1B9	+	−	+	+	+	+	+	−	−
Detected SNP, position*									
rs1138357, 238	G/A	A	G	G	G/A	G/A	G/A	A	A
rs1138358, 299	T/G	G	T	T	T/G	T/G	T/G	G	G
rs3826007, 427	G	G/A	G	G	G	G	G/A	G/A	G

Rt indicates recipient; Do, donor; UR, unrelated; +, yes; and −, no.
*Nucleotide positions are shown according to the NM_004092.2 mRNA sequence, available at http://www.ncbi.nlm.nih.gov/ as GEO accession GSE10044.

expression cloning,[8,9,11] there are differences in terms of power, sensitivity, and specificity. Direct sequencing of minor H antigen peptide guarantees that the purified peptide is surely present on the cell surface as antigen, but it requires highly specialized equipment and personnel. Expression screening of cDNA libraries is also widely used and has become feasible with commercially available systems. However, it depends highly on the quality of the cDNA library and expression levels of the target genes. In addition, it often suffers from false-positive results due to the forced expression of cDNA clones under a strong promoter. The current method of WGA/CTL genetically determines the relevant minor H antigen locus, not relying on highly technical protein chemistry using specialized equipment, or repetitive cell cloning procedures. It is also not affected by the expression levels of the target antigens.

As a genetic approach, the current method based on genetic association has several advantages over conventional linkage analysis: the mapping resolution has been greatly improved from several Mb in the conventional linkage analysis to the average haplotype block size of less than 100 kb,[17,25-27] usually containing a handful of candidate genes, compared with the dozens as typically found in linkage analysis. This means that the effort needed for the subsequent epitope mapping will be substantially reduced. In fact, the 115 kb region identified for CTL-2A12 contains 4 genes compared with 38 genes as revealed by the previous linkage study (data not shown), and the candidate gene was uniquely identified within the 26 kb region for CTL-1B9, for which linkage analysis had failed due to very rare segregating pedigrees among the CEPH panels with this trait (now ACC-1[C]; data not shown).[15,16] In addition, before moving on to epitope mapping, it would be possible to evaluate the clinical relevance of the minor H antigens by examining the tissue distribution of their expression, based on widely available gene expression databases such as Genomic Institute of the Novartis Research Foundation (GNF, http://symatlas.gnf.org/SymAtlas/).[28]

Second, the required sample size is generally small, and should be typically no more than 100 B-LCLs for common minor H alleles. This is in marked contrast to the association studies for common diseases, in which frequently thousands of samples are required.[17,25-27] In the current approach, sufficiently high test statistic values could be obtained for the relevant loci with a relatively small sample size, since the minor H allele is correctly segregated between the CTX^+ and CTX^- pools by the highly specific immunologic assay. Combined with high accuracy in allelic measurements, this feature allows for the use of pooled DNAs in WGAS, which substantially saves cost and time, compared with the genotyping of individual samples. Unexpectedly, our method allows for a considerable degree of error in the immunophenotyping, indicating the robustness of the current method; in fact, the minor H locus for CTL-2A12 was successfully identified in spite of the presence of 8 (~10%) immunophenotyping errors. When the minor H allele has an extreme allele frequency (eg, $< 5\%$ or $> 95\%$), which could be predicted by preliminary immunophenotyping, WGAS/CTL may not be an efficient method of mapping, due to the impractically large numbers of B-LCLs that would need to be screened to obtain enough CTX^+ or CTX^- B-LCLs. However, such minor H antigens would likely have limited clinical impact or applicability.

Sensitivity of the microarray analysis seems to be very high when the target SNP has good proxy SNPs on the array, because we were able to correctly identify the single SNP correlated with the target of CTL-1B9 from more than 500 000 SNP markers. On the other hand, genome coverage of the microarray is definitely important. In our experiments on CTL-2A12, the association was successfully identified by the marker SNPs showing r^2 values of approximately 0.74 with the target locus of ACC-6. Since the GeneChip 500 K array set captures approximately 65% of all the HapMap phase II SNPs with more than 0.74 of r^2,[29] and higher coverage will be obtained with the SNP 6.0 arrays having more than 1 000 K SNP markers, these arrays can be satisfactorily used as platforms for the WGA/CTL method.

