i.e., indented or multilobated, and were usually characterized by a mixed morphology, which was referred to as the polymorphic variant. Pure proliferation of immunoblasts was seen in only two patients (1%), and was termed the immunoblastic variant. Intravascular/sinusoidal infiltration was observed in 26% of the common variants, 62% of the giant cell-rich variants, 14% of the polymorphic variants, and 0% of the immunoblastic variants. The giant cell-rich variant was associated with intravascular/sinusoidal infiltration more frequently than the common variant (p=0.01).

Clinical features according to morphological variants

The patients' main characteristics and therapeutic results according to morphological categorization are summarized in Table 1. We compared the clinical characteristics between the current group of 120 patients with *de novo* CD5$^+$ DLBCL and 384 patients with CD5$^-$ DLBCL in our previous study.[11] Our previous findings on the clinical features of *de novo* CD5$^+$ DLBCL such as an older age, at onset, female predominance, frequent extranodal involvement, and higher International Prognostic Index (IPI)[31] score were confirmed in the current group of 120 patients (*data not shown*).

Table 1. Clinical features of the patients with *de novo* CD5$^+$ diffuse large B-cell lymphoma.

	Total (n=120) (%)	*Common (n=91) (%)*	*Giant cell-rich (n=13) (%)*	*Polymorphic (n=14) (%)*	*Immunoblastic (n=2) (%)*
Age at diagnosis, years.					
Median	66	66	63	67/71	62/69
Range	22-91	22-91	36-81	52-89	62,69
Over 60 years old	84 (70)	64 (70)	9 (69)	9 (64)	2 (100)
Sex (male:female)	58:62	40:51	9:4	8:6	1:1
Performance status >1	39 (33)	27 (30)	4 (31)	6 (43)	2 (100)
Serum LDH level >normal	85 (71)	61 (67)	11 (85)	11 (79)	2 (100)
Stage III/IV	73 (61)	54 (59)	9 (69)	8 (57)	2 (100)
Extranodal involvement	75 (63)	55 (60)	8 (62)	11 (79)	1 (50)
More than one site	29 (24)	20 (22)	4 (31)	5 (36)	0 (0)
International Prognostic Index					
Low	30 (25)	25 (27)	1 (8)	4 (29)	0 (0)
Low-intermediate	30 (25)	26 (29)	4 (31)	0 (0)	0 (0)
High-intermediate	19 (16)	11 (12)	4 (31)	4 (29)	0 (0)
High	41 (34)	29 (32)	4 (31)	6 (43)	2 (100)
B-symptoms present	49/117 (44)	35/88 (40)	5 (38)	7 (50)	2 (100)
Complete response rate	77/114 (68)	64/86 (74)	5/12 (42)	7/14 (50)	1/2 (50)
5-year OS rate	(38)	(44)	(15)	(21)	(0)

LDH: lactate dehydrogenase; OS: overall survival.

The clinical features, including the five factors of the IPI,[31] were not significantly different among the four morphological variants of *de novo* CD5$^+$ DLBCL. The bone marrow, liver, and spleen were the most frequently involved anatomical sites irrespective of the morphological variant (*data not shown*).

Atypical lymphocyte concentrations (range, 11 to 78%) were noted at presentation in the peripheral blood smear of four cases, whose white blood cell counts ranged from 6,000 to 41,000/mm^3. None of these patients showed marked splenomegaly and the morphology of leukemic cells differed from that of B-cell prolymphocytic leukemia cells.

Immunophenotypic features

BCL2 protein was expressed in 86 out of 96 tumors, and observed in more than 70% of the tumor cells in almost all positive cases (Figure 2B). This incidence was significantly higher than that in the CD5$^-$ DLBCL cases (105/150, 70%; p=0.0003).

As for the molecular classification system established by Hans *et al.*,[30] 36 of 44 cases (82%) of *de novo* CD5$^+$ DLBCL were classified as the non-germinal center B-cell type. Thirty patients (68%) showed the CD10$^-$BCL6$^-$MUM1$^+$ immunophenotype. CD10 was positive in seven patients (16%), BCL6 was negative in 79% of the cases examined (33/42), and MUM1 was positive in 95% of the cases (42/44). Only one patient showed the CD10$^+$BCL6$^+$MUM1$^-$ immunophenotype.

Among the four morphological variants, the common variant was positive for Ig-κ more frequently than either the giant cell-rich (p=0.05) or polymorphic (p=0.03) variant. As for other expression of other antigens there were no significant differences among the morphological variants of *de novo* CD5$^+$ DLBCL (*data not shown*).

Therapeutic outcome and long-term survival according to histopathological variants

Clinical follow-up data and information about the first-line therapy were available for all patients. The treatment consisted of chemotherapeutic regimens including anthracycline for 104 patients and without anthracycline for three. No patient was treated with rituximab in the first-line therapy. Seven patients with localized disease were treated with radiotherapy or surgical resection alone as first-line therapy. Six patients who did not receive any therapy because of their poor performance status all died of their disease. A complete response was achieved on first-line therapy in 77 (68%) out of the 114 patients who received treatment. Seven patients were lost to follow-up within 5 years after the diagnosis. The median observation time of surviving patients was 81 months. The 2-year overall survival rate of all 120 patients, estimated by the Kaplan-Meier method, was 52%, and the 5-year overall survival rate was 38% (Figure 3A).

We collected data on sites of involvement at relapse/progression. Among all 120 patients with *de novo* CD5$^+$ DLBCL, 16 patients (13%) developed central nervous system (CNS) recurrence (Table 2). All these patients were treated with anthracycline-containing chemotherapy as a front-line treatment. One patient had brain

involvement at diagnosis. She achieved a complete response following front-line therapy, but develop recurrence in the thoracic spinal cord. The other patients did not show any CNS involvement at diagnosis. Twelve patients experienced CNS relapse after achieving a complete response. Of these, eight experienced isolated CNS relapse while the CNS relapse was associated with a systemic relapse in the others. Four patients experienced CNS disease progression during the first-line treatment. The median age of all 16 patients with CNS relapse was 64 years (range, 28 to 85). Of note, all but three patients were over 60 years old. Seven were male and nine were female. The serum lactate dehydrogenase level was elevated in 13 of these patients and performance status was higher than one in seven patients. Five patients showed more than one extranodal site of involvement. Nine patients were categorized as having a high-intermediate or high risk, according to the IPI. The median time from diagnosis to CNS recurrence was 16 months. We compared therapeutic outcome and survival data in the 120 paients with *de novo* CD5⁺ DLBCL according to the morphological variants. The complete response rate was lowest (42%) in patients with the giant cell-rich variant of *de novo* CD5⁺ DLBCL, and was significant different from that in patients with the common variant (p=0.02, Table 1). Five-year overall survival rates for patients with common, giant cell-rich, polymorphic, and immunoblastic variants were 44%, 15%, 21%, and 0%, respectively (Table 1, Figure 3B). The survival curve of patients with the common variant was significantly better than that of patients with the other three variants combined (p=0.011, Figure 3C). The presence of intravascular/sinu-

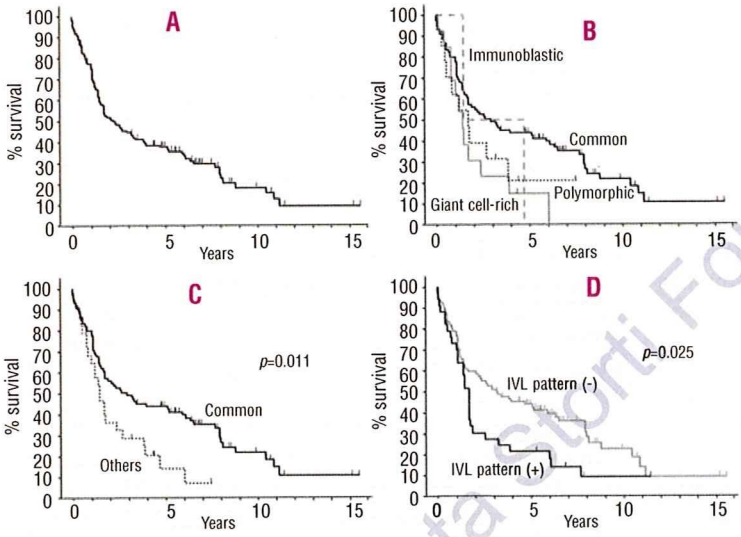

Figure 3. Survival according to the histological features of *de novo* CD5⁺ diffuse large B-cell lymphoma (DLBCL). (A) Overall survival in all 120 patients with *de novo* CD5⁺ DLBCL. (B) Overall survival of patients with different histological variants of *de novo* CD5⁺ DLBCL. (C) Patients with the common variant had a better survival than those with the other three variants of *de novo* CD5⁺ DLBCL. (D) The presence of intravascular/sinusoidal infiltration had an impact on the overall survival. IVL, intravascular/sinusoidal.

Table 2. Clinicopathological features of patients with *de novo* CD5⁺ diffuse large B-cell lymphoma who experienced central nervous system recurrence.