As shown in the current study, the intrinsic sensitivity and specificity of the WGA/CTL method in detecting associated SNPs were excellent. In other words, as long as target SNPs are captured in high r^2 values with one or more marker SNPs within the Affymetrix 500 K SNP set, there is a high likelihood of capturing the SNP with the current approach. To evaluate the probability of a given minor H antigen being captured in high r^2 with marker SNPs, we checked the maximum r^2 values of known minor H antigen SNPs with the Affymetrix 500 K SNPs, according to empirical data from the HapMap project (www.hapmap.org). Among 13 known minor H antigens, 7 have their entries (designated minor H SNP) in the HapMap phase II SNP set (HA-3,[30] HA-8,[31] HB-1,[11] ACC-1 and ACC-2,[7] LB-ADIR-1F,[10] and 7A7-PANE1[13]), and were used for this purpose (note that absence of their entries in the HapMap data set does not necessarily mean that they could not be captured by a particular marker SNP set). As shown in Table S4, all 7 minor H SNPs are captured by at least one flanking SNP that is included in the Affymetrix 500 K SNP set with r^2 values of more than 0.74 in at least one HapMap panel. The situation should be more favorable in the recently available SNP 6.0 array set with 1 000 K SNPs, indicating the genome coverage with currently available SNP arrays would be sufficient to capture typical minor H antigens with our approach.

Most patients who have received allo-HSCT could be a source of minor H antigen-specific CTL clones to be used for this assay, since the donor T cells are in vivo primed and many CTL clones could be established using currently available methods. In fact, substantial numbers of CTL clones have been established worldwide and could serve as the probes to identify novel minor H antigens.[32,33] Once constructed, a panel of B-LCLs, including those transduced with HLA cDNAs, could be commonly applied to immunophenotyping with different CTL clones, especially when

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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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

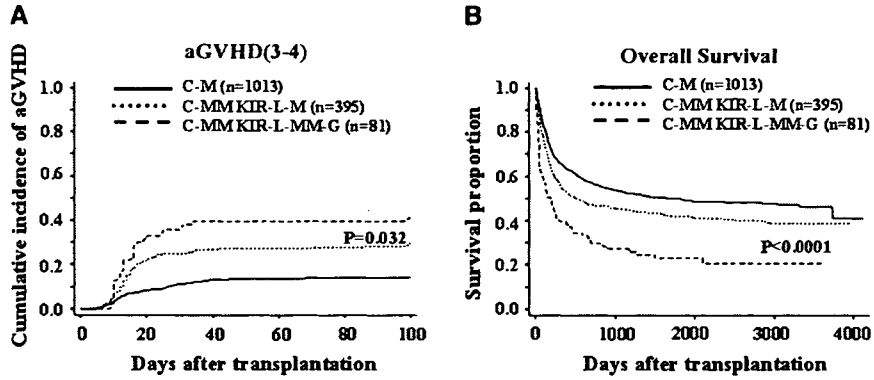

Figure 1. Effects of KIR ligand mismatch on transplantation outcome. Cumulative incidence of acute GVHD (grade III-IV) (A) and overall survival (B) by matching of KIR ligands in the GVHD direction. The directions of HLA-C mismatching were GVH and/or HVG. All patients were analyzed. The solid line represents 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			
	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							
Frequency	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

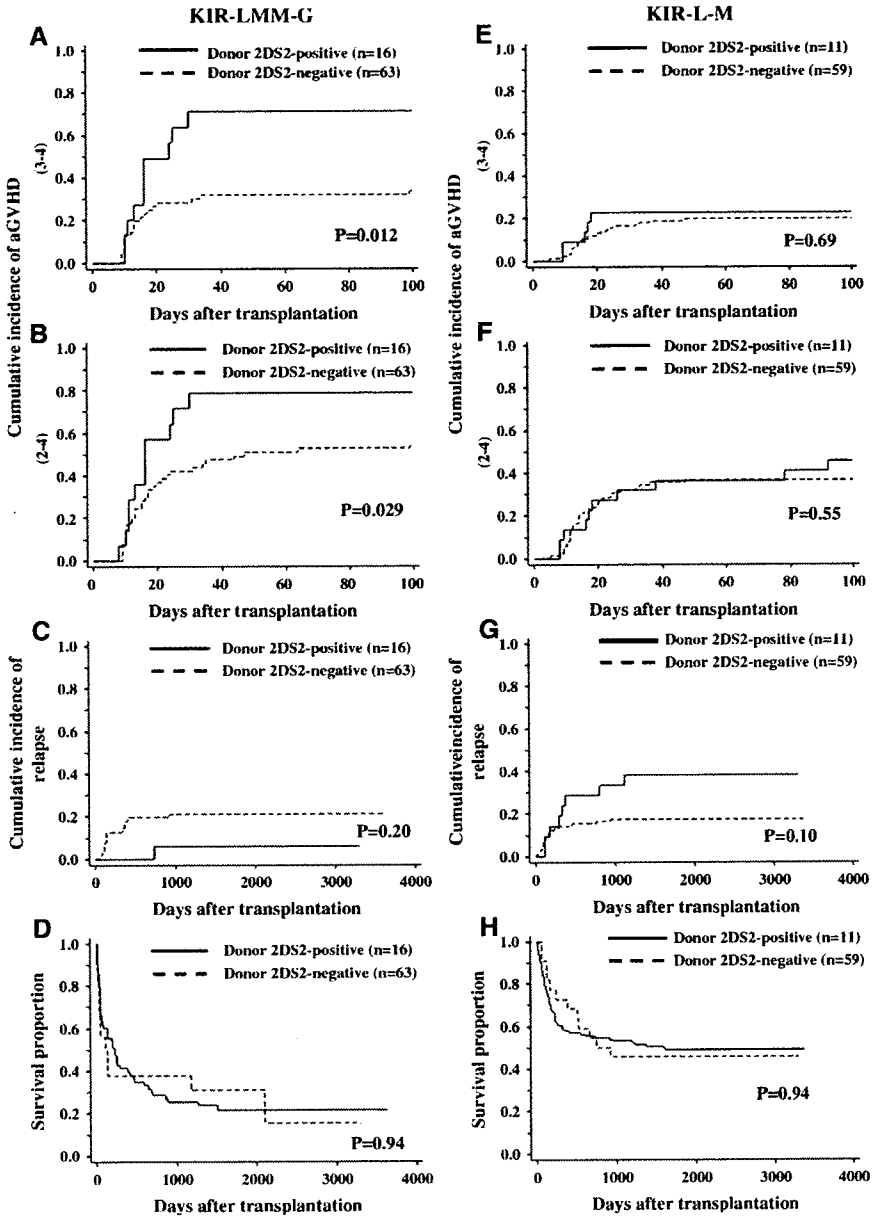

Figure 2. Effects of donor *KIR2DS2* in KIR ligand mismatch on transplantation outcome: Cumulative incidence of aGVHD, relapse, and overall survival with presence or absence of donor *KIR2DS2* gene in HLA-C–mismatched patients. Grade III-IV GVHD (A and E), grade 2-4 GVHD (B and F), relapse (C and G), 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