N.	*Age/sex*	*Stage*	*Sites of extranodal involvement*	*PS >1*	*LDH >N*	*IPI score*	*Histological variant*	*IVL pattern*	*CR*	*Sites of recurrence*	*Period from diagnosis to CNS recurrence (months)*	*Survival, (months) outcome*
1	62/M	IIIA	Lung, stomach, kidney, gingiva		Y	4	Common			CNS	2	8, DOD
2	77/M	IA		Y	Y	3	Polymorphic			CNS	2	4, DOD
3	76/M	IIA			Y	2	Common			CNS	3	9, DOD
4	61/F	IVB	BM	Y	Y	4	Common	Y	Y	CNS	5	9, DOD
5	67/M	IVB	Liver, BM	Y	Y	5	Common	Y	Y	CNS	6	23, DOD
6	85/M	IIIA		Y	Y	4	Common			CNS	<7	7, DOD
7	62/F	IIIA	Brain, pleura	Y	Y	5	Common	Y	Y	CNS	8	18, DOD
8	62/F	IIIB		Y	Y	4	Immunoblastic		Y	CNS, LN, liver, ascites, BM	8	18, DOD
9	38/F	IVB	BM		Y	2	Common		Y	CNS	24	72, DOD
10	66/F	III	Bone, uterus		Y	4	Common		Y	CNS (intraocular)	37	43, AWD
11	62/M	IVB	Liver, BM	Y	Y	5	Common		Y	Pelvis,CNS	39	40, DOD
12	28/F	IIA	Breast			0	Common		Y	CNS (intraocular)	57	86, AWD
13	50/M	IIIB			Y	2	Giant cell-rich	Y	Y	CNS	60	74, DOD
14	69/F	IA				1	Common		Y	CNS, etc.	71	80, DOD
15	67/F	IA			Y	2	Common	Y	Y	CNS (intraocular)	84	84, AWD
16	74/F	IA				1	Common		Y	CNS, LN	96	99, DOD

PS: performance status; LDH: lactate dehydrogenase; IVL: intravascular/sinusoidal; CR: complete response; Y: yes; BM: bone marrow; LN: lymph node; DOD: died of disease; AWD: alive with disease.

soidal infiltration also had an impact on survival (p=0.025, Figure 3D). The results of univariate and multivariate analyses to assess the impact of clinical and morphologic features on overall survival in *de novo* CD5+ DLBCL patients are shown in Table 3. Univariate analysis identified the five risk factors of IPI, morphological variants, and intravascular/sinusoidal infiltration as prognostic factors important for overall survival. The presence of either *snowman-like* cells or a higher mitotic ratio (> 4/one high-power field on average) was not associated with a reduced overall survival (*data not shown*). Multivariate analysis adjusted for the five risk factors of the IPI confirmed the independent prognostic significance of histological categorization for overall survival (Table 3). Among the prognostic factors, the morphologic variant, age, performance status, and serum lactate dehydrogenase level were significantly associated with survival.

Table 3. Prognostic factors affecting overall survival of patients with *de novo* CD5+ diffuse large B-cell lymphoma.

Variables	Unfavorable factor	Univariate HR	(CI)	p	Multivariate HR	(CI)	p
Comparison with risk factors							
Morphological variants	Not common	1.85	(1.14-3.01)	0.01	1.67	(1.02-2.75)	0.04
IVL pattern	Present	1.66	(1.06-2.60)	0.03		-	-
Age	>60 years	2.37	(1.44-3.92)	0.001	1.91	(1.15-3.19)	0.01
Performance status	2-4	2.81	(1.81-4.37)	<0.001	1.77	(1.11-2.85)	0.02
LDH	>Normal	3.71	(2.14-6.43)	<0.001	2.56	(1.43-4.61)	0.002
Stage	III/IV	2.34	(1.48-3.69)	<0.001		-	-
Extranodal diseases	>1 site	1.72	(1.07-2.77)	0.03		-	-
B symptoms	Present	2.09	(1.36-3.19)	<0.001		-	-
Comparison with IPI category							
Morphological variants	Not common	1.85	(1.14-3.01)	0.01	1.44	(0.87-2.36)	0.15
IPI category	HI/H	3.32	(2.14-5.15)	<0.001	3.14	(2.00-4.92)	<0.001
IVL pattern	Present	1.66	(1.06-2.60)	0.03	1.81	(1.14-2.86)	0.01
IPI category	HI/H	3.32	(2.14-5.15)	<0.001	3.46	(2.21-5.41)	<0.001

HR: hazard ratio; CI:confidence interval; HI/H: high-intermediate or high risk category of IPI; IVL: intravascular/sinusoidal; LDH, lactate dehydrogenase.

Discussion

We clarified detailed cytomorphological features of *de novo* CD5+ DLBCL. A German study also documented morphological features in their series of 13 cases of *de novo* CD5+ DLBCL, identifying eight centroblastic (62%), three immunoblastic (23%), and two unclassified DLBCL with irregular nuclei (15%).[13] Our findings generally appeared to be in keeping with those of the German study; however, the percentage of immunoblastic lymphoma cases (23%) was higher in the German study than in ours (2%). DLBCL developing in the setting of small lymphocytic lymphoma/chronic lymphocytic leukemia (Richter's syndrome) evidently tend to be characterized by an immunoblastic morphology and the expression of CD5.[32] In Japan, the incidence of chronic lymphocytic leukemia is one fifth of that in Western countries.[33,34] Moreover, CD5 expression was mainly examined using fresh material in the majority of studies of *de novo* CD5+ DLBCL in Japan, while it was examined in paraffin-embedded material in the studies in Western countries. In Japan, the incidence of *de novo* CD5+ DLBCL ranges from 4% (4/101)[35] to 10% (24/240),[36] which seems to be almost the same as that reported in Western series.[10,37] Since only two cases have been included in the current study, the clinicopathological features of the immunoblastic variant of *de novo* CD5+ DLBCL remain unknown. International cooperative studies are needed to verify the hypothesis that these facts may explain the conflicting data. Since *de novo* CD5+ DLBCL has various histopathological appearances, CD5 immunostaining should be performed routinely in cases of DLBCL.

In the current study, intravascular/sinusoidal patterns to various extents were observed in 38% of the cases of *de novo* CD5+ DLBCL. As Murase *et al.* demonstrated recently,[21] *de novo* CD5+ DLBCL with an intravascular/sinusoidal pattern showed intermediate features in terms of aggressive clinical behavior and prognosis between *de novo* CD5+ DLBCL without an intravascular/sinusoidal pattern and CD5+ intravascular large B-cell lymphoma, suggesting that a part of the two diseases overlaps. In the present study *snowman-like*, binucleated cells were frequently observed in *de novo* CD5+ DLBCL. Further studies in CD5- DLBCL and CD5+ intravascular large B-cell lymphoma are needed to evaluate their diagnostic significance in *de novo* CD5+ DLBCL.

The aggressive clinical feature of *de novo* CD5+ DLBCL that we previously reported[11] was confirmed by the current study and a recent study that was conducted using tumor specimens from patients with DLBCL uniformly treated with anthracycline-based chemotherapeutic regimens in a prospective, multi-center clinical trial.[37] In contrast, it has been reported that the expression of CD5 in DLBCL did not affect overall survival.[13] Recent studies revealed that patients with *de novo* CD5+ DLBCL with 8p21-associated chromosomal abnormalities[18] and with 9p21 loss in comparative genomic hybridization analysis[16] have an extremely short survival. The existence of these highly aggressive subgroups of *de novo* CD5+ DLBCL may explain the heterogeneity in the prognosis of this disease. The possible role of the CD5 molecule in the aggressiveness of *de novo* CD5+ DLBCL remains unknown. It has been reported that CD5 supports the survival of B cells by stimulating the production of interleukin-10 and by down-regulating B-cell receptor signaling.[38] This molecular basis may explain in part why *de novo* CD5+ DLBCL shows more aggressive clinical features than CD5- DLBCL.

According to the criteria established by Hans *et al.*,[30] 82% of the cases examined in the present study were non-germinal center B-cell DLBCL. Our results suggest that *de novo* CD5+ DLBCL is mainly classified into the non-germinal center B-cell type, and may provide a clue to clarify the aggressiveness of such DLBCL. Our present study also revealed that *de novo* CD5+ DLBCL typically shows the BCL2+ BCL6- immunophenotype.

Recent clinical studies suggest that the prognosis of DLBCL expressing BCL2 protein, BCL6 protein-negative DLBCL, and DLBCL of the non-germinal center B-cell subgroup is improved by rituximab-containing chemotherapy.[39-41] In our previous study published in 2002, no patients had been treated with rituximab.[11] In the present study, some patients had been treated with rituximab as a part of salvage therapy; however, the overall survival was almost the same as that in the previous study and was not clearly improved. The therapeutic impact of adding rituximab to first-line therapy in *de novo* CD5⁺ DLBCL needs to be evaluated in the setting of a well-designed clinical trial.

The overall incidence of CNS recurrence in aggressive non-Hodgkin's lymphoma excluding lymphoblastic lymphoma/acute lymphoblastic leukemia and Burkitt's lymphoma is approximately 5%,[42-44] and the incidence in DLBCL seems to be less than 5%. The incidence of CNS recurrence in the present study, 13%, was marked. Most of our patients with CNS recurrence had an elevated level of serum lactate dehydrogenase, which has been reported as a potential risk factor for CNS recurrence in aggressive lymphoma.[42] In contrast, most of the patients with CNS recurrence were over 60 years old, which was reported to be a favorable factor in a study of a large number of patients.[42] To establish an optimal therapeutic strategy for CNS prophylaxis in DLBCL, the relationship between CD5 expression and CNS recurrence in DLBCL should be examined in future studies.

In conclusion, our study provides new clinicopathological information on *de novo* CD5⁺ DLBCL. *De novo* CD5⁺ DLBCL shows many unique clinicopathological and genetic features. Further studies are needed to clarify molecular mechanisms in highly aggressive subgroups of *de novo* CD5⁺ DLBCL.

Appendix

List of participating institutes in the CD5⁺ DLBCL histology project: Akita University School of Medicine, Akita Kumiai General Hospital, National Miyagi Hospital, Saka General Hospital, Tohoku University School of Medicine, Sendai City Hospital, Furukawa City Hospital, Fukushima Medical College, Iwaki General Hospital, Ohta Nishinouchi General Hospital, Takeda General Hospital, Tokyo Women's Medical University Daini Hospital, Saitama Medical School, Matsudo Municipal Hospital, Higashi Matsudo Hospital, Kameda General Hospital, Niigata University, Toyama Prefectural Central Hospital, Kanazawa University, Noto General Hospital, Nagano Municipal Hospital, Nagano Red Cross Hospital, Hamamatsu Medical Center, Inazawa Municipal Hospital, Aichi Prefectural Hospital, Toyota Memorial Hospital, Fujita Health University School of Medicine, Nishio Municipal Hospital, Toyohashi Municipal Hospital, Okazaki Municipal Hospital, Ichinomiya Municipal Hospital, Japanese Red Cross Nagoya First Hospital, Nagoya Memorial Hospital, Nagoya City University Medical School, Nagoya Ekisaikai Hospital, Aichi Cancer Center, Suzuka Chuo General Hospital, Suzuka Kaisei General Hospital, Mie University School of Medicine, Matsusaka Municipal Hospital, Matsusaka Chuo General Hospital, Matsusaka Saiseikai General Hospital, Yamada Red Cross Hospital, Ise Municipal General Hospital, Kyoto University, Kyoto Prefectural University of Medicine, Rinku General Medical Center, Okayama University Medical School, Okayama Saiseikai General Hospital, Chugoku Central Hospital of the Mutual Aid Association of Public School Teachers, Okayama Red Cross General Hospital, Fukuoka University School of Medicine, Kyushu Cancer Center, Kyushu University, and University of the Ryukyus.

Authorship and Disclosures

MY, NN, RS, TM, and SN contributed to the design of the study, provided clinical data and samples, analyzed the data, and wrote the manuscript. YK, MO, RI, TY, JS, TM, IM, KO, MN, JT, and MT provided clinical data and samples and critically reviewed the manuscript. MH, YM, RU, and HS provided clinical data and gave critical advice on the study to improve its intellectual content.

The authors reported no potential conflicts of interest.

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DDX3Y encodes a class I MHC–restricted H-Y antigen that is expressed in leukemic stem cells

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The Y chromosome encodes male-specific minor histocompatibility (H-Y) antigens that stimulate T- and B-lymphocyte responses after sex-mismatched allogeneic hematopoietic cell transplantation (HCT). A CD8$^+$ cytotoxic T lymphocyte (CTL) clone that recognizes a novel HLA-B*2705–restricted H-Y antigen encoded by the *DDX3Y* gene was isolated from a male who had received a hematopoietic cell graft from his human leukocyte antigen (HLA)–identical sister. The antigenic peptide is a decamer that differs from the homologous *DDX3X*-encoded peptide at 4 positions. Expression of *DDX3Y* and of the H-Y epitope that it encodes was examined by quantitative polymerase chain reaction (PCR) and by CTL recognition assays. Expression of *DDX3Y* is detected in all myeloid and lymphoid leukemic cells that carry an intact Y chromosome. Moreover, the *DDX3Y*-encoded H-Y epitope is presented on the surface of both myeloid and lymphoid leukemic cells from male HLA-B*2705$^+$ patients. DDX3Y-specific CTLs prevent engraftment of human acute leukemia in nonobese diabetic/severe combined immune deficient mice, demonstrating that the *DDX3Y*-encoded H-Y antigen is also expressed in leukemic stem cells. These results demonstrate that CD8$^+$ T-cell responses against *DDX3Y* have the potential to contribute to graft-versus-leukemia (GVL) activity after female into male allogeneic HCT. This study is registered at http://clinicaltrials.gov as NCT00107354. (Blood. 2008;111:4817-4826)

Introduction

Sex-mismatched allogeneic hematopoietic cell transplantation (HCT) represents a unique situation wherein T-cell responses against Y-chromosome–encoded minor histocompatibility (H-Y) antigens can potentially contribute to graft rejection,[1,2] graft-versus-host disease (GVHD),[3,4] and graft-versus-leukemia (GVL)[5,6] activity. H-Y antigens in mice[7-12] and humans[1-3,13-19] are encoded by a specific class of Y-chromosome genes that have X-chromosome homologues and are expressed both in and variably outside the testis.[20,21] In humans, there are 15 such genes, 6 of which—*RPS4Y1*,[5,15] *USP9Y*,[1,14] *DDX3Y*,[3,4] *UTY*,[2,18,19] *TMSB4Y*,[16] and *SMCY*[13,17]—have been shown to encode H-Y antigens. Although most of the human H-Y antigens identified to date are presented by major histocompatibility complex (MHC) class I molecules and recognized by CD8$^+$ T lymphocytes, the *DDX3Y*[3,4] and *RPS4Y1*[15] genes have been shown to encode H-Y antigens that are presented by MHC class II molecules and recognized by CD4$^+$ T lymphocytes.

The clinical significance of H-Y–specific T-cell responses in female into male (F→M) HCT has not been completely defined. CD8$^+$ SMCY peptide–specific T-cell clones mediated histologic changes of skin GVHD in an in vitro skin explant model.[22] Studies using MHC class I HLA-A2 or HLA-B7 tetramers complexed with SMCY peptides also demonstrated that SMCY-specific CD8$^+$ T cells were detectable in the peripheral blood of HLA-A2$^+$ or HLA-B7$^+$ F→M hematopoietic cell transplant recipients who developed acute GVHD.[23] CD4$^+$ T cells recognizing MHC class II–restricted peptides encoded by *DDX3Y* have been isolated from patients with severe acute[3] and extensive chronic[4] GVHD. In the latter case, the DDX3Y peptide–specific CD4$^+$ T-cell response was temporally associated with a high-titer DDX3Y-specific IgG antibody response, demonstrating a coordinated B- and T-cell response to DDX3Y after sex-mismatched, F→M HCT. These observations have implicated SMCY- and DDX3Y-specific T-cell responses in GVHD that occurs in F→M transplant recipients. A role for H-Y–specific T-cell responses in GVHD is also supported by the observation that male recipients of female hematopoietic cell grafts exhibit the highest rates of both acute and chronic GVHD of any donor-recipient sex combination.[24-26]

Clinical observations also suggest, however, that H-Y–specific T-cell responses contribute to GVL responses. High frequencies in the peripheral blood of CD8$^+$ SMCY peptide–specific cytotoxic T lymphocytes (CTLs) have been associated with regression of blast-phase chronic myelogenous leukemia (CML) after donor lymphocyte infusion.[6] F→M hematopoietic cell transplant recipients also experience a lower risk of relapse than that observed in any other donor-recipient sex combination, which is apparent even after controlling for GVHD.[24,26,27] The selective GVL effect seen in male recipients of female grafts is observed in patients who underwent transplantation for CML, acute myelogenous leukemia

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(AML), and acute lymphoblastic leukemia (ALL),[26] and could potentially be explained by expression of some H-Y antigens in leukemic stem cells. This hypothesis is supported by previous studies in our laboratory, which demonstrated that $CD8^+$ CTLs specific for an HLA-B8–restricted H-Y antigen encoded by *UTY* inhibited the engraftment of male AML in nonobese diabetic/severe combined immune deficient (NOD/SCID) mice.[28]

In this report, we describe a novel MHC class I–restricted H-Y antigen that is encoded by *DDX3Y* and recognized by $CD8^+$ CTLs. In contrast to previous studies that reported the isolation of $CD4^+$, DDX3Y-specific T cells from patients with severe GVHD,[3,4] the $CD8^+$ DDX3Y-specific CTL clone was derived from a male who had received a hematopoietic cell graft from his MHC-identical sister but did not develop histologic evidence of GVHD. We therefore investigated the expression of the *DDX3Y*-encoded H-Y antigen recognized by these CTLs to gain insight into the extent to which it might serve as a target for GVL and GVHD responses after F→M HCT.

Methods

Human subjects

After written informed consent was obtained in accordance with the Declaration of Helsinki, blood samples and skin biopsies were collected from 2 male patients—one (UPN 19492) with acute lymphoblastic leukemia (ALL) in first complete remission, and another (UPN 21234) with myelodysplasia/refractory anemia with excess blasts–2 (MDS/RAEB-2)—and from their MHC-identical sisters, all of whom were enrolled in a clinical trial approved by the Institutional Review Board at the Fred Hutchinson Cancer Research Center.

Cell culture

The $CD8^+$, HLA-B*2705–restricted, donor-derived, recipient-specific CTL clone 68H7-819 was isolated from peripheral blood mononuclear cells (PBMCs) obtained on day +42 after a myeloablative, unmodified peripheral blood stem cell transplantation, and was maintained in vitro as previously described.[29] Phytohemagglutinin (PHA)–stimulated T-cell blasts (PHA blasts),[29] Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines (EBV-LCLs),[30] and fibroblast lines[31] were generated and maintained as described. COS-7[32] and WEHI 164 clone 13 cells[33] were maintained in vitro as previously described.

Cytotoxicity assays

Cytotoxicity was measured in ^{51}Cr release assays as previously described[29] using target cells labeled overnight at 37°C with 100 μCi (3.7 MBq) ^{51}Cr. EBV-LCL lines derived from males carrying constitutional deletions of the Y chromosome were infected at a multiplicity of infection of 10:1 with a recombinant vaccinia virus encoding HLA-B*2705 concurrent with ^{51}Cr labeling. Specific lysis was calculated using the standard formula.[31] H-Y peptides for epitope reconstitution studies were synthesized using standard Fmoc chemistry (SynPep, Dublin, CA) and pulsed onto ^{51}Cr-labeled donor-derived EBV-LCLs for 30 minutes at 37°C before addition of CTLs.

Subgenic cloning and transfection studies

Subgenic fragments of the *DDX3Y* or *USP9Y* genes were amplified by polymerase chain reaction (PCR) and cloned into pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA). The *HLA-B*2705* cDNA was amplified by PCR and cloned into pEAK10 (Edge Biosystems, Gaithersburg, MD). To identify the nucleotide sequence that encodes the H-Y epitope recognized by CTL 68H7-819, COS-7 cells previously seeded in 96-well flat-bottom plates at 1.5×10^4/well were transiently cotransfected in duplicate with plasmids encoding *DDX3Y* or *USP9Y* minigenes and the *HLA-B*2705*/pEAK10 plasmid. Transfection was carried out in Dulbecco modified Eagle medium containing 2 ng/μL each plasmid construct, 800 μg/mL diethylaminoethyl-dextran, and 200 μM chloroquine, at 37°C for 4 hours, followed by a 2-minute hyperosmotic shock with 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO) in phosphate-buffered saline (PBS; Invitrogen). The day following transfection, the medium was removed from the COS-7 transfectants, and 5×10^3 to 1×10^4 CTL 68H7-819 suspended in 100 μL COS-7 medium[32] supplemented with 25 IU/mL interleukin-2 (IL-2; Chiron, Emeryville, CA) was added to each well. After 20 hours of coculture, the supernatants were evaluated for interferon-γ (IFN-γ) by enzyme-linked immunosorbent assay (ELISA) or for tumor necrosis factor by cytotoxicity.[33]

Quantitative real-time reverse transcription–PCR (RT-PCR)

Total RNA was purified from cultured cells, primary leukemic cells, and human tumor cell lines using the RNeasy Mini Kit (QIAGEN, Valencia, CA) or Trizol (Invitrogen). First-strand cDNA was synthesized from 1 to 4 μg total RNA using Oligo-dT and SuperScript II reverse transcriptase, initially treated with Dnase I (all from Invitrogen). First-strand cDNA from poly $(A)^+$ RNA from selected normal human tissues and blood cell subsets, pooled from both males and females, was commercially obtained (human Multiple Tissue cDNA panels I and II, and human blood fractions; BD Biosciences/Clontech, Palo Alto, CA). Expression of *DDX3Y* was evaluated in triplicate and normalized to *GAPDH* expression. *DDX3Y* transcript level was expressed relative to the male AML cell line KG-1a (ATCC, Manassas, VA). Amplification was performed on 25 ng initial RNA for primary leukemic samples, or 2 ng commercially prepared cDNA, using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a total reaction volume of 25 μL. Primers were at a final concentration of 400 nM, and the sequences were as follows: *DDX3Y*: 5′-GACAGTTCAGGTTGGAGTTGC-3′ and 5′-TCACACCAACGACTATGTCCA-3′; *GAPDH*: 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′. Amplification was performed on a Stratagene Mx3000P (La Jolla, CA), and relative expression was determined with the comparative C_t method,[34] using the average C_t values for *GAPDH* and *DDX3Y*.