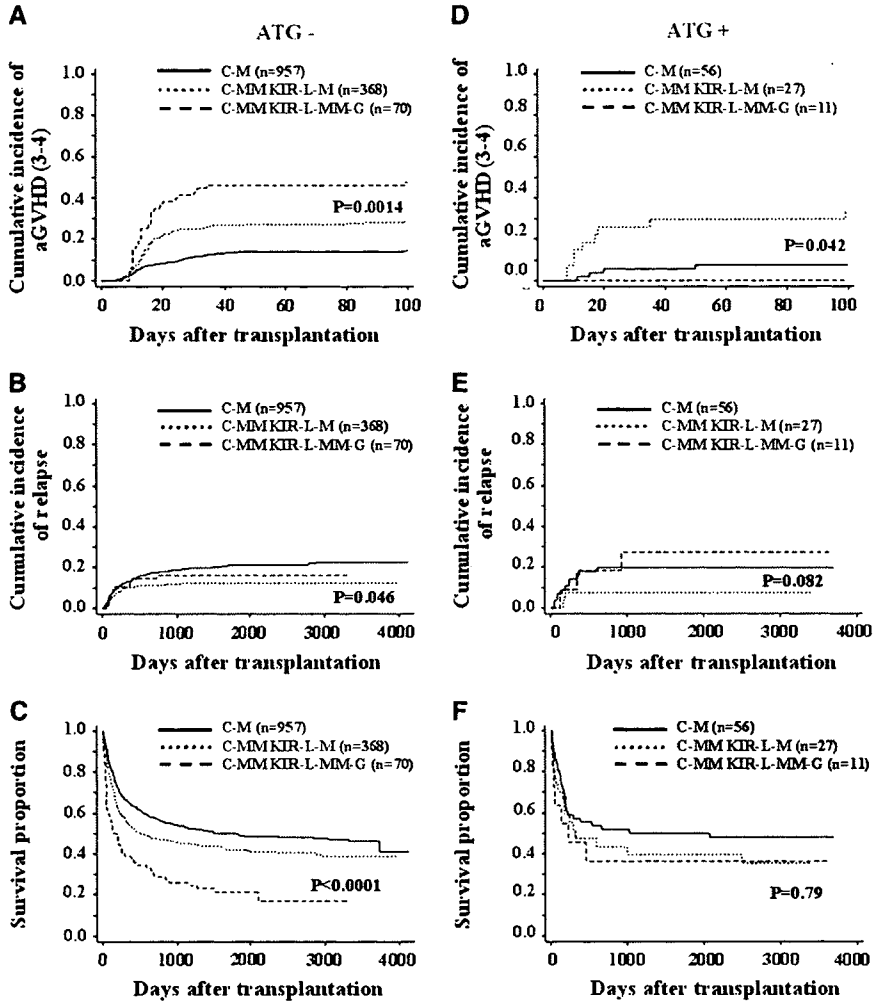

Figure 3. Effects of ATG preadministration in KIR ligand mismatch on transplantation outcome: Cumulative incidence of aGVHD, relapse, and overall survival of patients not receiving ATG (A-C) and those receiving ATG (D-F). The solid line represents HLA-C match (C-M), the thick dotted line represents HLA-C 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 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

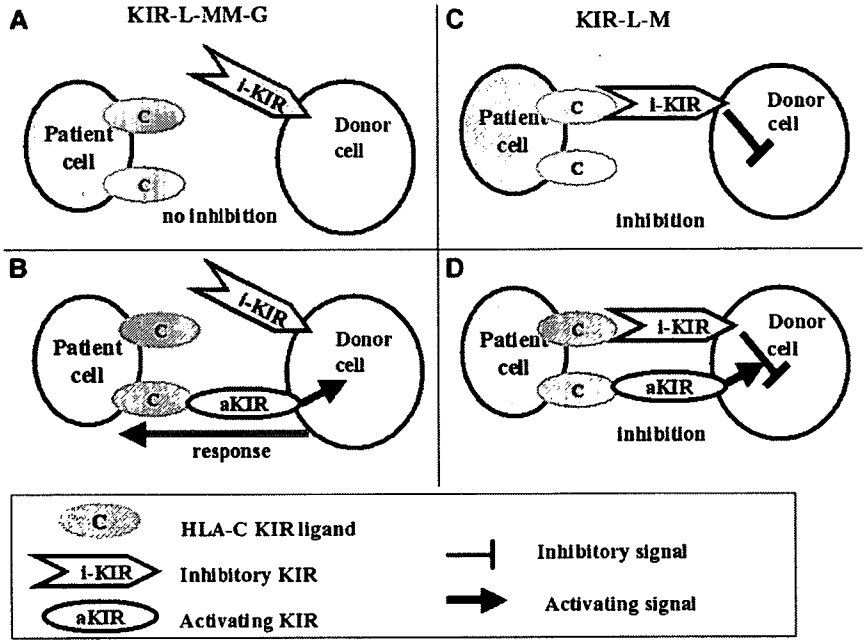

Figure 4. Model of interaction between activating KIR and cognate KIR ligand. Donor-activating KIR transduces an activating signal on recognition of the cognate KIR ligand of the patient cell in KIR-L-MM-G case (B). The activating signal is canceled by an inhibitory signal from inhibitory KIR, which 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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Potential Role of a Mismatched HLA-Specific CTL Clone Developed Pre-Transplant in Graft Rejection following Cord Blood Transplantation

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ABSTRACT

Graft rejection is a serious complication in cord blood transplantation (CBT), but little is known about the mechanism of rejection. To investigate the potential role of T lymphocytes in graft rejection, we isolated a $CD8^+$ cytotoxic T lymphocyte (CTL) clone of recipient origin from blood obtained from a patient with graft rejection after CBT from an HLA-mismatched unrelated donor. The isolated CTL clone specifically recognized an HLA-B*1501 molecule as an alloantigen, which was expressed in donor cells but not in recipient cells. The results of a microchimerism analysis specific for HLA-B*1501 and a polymerase chain reaction assay specific for the T cell receptor on DNA from pretransplant peripheral blood mononuclear cells revealed that the patient was exposed to HLA-B*1501 prior to CBT, and that the CTL clone was in the patient's blood prior to transplantation. The present study demonstrates a potential role for pretransplant CTL in graft rejection following CBT.