HLA typing

High-resolution HLA typing of UPN 19492 and UPN 21234 was performed by the Clinical Immunogenetics Laboratory of the Seattle Cancer Care Alliance. Identification of HLA-B*2705$^+$ primary leukemia samples was performed using the Dynal RELI Sequence-Specific Oligonucleotide (SSO) HLA-A,B,C DRB1 and DQB1 Line Strip Typing System (Invitrogen) to determine HLA-B locus genotype from genomic DNA that was isolated using the Versagene Genomic DNA Purification Kit (Qiagen [formerly Gentra], Valencia, CA).

NOD/SCID leukemic engraftment assay

Primary leukemic cells were thawed, washed twice, and then cultured for 16 hours at 37°C in CTL medium[29] supplemented with 25 IU/mL IL-2, in the absence or presence of DDX3Y-specific or irrelevant CTLs at a CTL/leukemia ratio of 5:1. Leukemia cells and CTL/leukemia cell mixtures were then washed once, resuspended in PBS, and injected via the tail vein into cohorts of 5 to 6 sublethally irradiated (350 cGy delivered by linear accelerator at 20 cGy/min) NOD/SCID mice. Control mice were injected with 200 μL PBS. For experiments using AML cells, NOD/SCID mice were injected with purified rat antimouse CD122 antibody (200 μg/mouse) into the peritoneal cavity immediately following irradiation to facilitate the engraftment of human leukemic cells.[35] Mice were killed 6 weeks after injection, and bone marrow mononuclear cells (BMMCs), peripheral blood, spleen, liver, kidney, lung, and thymus were harvested and analyzed for engraftment by flow cytometry or by PCR using human Y-chromosome–specific primers.

Mouse BMMCs were washed and incubated with fluorescein isothiocyanate (FITC)–conjugated anti–HLA-B27 (One Lambda, Canoga Park, CA) and phycoerythrin (PE)–conjugated anti-CD33 or anti-CD45 (BD Pharmingen, San Diego, CA) antibodies for 30 minutes on ice, washed twice, and analyzed on a FACScan flow cytometer (BD Biosciences, Mountain View,

CA). The data were analyzed with CellQuest software (BD Biosciences). PE- or FITC-conjugated isotype-matched antibodies were used as controls.

Genomic DNA was isolated using the QIAamp DNA Blood Mini Kit or QIAamp DNA Mini Kit (Qiagen) and analyzed by PCR with human Y-chromosome–specific sequence-tagged site sY14 primers. PCR was carried out using ABI Fast PCR mix (Applied Biosystems), 500 nM sY14 primers,[36] deionized H_2O, and approximately 100 ng genomic DNA. Cycling conditions were 95°C for 10 seconds, 40 cycles of 95°C for 0 seconds, 61°C for 15 seconds, and 72°C for 30 seconds, followed by 72°C for 1 minute.

Quantitation of DDX3Y-specific CD8+ and CD4+ T-cell responses

Detection and enumeration of DDX3Y-specific T-cell responses were performed by IFN-γ enzyme-linked immunosorbent spot (ELISpot) assay. Assessment of subject UPN 19492 for T cells specific for the $DDX3Y_{74-83}$ epitope presented by HLA-B*2705 was performed directly ex vivo, without in vitro stimulation, on PBMCs obtained on days +42, +62, +93, and +109 after transplantation. This patient was also evaluated for T-cell responses against the 2 previously identified MHC class II–restricted *DDX3Y*-encoded H-Y epitopes, $DDX3Y_{30-48}$,[4] and $DDX3Y_{176-187}$,[3] using PBMCs obtained on days +42, +62, +100, and +109 after transplantation that were analyzed both directly ex vivo and after 2 to 6 weekly in vitro stimulations with $DDX3Y_{30-48}$- and $DDX3Y_{176-187}$-pulsed (1 μM), γ-irradiated (35 Gy) recipient pretransplantation PBMCs. The in vitro stimulations were performed in CTL medium[29] supplemented with 10 IU/mL IL-2. ELISpot assays were performed in 96-well Multi-Screen-IP plates (MAIPS4510; Millipore, Bedford, MA) coated overnight with antihuman IFN-γ antibody (M700A; Thermo Fisher Pierce Endogen, Rockford, IL) by culturing PBMC responders with peptide (2 μM)–pulsed donor PBMCs pulsed at a responder-stimulator ratio of 5:1 for 24 to 48 hours in 200 μL LCL medium.[30] Spots were visualized by addition of an antihuman biotin-labeled antibody (M701B; Thermo Fisher Pierce Endogen), PolyHRP20-streptavidin (Fitzgerald Industries International, Concord, MA), and Vectastain AEC substrate (Vector Laboratories, Burlingame, CA), and counted on an automated ELISpot reader (Immuno Biosys, The Colony, TX).

Identification of novel DDX3Y-specific T-cell responses in subject UPN 21234 was performed by stimulating PBMCs obtained on day +127 after transplantation with γ-irradiated (80 Gy) donor-derived EBV-LCLs pulsed with a mixture of 36 overlapping pentadecapeptides[37] that collectively encompassed the N-terminal 195 residues of DDX3Y (Table 1; NMI Peptides, Reutlingen, Germany). Stimulations were performed in 24-well plates at a responder/stimulator ratio of 4:1 in CTL medium[29] supplemented with 12.5 IU/mL IL-2, 5 ng/mL IL-7, and 10 ng/mL IL-15, and with each peptide present at a final concentration of 2.5 μM. Responder cells were tested by ELISpot on days +7 to +9 after the second and third stimulations, against 12 pools of 6 pentadecapeptides each, with each pool derived from 1 of the 6 rows or 6 columns of a 6 × 6 array of the 36 pentadecapeptides (Table 2). The concentration of the constituent pentadecapeptides in each pool was 1.25 μM.

Results

CD8+ CTL clone 68H7-819 recognizes a HLA-B*2705–restricted H-Y antigen encoded by *DDX3Y*

The CD8+ CTL clone 68H7-819 was isolated from UPN 19492, a male patient with ALL in first complete remission who had received an unmodified hematopoietic cell graft from his MHC-identical sister. The CTLs efficiently lysed EBV-LCLs, PBMCs, and PHA-stimulated T-cell blasts derived from the recipient, did not lyse EBV-LCLs derived from the donor, and weakly recognized recipient- but not donor-derived fibroblasts (Figure 1A). The CTLs also recognized EBV-LCLs derived from male but not female HLA-B27+ individuals (Figure 1B), suggesting that the clone was specific for a peptide encoded by a Y-chromosome gene and presented by HLA-B27. To identify the gene encoding this novel H-Y antigen, CTL 68H7-819 was tested for recognition of EBV-LCLs derived from individuals with constitutional Y-chromosome deletions[18,36] after infection with a recombinant vaccinia virus encoding HLA-B*2705. CTL 68H7-819 showed no recognition of vac/HLA-B*2705–infected WHT2996, an EBV-LCL carrying a proximal Yq deletion encompassing *USP9Y* and *DDX3Y* in the

Table 1. Overlapping DDX3Y pentadecapeptides for identification of novel epitopes

Peptide	Sequence	Residues
1	MSHVVVKNDPELDQQ	1-15
2	VVKNDPELDQQLANL	5-19
3	DPELDQQLANLDLNS	9-23
4	DQQLANLDLNSEKQS	13-27
5	ANLDLNSEKQSGGAS	17-31
6	LNSEKQSGGASTASK	21-35
7	KQSGGASTASKGRYI	25-39
8	GASTASKGRYIPPHL	29-43
9	IPPHLRNREASKGFH	39-53
10	LRNREASKGFHDKDS	43-57
11	EASKGFHDKDSSGWS	47-61
12	GFHDKDSSGWSCSKD	51-65
13	KDSSGWSCSKDKDAY	55-69
14	GWSCSKDKDAYSSFG	59-73
15	SKDKDAYSSFGSRDS	63-77
16	DAYSSFGSRDSRGKP	67-81
17	SFGSRDSRGKPGYFS	71-85
18	RDSRGKPGYFSERGS	75-89
19	GKPGYFSERGSGSRG	79-93
20	YFSERGSGSRGRFDD	83-97
21	RFDDRGRSDYDGIGN	94-108
22	RGRSDYDGIGNRERP	98-112
23	DYDGIGNRERPGFGR	102-116
24	IGNRERPGFGRFERS	106-120
25	ERPGFGRFERSGHSR	110-124
26	FGRFERSGHSRWCDK	114-128
27	ERSGHSRWCDKSVED	118-132
28	HSRWCDKSVEDDWSK	122-136
29	CDKSVEDDWSKPLPP	126-140
30	VEDDWSKPLPPSERL	130-144
31	YDDIPVEATGSNCPP	161-175
32	PVEATGSNCPPHIEN	165-179
33	TGSNCPPHIENFSDI	169-183
34	CPPHIENFSDIDMGE	173-187
35	IENFSDIDMGEIIMG	177-191
36	SDIDMGEIIMGNIEL	181-195

Underlined amino acids indicate the positions at which there is nonidentity between the *DDX3Y*-encoded pentadecapeptide and the homologous peptide encoded by *DDX3X*.

Table 2. DDX3Y pentadecapeptide grid

Peptide pool #	7	8	9	10	11	12
1	1	2	3	4	5	6
2	7	8	9	10	11	12
3	13	14	15	16	17	18
4	19	20	21	22	23	24
5	25	26	27	28	29	30
6	31	32	33	34	35	36

DDX3Y pentadecapeptide grid illustrating the 12 peptide pools, each comprising one row or one column of the grid, used in ELISpot analysis to determine the pentadecapeptide specificity of responding PBMC. The sequences of peptides 1 through 36 are indicated in Table 1.

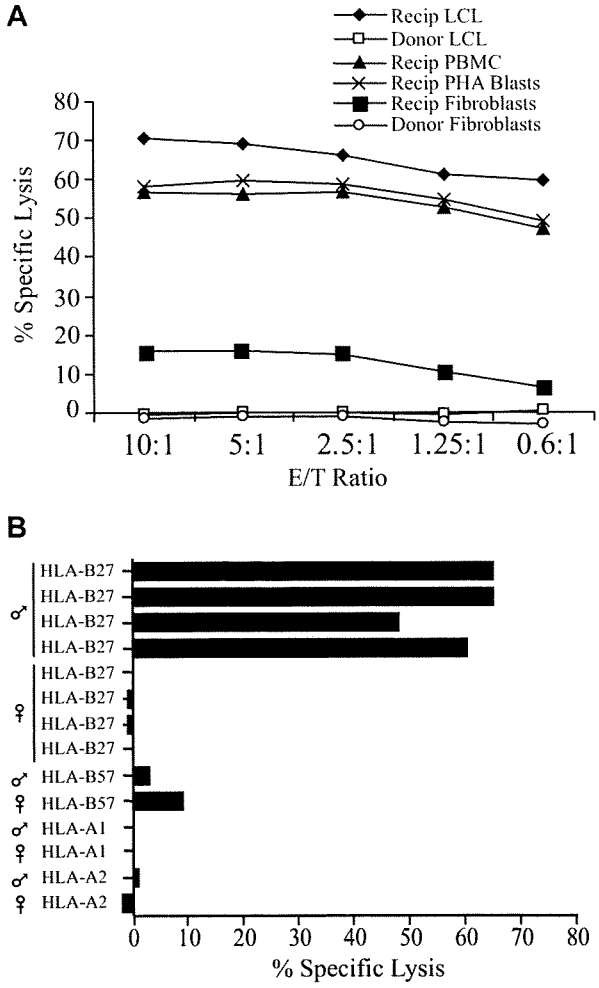

Figure 1. CTL 68H7–819 recognizes a male-specific minor histocompatibility (H-Y) antigen presented by HLA-B*2705. (A) ^{51}Cr release assay at the indicated effector-target (E/T) ratios showing cytolytic activity of CTL 68H7-819 against donor- and recipient-derived EBV-LCLs, recipient-derived unfractionated PBMCs and PHA-stimulated T-cell blasts, and donor- and recipient-derived dermal fibroblasts. (B) ^{51}Cr release assay at E/T 5:1 showing cytolytic activity of CTL 68H7-819 against a panel of EBV-LCLs derived from unrelated male and female individuals who shared a single MHC class I allele with the donor-recipient pair from which CTL 68H7-819 was isolated. The shared MHC class I allele and sex of the individual is indicated.

AZFa locus, but did recognize vac/*HLA-B*2705*–infected WHT2780, which contains a point mutation in *USP9Y* that results in 90% truncation (Figure 2A).[38] CTL 68H7–819 also recognized vac/*HLA-B*2705*–infected WHY26, which carries a Y-chromosomal break in *UTY*. These results suggested that the epitope recognized by CTL 68H7-819 is encoded by *DDX3Y*. This was confirmed by CTL 68H7-819 recognition of COS-7 cells cotransfected with *HLA-B*2705* and *DDX3Y* cDNAs (Figure 2B).

Testing recognition of CTL 68H7-819 against COS-7 cells cotransfected with *HLA-B*2705* cDNA and a panel of overlapping *DDX3Y* minigenes revealed recognition of a minigene encoding residues 1 through 93, but not a minigene encoding residues 1 through 72 (data not shown). An epitope prediction algorithm[39] was used to identify nonameric or decameric peptides in this region expected to bind to HLA-B*2705 with high affinity. Minigenes encoding 3 such peptides—DDX3Y$_{75\text{-}83}$ (RDSRGKPGY), DDX3Y$_{74\text{-}83}$ (SRDSRGKPGY), and DDX3Y$_{74\text{-}84}$ (SRDSRGKPGYF)—were recognized by CTL 68H7-819 in an *HLA-B*2705*–dependent manner (Figure 2B). Donor EBV-LCLs pulsed with

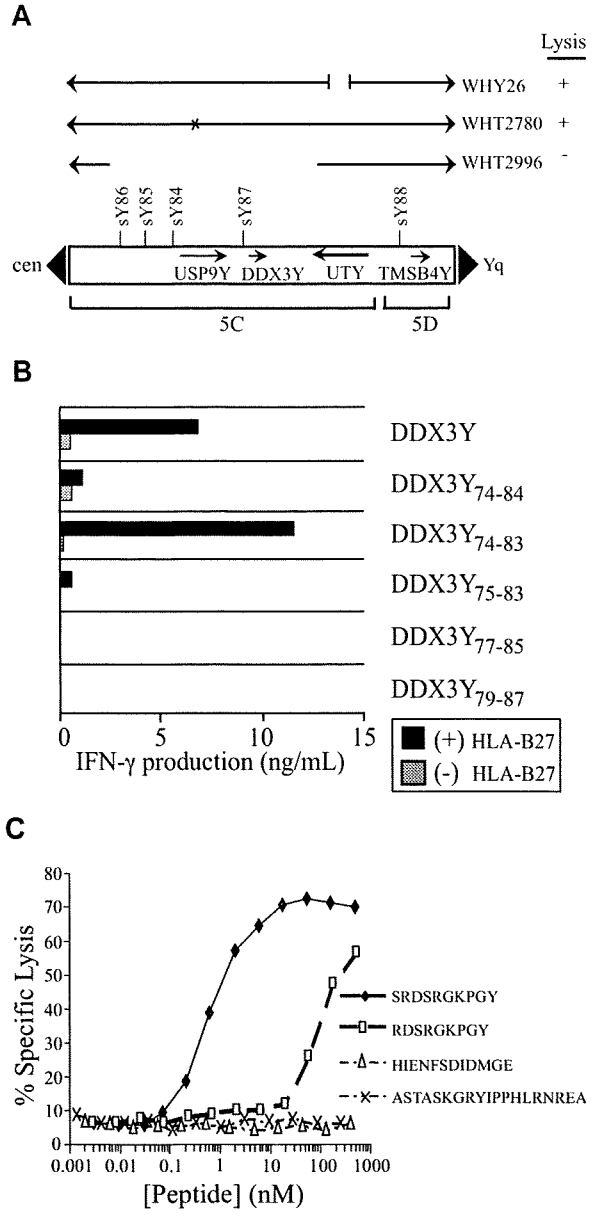

Figure 2. *DDX3Y* encodes the H-Y antigen recognized by CTL clone 68H7-819. (A) Localization by Y-chromosome deletion mapping of the gene encoding the H-Y antigen recognized by CTL 68H7-819. The CTLs were tested for recognition of EBV-LCLs derived from males carrying Y chromosomes with constitutional deletions in a ^{51}Cr release assay at E/T 10:1. EBV-LCLs were infected the day before the assay with a recombinant vaccinia virus carrying an *HLA-B*2705* transgene. EBV-LCL WHT2996 is derived from an individual with a deletion encompassing genes *DDX3Y* and *USP9Y*.[38] WHT2780 is derived from an individual with a splice site deletion that results in 90% truncation of the *USP9Y* gene, indicated by an X.[38] WHY26 is from an individual with a chromosomal break in *UTY*. Arrows indicate the intact and aberrant segments of the Y chromosome. Y-chromosome landmarks include the boundaries of deletion intervals 5C and 5D, and selected sequence-tagged sites. + indicates lysis of 35% or more, and – indicates lysis of 4% or less. (B) Plasmids encoding *DDX3Y* minigenes were cotransfected into COS-7 cells with a plasmid encoding *HLA-B*2705*. On the following day, CTL 68H7-819 was added to the COS-7 transfectants, and IFN-γ release was measured in the supernatants by ELISA after 20 hours of coculture. (C) Epitope reconstitution assay to determine CTL 68H7-819 recognition of donor EBV-LCLs that had been pulsed for 30 minutes with the indicated synthetic peptides over the indicated range of concentrations; 4-hour ^{51}Cr release assay, E/T 5:1. (D) Partial sequence alignment of the DDX3Y and DDX3X proteins spanning the region that includes the epitope recognized by CTL 68H7-819. Asterisks indicate disparate residues.