KEY WORDS

Graft failure • HLA class I antigen • T lymphocyte • Microchimerism • Reduced-intensity stem cell transplantation

INTRODUCTION

Although cord blood transplantation (CBT) from unrelated donors is an attractive treatment for patients with hematologic disease [1-6], graft rejection is a serious complication and is associated with a high rate of mortality [3,4,7,8]. The mechanisms of graft rejection have been extensively studied in bone marrow transplantation (BMT). One mechanism proposed by the studies in human and animal models is an immunologic response in which the host-derived T lymphocytes recognize donor-specific antigens [9-14]. For CBT, however, the role of the host-derived T lymphocytes in graft rejection has never been demonstrated in humans.

Here, we demonstrate a potential role of the host-derived cytotoxic T lymphocytes (CTLs) for graft rejection after CBT.

MATERIALS AND METHODS

Study Patient

A 59-year-old woman with acute myelomonocytic leukemia received CBT from an unrelated male donor. The details of the CBT procedure and the clinical course of the patient were reported previously [15]. Briefly, the preparative regimen consisted of 125 mg/m^2 fludarabine and 180 mg/m^2 melphalan, and 2.9×10^7/kg nuclear cells were infused. White blood cell (WBC) and neutrophil counts increased to 100/μL and 60/μL, respectively, with 65% of donor chimerism on day 16, but both counts subsequently decreased to less than the detection limit of the autohemocytometer on day 26. Graft rejection was diagnosed based on severe marrow hypoplasia and a complete loss of donor chimerism in bone marrow cells on day 28. A second

CBT from an unrelated female donor was conducted on day 35. The infused nuclear cell dose was 2.9×10^7/kg. DNA typing of the HLA-A, -B, and -DRB1 loci demonstrated that the recipient was A*1101/A*2402, B*4404/B*5603, and DRB1*1201/DRB1*1302, the first CBT donor was A*1101/A*2402, B*1501/B*5603, and DRB1*0901/DRB1*1201, and the second CBT donor was A*2402/A*3303, B*4403/B*5101, and DRB1*1201/DRB1*1302. The patient had HLA antibodies, including those against HLA-A33, and broad HLA-DR antigens prior to transplantation, whereas HLA antibody against HLA-B*1501 was not detected. The patient has one 19-year-old daughter. DNA typing of the HLA-A, -B, and -DRB1 loci demonstrated that her daughter was A*1101/A*2601, B*1501/B*5603, and DRB1*0405/DRB1*1201.

Cell Culture

CTL clones were isolated from a blood sample as described previously [16]. Briefly, peripheral blood mononuclear cells (PBMCs) obtained from the patient on day 20 were cultured in interleukin-2-containing media without stimulator cells for 14 days, and T lymphocyte clones were isolated by limiting dilution.

Transfection and CTL Stimulation Assays

The patient's B-LCL were transfected by electroporation with the pEAK10 plasmid (Edge BioSystems, Gaithersburg, MD) encoding *HLA-B*1501* cDNA, selected for 3 days with 0.8 μg/mL of puromycin, and assayed as a target for N19D8 CTL. Cytotoxicity was determined using a chromium release assay [16].

Interferon-γ release assays were conducted as previously described [16]. Briefly, COS cells were transfected with a plasmid encoding *HLA-B*1501*, *B*5603* (negative control), or *B*4403* (negative control) cDNA using the FuGENE transfection reagent (Roche, Indianapolis, IN). COS transfectants were cocultured with N19D8 CTL, and interferon-γ production was measured in the supernatant after 24 hours using an enzyme-linked immunosorbent assay (Endogen, Pierce, Rockford, IL).