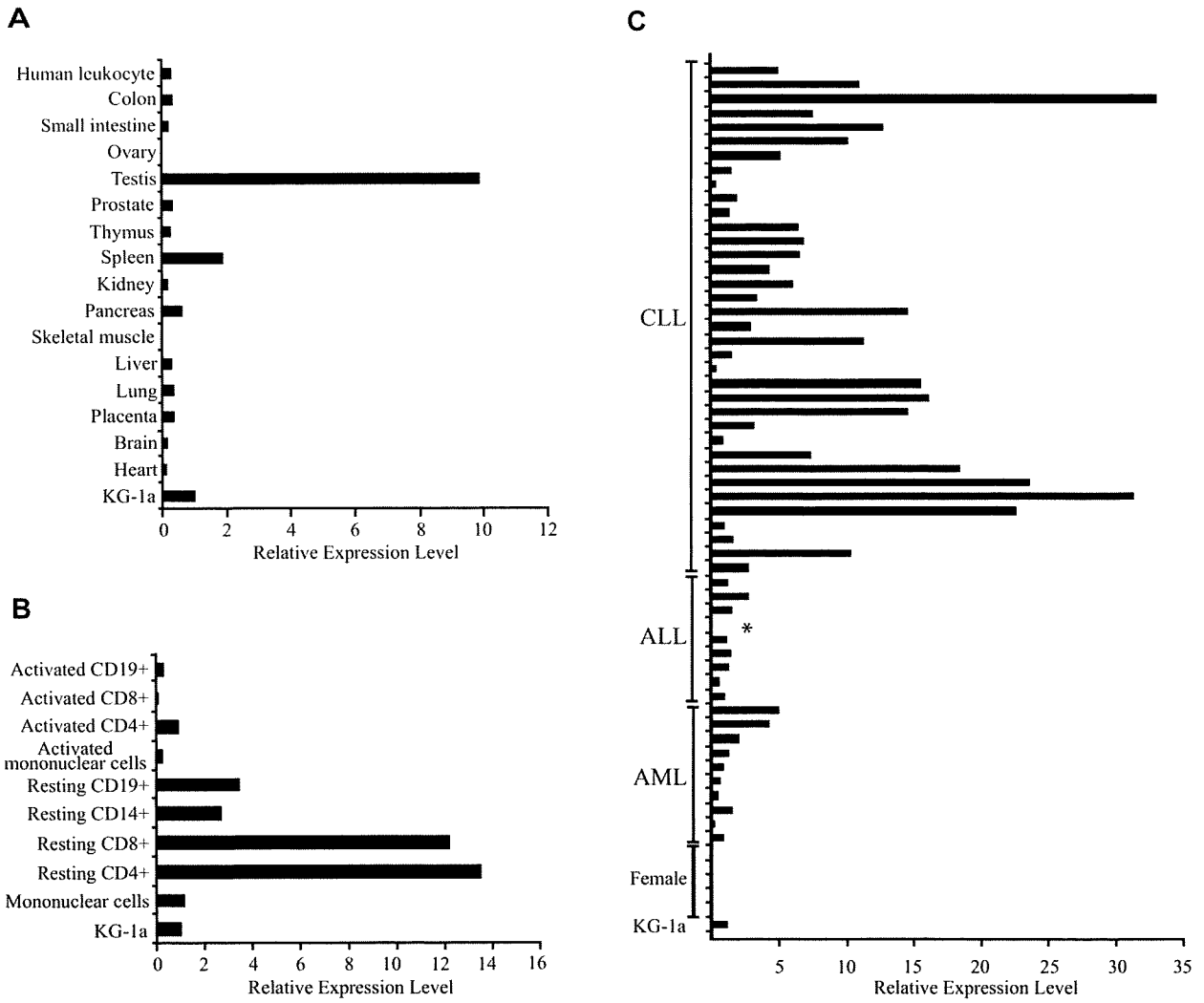

Figure 3. *DDX3Y* is transcribed outside the testis and is universally expressed in myeloid and lymphoid leukemia cells that carry a Y chromosome. Relative expression of *DDX3Y* in a panel of normal human tissues (A), normal blood cell fractions (B), and primary male ALL, CLL, and AML samples (C). Quantitative real-time PCR using SYBR green was carried out as described in "Methods." Analysis of *GAPDH* expression was used to standardize samples for RNA quality and quantity, and the relative *DDX3Y* expression in the KG-1a AML cell line was arbitrarily defined as 1. The asterisk in panel C indicates a primary male ALL sample that had clonal loss of the Y chromosome by cytogenetic analysis and no detectable *DDX3Y* transcript. Five of 14 female samples are shown, all of which were negative.

serial dilutions of synthetic RDSRGKPGY and SRDSRGKPGY peptides were recognized by CTL 68H7-819 with half-maximal recognition seen at both 60 nM and 600 pM, respectively (Figure 2C). No recognition by CTL 68H7-819 of donor EBV-LCLs pulsed with either of the synthetic peptides corresponding to the 2 previously described *DDX3Y*-encoded, MHC class II–restricted H-Y epitopes[3,4] was observed (Figure 2C). These data demonstrate that SRDSRGKPGY represents the HLA-B*2705–restricted H-Y antigen recognized by CTL 68H7-819. Comparison of the predicted DDX3Y protein sequence with that of the homologous X-chromosome–encoded DDX3X protein (Figure 2D) revealed that the SRDSRGKPGY epitope differs at 4 positions with the homologous DDX3X peptide.

DDX3Y expression in cultured cells, primary tissues, and primary leukemic cells

DDX3Y expression was evaluated by semiquantitative real-time PCR of cDNA from normal and malignant tissues of both hematopoietic and nonhematopoietic origin. Analysis of cDNA from a panel of normal human tissues, derived from a pool containing both males and females, revealed that *DDX3Y* transcript was detectable in all normal tissues tested except ovary and skeletal muscle, with the highest levels of expression seen in testis, followed by the spleen and pancreas (Figure 3A). *DDX3Y* transcript was also detectable in all subsets of resting and activated human blood cells, with the highest levels seen in resting blood fractions, and approximately 10-fold lower expression in activated fractions (Figure 3B). *DDX3Y* expression was detected in 54 of 55 primary male leukemic samples (Figure 3C). The relative *DDX3Y* expression levels were highest in male CLL samples (35 samples; mean: 9.1; median: 6.3; range: 0.4-33.5), followed by AML (11 samples; mean: 1.7; median: 1.1; range: 0.2-5.0) and ALL samples (9 samples; mean: 1.4; median: 1.3; range: 0.6-2.8). *DDX3Y* expression was undetectable in all 14 female malignant samples tested (Figure 3C and data not shown). *DDX3Y* expression in the recipient fibroblasts from which CTL 68H7-819 was derived was 0.3 compared with the reference sample KG-1a, and undetectable in the female donor fibroblast sample (data not shown). One leukemic sample obtained from a male patient with ALL did not express

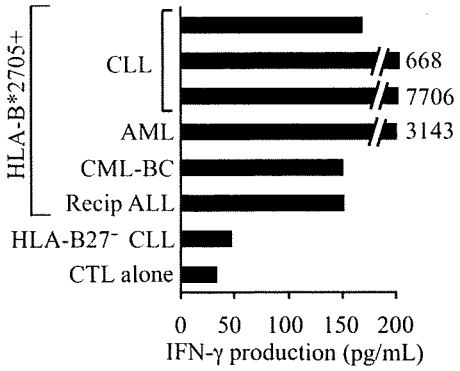

Figure 4. The *DDX3Y*-encoded H-Y antigen recognized by CTL 68H7-819 is expressed on the surface of HLA-B*2705$^+$ leukemic cells. CTL 68H7-819 recognition of primary leukemic samples from males carrying the HLA-B*2705 allele was tested by IFN-γ ELISA after overnight coculture with CTL at a CTL/leukemia ratio of 1:5. The 6 samples consisted of BMMCs derived from the hematopoietic cell transplant recipient from whom CTL 68H7-819 was derived (Recip ALL), PBMCs from one patient each with primary refractory AML and with CML in T-lymphoid blast crisis (CML-BC), and 3 PBMC samples from CLL patients. Negative controls included a CLL sample that did not carry the *HLA-B*2705* allele, and CTL 68H7-819 alone.

detectable *DDX3Y* transcript (indicated by asterisk in Figure 3C). Cytogenetic analysis of this sample revealed clonal loss of the Y chromosome (data not shown).

The *DDX3Y*-encoded H-Y epitope is presented on the surface of HLA-B*2705$^+$ leukemias

To investigate the expression of the *DDX3Y*-encoded H-Y epitope on the surface of primary leukemic cells, leukemic samples that natively expressed the restricting molecule HLA-B*2705 were identified by genotyping the HLA-B locus in each of the 54 primary male leukemic samples that carried a Y chromosome. Of these, 6 carried a HLA-B*2705 allele, consistent with the low frequency of this allele in the white population from which most of the samples were obtained.[40] The 6 HLA-B*2705$^+$ leukemic samples included BMMCs from the ALL patient from whom CTL clone 68H7-819 was derived, as well as PBMCs from 3 patients with CLL, 1 patient with CML in T-lymphoid blast crisis (CML-BC), and 1 patient with primary refractory AML. Each sample contained between 80% to 90% leukemic cells, and were reproducibly recognized by CTL 68H7-819 in an IFN-γ ELISA assay (representative data in Figure 4); no recognition of HLA-B*2705$^-$ or female leukemic samples was seen. These results demonstrate that the *DDX3Y*-encoded H-Y epitope is expressed on the surface of both acute and chronic leukemia cells of both myeloid and lymphoid lineage.

The *DDX3Y*-encoded H-Y epitope is expressed by leukemic stem cells

To determine whether the *DDX3Y* epitope is expressed on the putative leukemic stem cell that can establish human acute leukemia in NOD/SCID mice, CTL clone 68H7-819 was tested for its ability to inhibit the engraftment of HLA-B27$^+$ primary leukemic cells. PBMCs from the 2 HLA-B*2705$^+$ patients with T-lymphoid blast crisis of CML and primary refractory AML were used for these experiments. CTL 68H7-819 demonstrated in vitro recognition of both leukemic samples by both IFN-γ ELISA assay (Figure 4) as well as by chromium release assay (Figure 5A, and data not shown).

In the first NOD/SCID experiment, control mice injected with CML-BC cells alone did not survive beyond 24 days after injection, but mice that received equal doses of CML-BC cells that had been cultured overnight with CTL 68H7-819 uniformly survived to day 35, at which point they were killed (Figure 5B). Analysis of the 2 groups of mice for engraftment with human cells by flow cytometry and PCR with human Y-chromosome–specific primers confirmed the presence of human leukemic cells (Figure 5C) and human Y-chromosome DNA (Figure 5D) in the BMMCs of all mice that received CML-BC cells alone and survived at least 15 days after injection. In contrast, all mice that received CML-BC cells cocultured with CTL 68H7-819 had 0.01% or less human cells by flow cytometry (Figure 5C) and no detectable human Y-chromosome DNA by PCR (Figure 5D). CTL 68H7-819 had no inhibitory effect on the engraftment of leukemic cells obtained from a female AML patient (data not shown).

In the second NOD/SCID experiment, mice that received injections of AML cells from an HLA-B*2705$^+$ male that were cultured overnight in medium alone exhibited high levels of bone marrow engraftment, ranging from 51% to 87% when assessed at the time of death at 6 weeks after injection (Figure 6). PCR analysis revealed human Y-chromosome DNA in bone marrow, peripheral blood, spleen, thymus, lung, liver, and kidney. In contrast, all mice injected with AML cells that had been cultured overnight with CTL 68H7-819 showed no detectable human cells in the bone marrow by flow cytometry and no detectable human Y-chromosome DNA by PCR (Figure 6), comparable with mice injected with PBS alone. Engraftment of AML cells was not inhibited by overnight culture with an irrelevant CD8$^+$ CTL clone specific for a distinct minor histocompatibility antigen not expressed by the AML (Figure 6), ruling out nonspecific inhibition of engraftment by overnight coculture with CTLs. Moreover, CTL 68H7-819 once again did not inhibit the engraftment of a control AML sample derived from a female, demonstrating that the inhibitory effect was male specific (data not shown).

Quantitation of DDX3Y-specific CD8$^+$ and CD4$^+$ T-cell responses in UPN 19492

IFN-γ ELISpot analysis was performed to estimate the frequency of $DDX3Y_{74\text{-}83}$-specific T cells in the peripheral blood of UPN 19492 at intervals during the first 100 days after transplantation, during which time he did not have histologic evidence of GVHD and also remained in complete remission. Analysis of CTL 68H7-819 serially diluted into polyclonal PBMCs revealed that the sensitivity of the ELISpot assay was approximately 1 in 3000 cells. Direct ex vivo analysis of PBMCs obtained on days +42, +62, +93, and +109 after transplantation did not detect $DDX3Y_{74\text{-}83}$-specific responses, indicating that the frequency of $DDX3Y_{74\text{-}83}$-specific T cells in the blood at these time points was less than 0.03%.

HLA typing of UPN 19492 revealed that he also expressed the MHC class II molecules, HLA-DQ5 and HLA-DRB1*1501, that have been shown in previous studies[3,4] to present other *DDX3Y*-encoded H-Y peptides ($DDX3Y_{176\text{-}187}$ and $DDX3Y_{30\text{-}48}$, respectively) to CD4$^+$ T cells. Since the CD8$^+$ $DDX3Y_{74\text{-}83}$-specific response was detected in PBMCs obtained on day +42 after transplantation, day +42 PBMCs were analyzed both directly ex vivo and after in vitro stimulation with the $DDX3Y_{176\text{-}187}$ and $DDX3Y_{30\text{-}48}$ peptides in ELISpot assays to determine whether CD4$^+$ T-cell responses to the 2 MHC class II–restricted DDX3Y epitopes had developed concurrently. The DDX3Y-specific CD4$^+$ T-cell responses were not detected in PBMCs obtained at days +42, +62, +100, and +109 after transplantation, when analyzed both directly ex vivo and after multiple (up to 6) cycles of in vitro

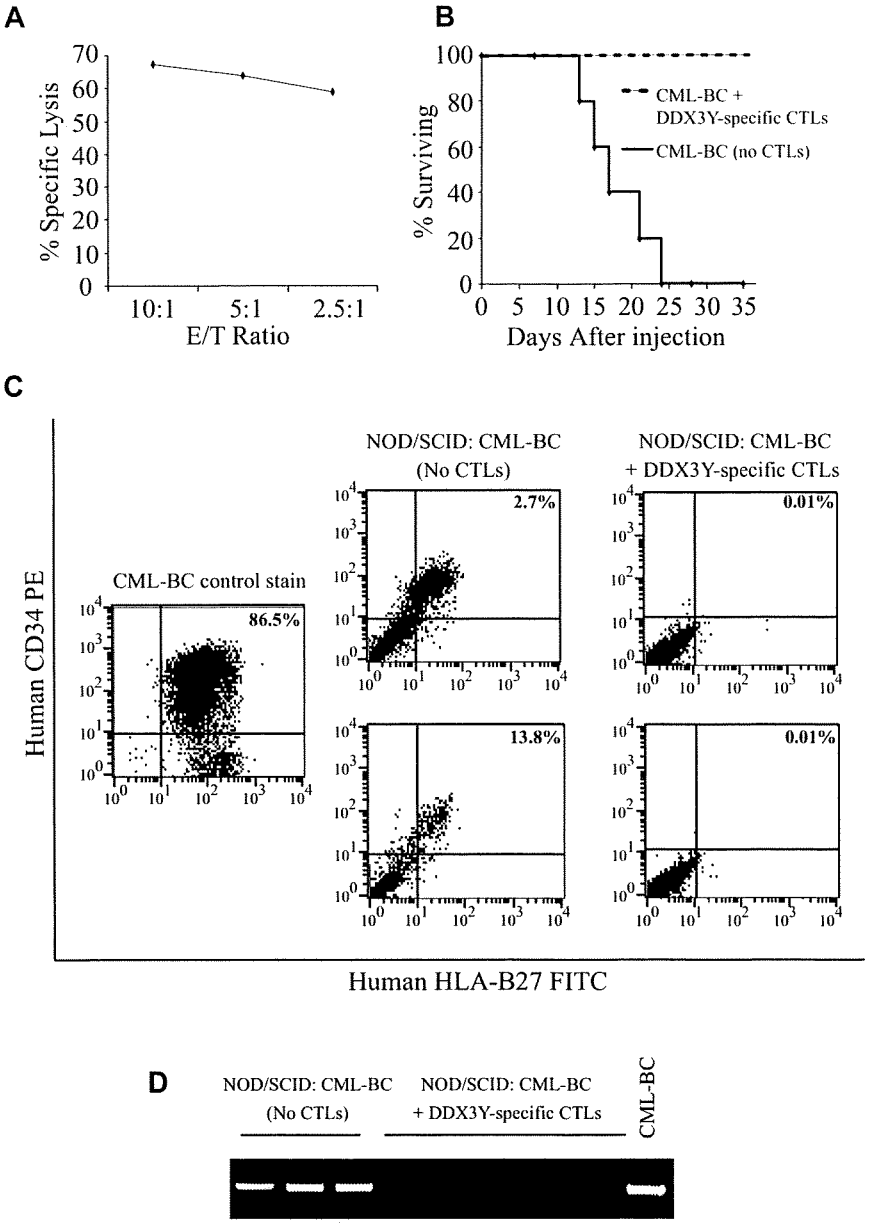

Figure 5. CTL 68H7-819 targets the leukemic stem cell in T-lymphoid blast phase CML (CML-BC). (A) Recognition of CML-BC cells by CTL 68H7-819 in a ^{51}Cr release assay at the indicated E/T ratios. (B) Survival analysis of mice injected with CML-BC cells cultured overnight in medium alone or with CTL 68H7-819. Each group was composed of 5 mice. (C) Flow cytometric analysis using human PE-conjugated antihuman CD34 and FITC-conjugated anti–HLA-B27 antibodies of BMMCs from representative mice injected with CML-BC cells that had been cultured overnight in medium alone or with CTL 68H7-819. Uninjected CML-BC cells were used as a positive control. (D) Human Y-chromosome PCR analysis of genomic DNA extracted from mouse BMMCs to detect human male leukemic cells. Flow cytometric and PCR analysis was performed on mice that survived at least 15 days after injection.

stimulation with the $DDX3Y_{176-187}$ and $DDX3Y_{30-48}$ peptides. Therefore, T-cell responses to the MHC class II–restricted epitopes $DDX3Y_{176-187}$ and $DDX3Y_{30-48}$ were either not present in the recipient's peripheral blood at the indicated time points, or were present at a frequency below the detection limit of the ELISpot assay.

Identification of novel DDX3Y-specific T-cell responses in a F→M hematopoietic cell transplant recipient with active GVL

To determine whether DDX3Y-specific T cells are detectable in F→M hematopoietic cell transplant recipients who are actively experiencing GVL activity, we analyzed posttransplantation PBMCs from an HLA-B*2705–negative patient (UPN 21234) with MDS/RAEB-2 who relapsed on day +62 after he received a hematopoietic cell transplant from his MHC-identical sister, underwent withdrawal of immune suppression, and subsequently achieved a complete remission of his disease by day +160 after transplantation. Recipient PBMCs obtained on day +127, after relapse and withdrawal of immune suppression but before documentation of complete remission, were stimulated in vitro with a mixture of 36 DDX3Y-derived, overlapping pentadecapeptides that collectively spanned the amino-terminal 195 residues of the 660-residue protein (Table 1). Responder PBMCs were analyzed by ELISpot for reactivity against 12 peptide pools comprising the peptides in the 6 rows and 6 columns of a 6 × 6 array of the 36 pentadecapeptides (Table 2). Significant peptide-specific responses were detected against pools 3, 5, 10, and 11, with the largest responses against the orthogonal pools 3 and 11 (Figure 7A). These 2 pools share pentadecapeptide no. 17, with the sequence SFGSRDSRGKPGYFS in common (Tables 1,2). An epitope prediction algorithm[39] was then used to identify any peptides within this pentadecameric sequence that were predicted to bind to the recipient's MHC molecules, which included HLA-A*01, -A*02, -B*07, -B*15, -DRB1*0401/0404, and -DQB1*03 (data not shown). The peptide with the highest predicted binding affinity was the decamer SRDSRGKPGY ($DDX3Y_{74-83}$), identical to the HLA-B*2705–

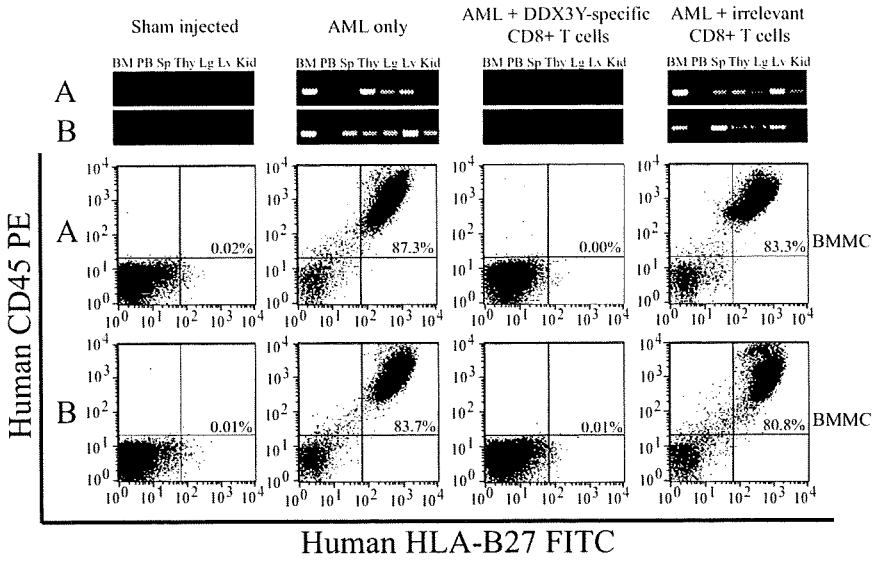

Figure 6. CTL 68H7-819 targets the leukemic stem cell in AML. Flow cytometric analysis using human PE-conjugated antihuman CD45 and FITC-conjugated anti–HLA-B27 antibodies of BMMCs from representative mice injected with PBS (sham), or with AML cells that had either been cultured overnight in medium alone, with CTL 68H7-819, or with an irrelevant CD8+ CTL clone that did not recognize the AML in vitro. Top panels show human Y-chromosome–specific PCR analysis of genomic DNA isolated from BMMCs (BM), peripheral blood (PB), spleen (Sp), thymus (Thy), lung (Lg), liver (Lv), and kidney (Kid). The flow cytometry and PCR data from 2 representative mice from each group are shown, and are designated as A or B.

restricted epitope recognized by CTL 68H7-819, which was predicted to bind to HLA-A*01. An aliquot of the T-cell line that had been stimulated with the pool of 36 DDX3Y-derived pentadecapeptides was then restimulated with the SFGSRDSRGKPGYFS pentadecamer, and subsequent ELISpot analysis demonstrated specific reactivity with the SRDSRGKPGY decamer (Figure 7B).