Polymerase Chain Reaction (PCR) Specific for the T Cell Receptor

The T cell receptor Vβ repertoire was determined by flow cytometry using an IOTest Beta Mark Kit (Beckman Coulter, Fullerton, CA). The nucleotide sequences of the CTL clone's uniquely rearranged T cell receptor Vβ chain gene were determined by direct DNA sequencing of the amplified PCR product [17]. To determine the presence of the N19D8 clone-specific T cell receptor rearrangement, nested PCR was performed on genomic DNA extracted from a CTL clone N19D8 and PBMCs using a T cell receptor Vβ17-specific primer set for the first PCR: 5′-TTTCAGAAAGGAGATATAGCT-3′ (sense) and 5′-TTCTGATGGCTCAAACAC-3′ (antisense) followed by a second primer set specific for the N19D8 clone T cell receptor: 5′-GGAGATATAGCTGAAG GGTA-3′ (sense) and 5′-CCCCGCAAAGCTCTCA-3′ (antisense). PCR products were sequenced and confirmed to be identical in sequence to the N19D8-specific T cell receptor rearrangement. The PCR was performed with thermalcycler (Model 9600; Perkin-Elmer, Boston, MA) for 35 cycles under the following conditions: denaturation at 95°C for 1 minute, primer annealing at 55°C for 1 minute, and primer extension at 72°C for 1 minute in the first step, and denaturation at 95°C for 1 minute, primer annealing at 67°C for 15 seconds, and primer extension at 72°C for 1 minute in the second step. Each reaction contained 0.4 mL of Advantage 2 Polymerase Mix (Clontech Laboratories Inc., Palo Alto, CA).

Microchimerism Analysis

The presence of the microchimerism was determined using a nested PCR approach on genomic DNA extracted from a fingernail sample and PBMCs as previously described [18]. Briefly, nested PCR was performed on genomic DNA using an HLA-B-specific primer set for the first PCR: 5′-GGCGGGGGCG CAGGACCTGA-3′ and 5′-GGCGGGGGCGCAG GACCCGG-3′ (1:1 mixture; sense) and 5′-GAGGC CATCCCCGGCGACCTAT-3′ (antisense) followed by a second primer set specific for HLA-B*1501: 5′-A CCGGGAGACACAGATCTC-3′ (sense) and 5′-CT TGCCGTCGTAGGCGG-3′ (antisense). The touchdown procedure [19] was performed as first-step PCR under the following conditions: (1) denaturation at 96°C for 20 seconds and primer annealing at 72°C for 2 minutes for 5 cycles, (2) denaturation at 96°C for 20 seconds and primer annealing at 70°C for 2 minutes for 5 cycles, (3) denaturation at 96°C for 20 seconds and primer annealing at 68°C for 2 mintues for 4 cycles, and (4) denaturation at 96°C for 20 seconds and primer annealing at 72°C for 2.15 minutes for 15 cycles. The second-step PCR was performed for 28 cycles under the following conditions: denaturation at 94°C for 1 minute, primer annealing at 62°C for 1 minte, and primer extension at 72°C for 1 minute. FastStart Taq DNA Polymerase (Roche) and AmpliTaq Gold PCR Master Mix (Applied Byosistems, Foster City, CA) were used in the first- and the second-step PCR, respectively.

RESULTS

Isolation of CTL Clone N19D8

Two CTL clones were isolated from the peripheral blood of the patient just after the onset of graft rejection. One clone lysed B-LCL from the patient but failed to lyse B-LCL from the donor of the first CBT. The other clone, designated N19D8, lysed

B-LCL from the donor but not from the patient (Figure 1A). Thus, we further investigated the N19D8 clone because this clone had the possibility of being involved in immunologic graft rejection. Flow cytometry analysis and sex chromosome fluorescein in situ hybridization revealed that the N19D8 clone was a $CD3^+CD4^-CD8^+$ T cell and originated from the patient (data not shown).

CTL Clone N19D8 Recognizes the HLA-B*1501 Molecule

In a cytotoxicity assay using a panel of B-LCL derived from unrelated individuals, N19D8 CTL lysed