Discussion

The human *DDX3Y* gene is expressed at high levels in the testis and encodes a 660-residue protein that has only 92% sequence identity with the protein encoded by its X-chromosome homologue, *DDX3X*. Although the precise function of the DDX3Y protein is unknown, deletion of the AZFa locus on the Y chromosome that includes *DDX3Y* results in Sertoli-cell-only (SCO) syndrome and azoospermia.[41] *DDX3Y* orthologues in *C elegans*,[42] *Drosophila*,[43] *Xenopus*,[44] mice,[45] and zebrafish[46] are also implicated in the development or function of germ cells. *DDX3Y*, however, is also expressed outside the immunologic sanctuary of the testis, in both normal as well as malignant tissues, which enables presentation of DDX3Y to the afferent and efferent arms of the immune system. Consequently, in the setting of allogeneic F→M HCT, the female immune system encounters DDX3Y protein in extratesticular tissues, and this encounter can elicit CD4+ T-cell,[3,4] B-cell,[47,48] and, as shown for the first time in this study, CD8+ T-cell immunity.

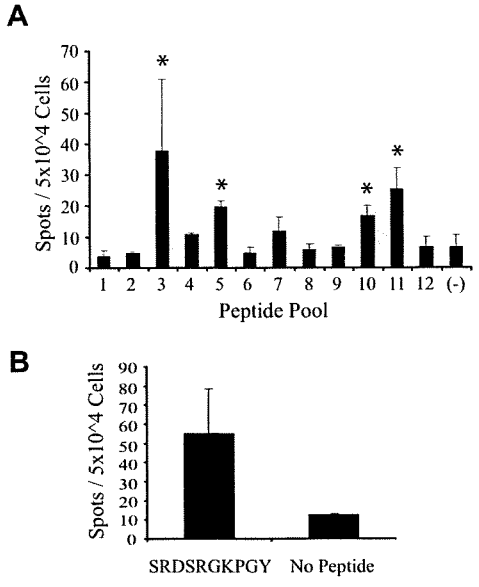

Figure 7. DDX3Y peptide–specific T-cell responses in a F→M hematopoietic cell transplant recipient experiencing GVL. PBMCs obtained from UPN 21234 (HLA-A*01, -A*02, -B*07, -B*15, -DRB1*0401/0404, and -DQB1*03) on day +127 after transplantation were stimulated in vitro with a pool of 36 overlapping DDX3Y-derived pentadecapeptides pulsed onto donor-derived EBV-LCLs and subsequently analyzed by IFN-γ ELISpot for DDX3Y peptide–specific responses (Tables 1,2). (A) ELISpot analysis of day +127 PBMCs that had been stimulated in vitro with the entire pool of 36 DDX3Y-derived pentadecapeptides. Asterisks indicate the 4 peptide pools that stimulated spot formation above background. (B) An aliquot of the T-cell line analyzed in panel A was restimulated with pentadecapeptide no. 17, and subsequently analyzed by ELISpot for reactivity with the SRDSRGKPGY decamer, encompassed within the sequence of pentadecapeptide no. 17.

Although the identification of a MHC class I–restricted H-Y antigen encoded by *DDX3Y* further illustrates the immunogenicity of its gene product in F→M HCT, the results of our study do not permit drawing any firm conclusions about the extent to which CD8+ DDX3Y-specific T-cell responses may contribute to GVHD. The hematopoietic cell transplant recipient from whom CTL clone 68H7–819 was isolated did not develop histologic evidence of either acute or chronic GVHD during the first 100 days after transplantation, but did receive a short course of prednisone therapy for diarrhea that was initially attributed to GVHD but subsequently attributed to *C difficile* infection. However, ELISpot analysis demonstrated that the frequency in the peripheral blood of T cells reactive with the *DDX3Y*-encoded SRDSRGKPGY peptide remained quite low (< 0.03%) throughout this interval. Thus, it is quite possible that the magnitude of the CD8+ DDX3Y-specific T-cell response was insufficient to trigger clinically significant manifestations of GVHD. Previous studies have reported an association of CD4+ T-cell responses to MHC class II–restricted epitopes derived from *DDX3Y* with severe acute[3] or extensive chronic[4] GVHD in F→M hematopoietic cell transplant recipients. ELISpot analysis did not detect T-cell responses against these MHC class II–restricted DDX3Y epitopes in UPN 19492, suggesting that any CD4+ DDX3Y-specific T-cell response that may have developed in this patient may also have been of insufficient magnitude to trigger clinically significant GVHD. Definitive assessment of the extent to which CD8+ DDX3Y-specific T cells can contribute to GVHD will likely require adoptive transfer studies[49] in which the GVHD potential of the DDX3Y-specific cells

can be more confidently distinguished from the effects of simultaneous T-cell responses against other alloantigens.

Several lines of evidence suggest that CD8+, MHC class I–restricted T-cell responses to *DDX3Y* could potentially contribute to GVL activity after F→M HCT. First, *DDX3Y* transcripts are detected in all male leukemic samples that retain a Y chromosome. Second, the $DDX3Y_{74\text{-}83}$ peptide recognized by CTL 68H7-819 is expressed on the surface of both myeloid and lymphoid leukemic cells from *HLA-B*2705*+ males. Third, the HLA-B*2705–restricted $DDX3Y_{74\text{-}83}$ epitope is expressed on the surface of the putative leukemic stem cell(s) that can establish AML and T-lymphoid blast phase CML in NOD/SCID mice. It is unclear whether UPN 19492, the patient from whom CTL 68H7-819 was isolated, might have benefited from any antileukemic effect mediated by CD8+ DDX3Y-specific T cells, since he underwent transplantation in complete remission, and remained in remission throughout the period of observation. Therefore, to determine whether DDX3Y-specific T-cell responses could be detected in F→M hematopoietic cell transplant recipients experiencing active GVL responses, we looked for DDX3Y-specific T cells in an HLA-B*2705–negative patient, UPN 21234, who suffered relapse of MDS/RAEB-2 on day +62 after transplantation and subsequently achieved a complete remission after withdrawal of immune suppression. Although the scope of this analysis was limited to less than a third of the total DDX3Y protein sequence, it demonstrated the presence of T cells reactive with several pools of DDX3Y-derived peptides and indicates that additional *DDX3Y*-encoded T-cell epitopes remain to be identified. In addition, the identification of a T-cell response specific for $DDX3Y_{74\text{-}83}$ in a HLA-B*2705–negative male hematopoietic cell transplant recipient indicates that this peptide can be presented by additional MHC allele(s). Identification of these alleles and characterization of other novel *DDX3Y*-encoded epitopes, and their expression in normal and malignant tissues, are the focus of active research in our laboratory.

All human H-Y antigens identified to date[50] are encoded by a specific class of Y-chromosome genes that have X-chromosome homologues and are expressed both in and variably outside the testis.[15,20,21] There are 15 such genes on the human Y chromosome, and sequence analysis of their predicted protein products reveals 990 residues at which there is sequence nonidentity between the Y- and the X-encoded isoforms. Thus, it is very likely that the Y chromosome encodes a large number of H-Y antigens that remain to be identified, all of which would be expected to be in perfect linkage disequilibrium. The predicted 660-residue DDX3Y protein sequence, for example, contains more than 50 contiguous peptide sequences that (1) differ by one or more residues with the homologous peptides in DDX3X, and (2) are predicted to bind with high affinity to common class I MHC alleles, including HLA-A*0101, -A*0201, -A*0301, -B*0702, -B*0801, -B*4402, and -B*4403 (K.V.R. and E.H.W., unpublished observations, March 2007). The demonstration that a DDX3Y-specific CD8+ T-cell response can contribute to GVL activity provides an experimental basis for the clinical observation of a selective GVL effect associated with F→M HCT,[24,26,27] and raises the prospect that further studies of H-Y immunity could eventually lead to strategies for selectively enhancing the GVL effect in recipients of sex-mismatched transplants.

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Authorship

Contribution: K.V.R., N.F., J.K.M., K.K.W.K., S.M.X., and E.H.W. performed experiments; O.S.-T., J.S.G., J.P.R., Y.A., and B.J.V.E. contributed vital new reagents; E.H.W., K.V.R., N.F., and S.R.R. analyzed the data; E.H.W. and K.V.R. designed the research, wrote the paper, and made the figures; all authors edited the paper.

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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

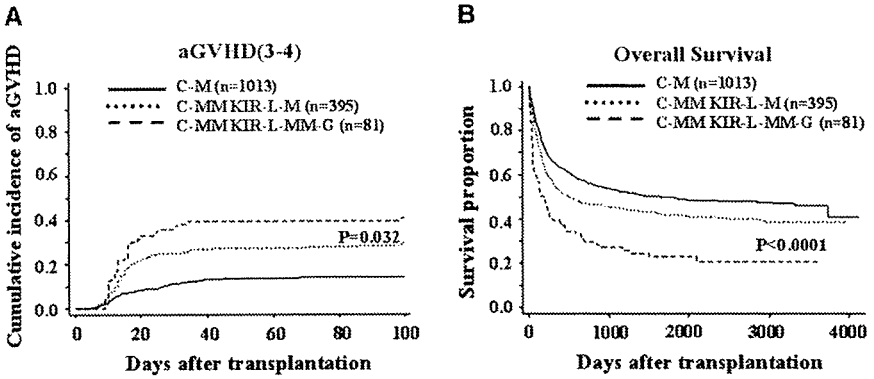

Figure 1. Effects of KIR ligand mismatch on transplantation outcome. Cumulative incidence of acute GVHD (grade III-IV) (A) and overall survival (B) by matching of KIR ligands in the GVHD direction. The directions of HLA-C mismatching were GVH and/or HVG. All patients were analyzed. The solid line represents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