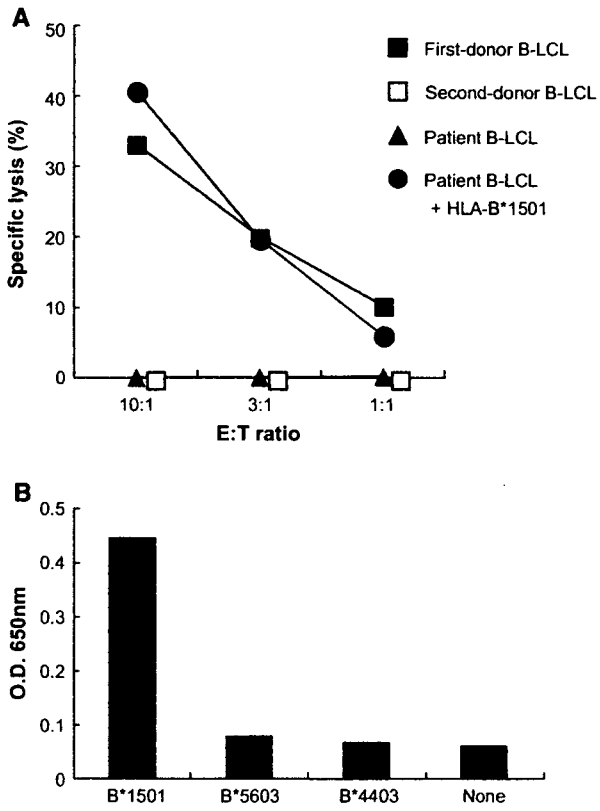

Figure 1. The N19D8 CTL clone recognizes HLA-B*1501. (A) Transfection of *HLA-B*1501* cDNA into the B-LCL from the patient restored recognition by N19D8 CTL. The patient's B-LCL were transfected with a plasmid encoding *HLA-B*1501* cDNA and assayed as a target for N19D8 CTL. Cytotoxicity was determined using a chromium release assay. Lysis of the first-donor B-LCL (closed squares), second-donor B-LCL (open squares), patient B-LCL (triangles), and HLA-B*1501-transfected patient B-LCL (circles) are shown as the mean of triplicate cultures at various E:T ratios. (B) *HLA-B*1501* cDNA stimulated interferon-γ production by N19D8 CTL. COS cells were transfected with a plasmid encoding *HLA-B*1501*, *B*5603* (negative control), or *B*4403* (negative control) cDNA. COS transfectants were cocultured with N19D8 CTL, and interferon-γ production was measured in the supernatant, an enzyme-linked immunosorbent assay. Data is shown as the mean of triplicate determinations.

all of 4 B-LCL lines from unrelated individuals that shared HLA-B*1501 but failed to lyse B-LCL from the donor of the second CBT and 7 unrelated individuals without B*1501 (Table 1). These results indicated that N19D8 CTL recognized the HLA-B*1501 molecule as an alloantigen, which was only expressed in donor cells, or a minor histocompatibility antigen presented by HLA-B*1501 molecule.

To determine if the lack of recognition by N19D8 CTL was solely because of the absence of HLA-B*1501 gene expression, the patient's B-LCL were transfected with *HLA-B*1501* cDNA and used as a target in cytotoxicity assays. The B*1501-transfected patient B-LCL was lysed almost as well as the first CBT donor's B-LCL (Figure 1A). Furthermore, COS cells transfected with *HLA-B*1501* cDNA alone stimulated interferon-γ production by N19D8 CTL (Figure 1B). Thus, we concluded that the N19D8 CTL clone recognizes mismatched HLA-B*1501 as an alloantigen.

The Presence of the N19D8 CTL Clone and HLA-B*1501 Microchimerism Prior to Transplantation

We next determined whether the N19D8 clone developed prior to transplantation using nested PCR assays specific for the CTL clone's uniquely rearranged T cell receptor Vβ17 chain gene. PCR products were detected by amplification of DNA from pretransplant as well as posttransplant PBMCs (Figure 2), demonstrating that the N19D8 CTL clone developed in the patient prior to the first CBT. We further tested pretransplant PBMCs for chimeric cells with the HLA-B*1501 gene using PCR. Indeed, the HLA-B*1501 microchimerism was detected by amplification of DNA from the pretransplant patient PBMCs using standard PCR as well as nested PCR (Figure 3), indicating that the patient was exposed to HLA-B*1501 prior to CBT. The HLA-A*2601 microchimerism was also detected from the pretransplant patient PBMCs. From the patient PBMCs 3 months after transplantation, HLA-A*2601 and HLA-B*1501 microchimerisms were detected (data not shown).

DISCUSSION

Although direct verification would be difficult, it is reasonable to conclude that the N19D8 clone was involved in the graft rejection based on the following clinical and laboratory findings. First, the patient rejected the first cord blood graft with HLA-B*1501, whereas the second graft without B*1501 was successfully engrafted. This clinical course is consistent with allo-activity of the N19D8 clone. Second, $CD3^+$ $CD4^-CD8^+$ T lymphocytes accounted for the majority of the lymphocytes in the patient's blood just after the onset of graft rejection (84.8%, data not shown). Third, no clones that recognize the other mismatched HLA antigens or minor histocompatibility antigens