Table 3. Results of multivariate analysis of overall survival according to disease status at transplantation

	First complete remission			Second or after complete remission			More advanced		
Overall survival	**n**	**RR (95% CI)**	***P***	**n**	**RR (95% CI)**	***P***	**n**	**RR (95% CI)**	***P***
AML									
UBMT	130	1.00		82	1.00		95	1.00	
UCBT	50	2.92 (1.38-6.18)	.005	39	1.24 (0.51-3.04)	.63	81	1.29 (0.84-1.98)	.25
ALL									
UBMT	130	1.00		48	1.00		42	1.00	
UCBT	63	1.60 (0.84-3.05)	.16	21	0.62 (0.22-1.74)	.36	30	0.80 (0.38-1.69)	.57

RR indicates relative risk; CI, confidence interval; UBMT, unrelated bone marrow transplantation; and UCBT, unrelated cord blood transplantation.

The unadjusted cumulative incidence of platelet recovery greater than 50 000/μL at 4 months was significantly lower among CB recipients for both AML (59% vs 85%) and ALL (61% vs 83%) compared with that of BM recipients ($P < .001$ for both). The difference was also significant on multivariate analyses for both AML (RR = 0.3, 95% CI = 0.3-0.4, $P < .001$) and ALL (RR = 0.4, 95% CI = 0.3-0.6, $P < .001$; Table 2).

Acute GVHD. The unadjusted cumulative incidence of grade 2 to 4 acute GVHD was lower among CB recipients compared with that among BM recipients (32% vs 35% in AML, 28% vs 42% in ALL); the difference was significant in patients with ALL ($P = .39$ in AML, $P = .008$ in ALL). The difference was also significant on multivariate analyses in ALL (RR = 0.6, 95% CI = 0.4-1.0, $P = .028$). There was no significant difference in patients with AML (RR = 0.8, 95% CI = 0.6-1.2, $P = .23$; Table 2).

Chronic GVHD. The unadjusted cumulative incidence of chronic GVHD at 1 year after transplantation did not significantly differ between CB recipients and BM recipients in both AML (28% vs 32%, $P = .46$) and ALL (27% vs 30%, $P = .50$). The cumulative incidence of extensive-type chronic GVHD was significantly lower among CB recipients compared with that among BM recipients in both AML (8% vs 20%, $P < .001$) and ALL (10% vs 17%, $P = .034$). On multivariate analyses, the risk of developing chronic GVHD was similar in CB recipients and BM recipients in both AML (RR = 0.9, 95% CI = 0.6-1.4, $P = .79$) and ALL (RR = 1.1, 95% CI = 0.7-1.8, $P = .77$). The risk of developing extensive chronic GVHD was lower in CB recipients compared with BM recipients (RR = 0.4, 95% CI = 0.2-0.7, $P = .004$ in AML, and RR = 0.6, 95% CI, 0.3-1.2, $P = .14$ in ALL) and was significantly different in patients with AML (Table 2).

Discussion

The objective of our study was to investigate the outcomes of HLA-A, -B, low-resolution, and -DRB1 high-resolution 0 to 2 mismatched single-unit unrelated CBT in adult patients with acute leukemia compared with those of HLA-A, -B, -C, and -DRB1 (8 of 8) allele-matched unrelated BMT. Although AML and ALL are different diseases, previous comparisons of unrelated BMT and

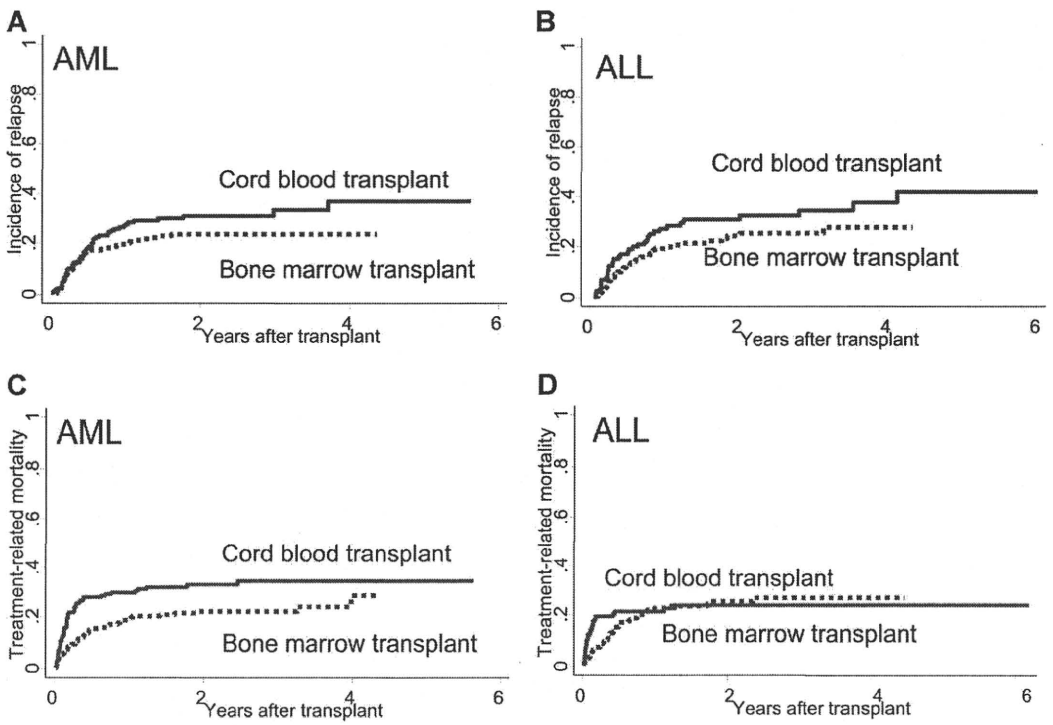

Figure 2. Cumulative incidence of relapse or TRM of recipients of CB or BM among patients with AML or ALL. For patients with AML, the cumulative incidence of (A) relapse (CB vs BM = 31% vs 24% at 2 years, $P = .068$) and (C) TRM (CB vs BM = 33% vs 22% at 2 years, $P = .004$) was higher in CB recipients. For patients with ALL, the cumulative incidence of relapse (B) was higher in CB recipients with marginal significance (CB vs BM = 31% vs 24% at 2 years, $P = .085$), but the incidence of TRM (D) was similar in CB and BM recipients (CB vs BM = 24% vs 25% at 2 years, $P = .83$).

Table 4. Causes of death after transplantation of unrelated cord blood or unrelated bone marrow among patients with acute myeloid leukemia or acute lymphoblastic leukemia

	Acute myeloid leukemia		Acute lymphoblastic leukemia	
Cause of death	UCBT	UBMT	UCBT	UBMT
Recurrence of disease	35 (37)	34 (33)	18 (36)	34 (41)
Graft failure/rejection	3 (3)	4 (4)	0 (0)	3 (4)
Graft-versus-host disease	6 (6)	7 (7)	3 (6)	5 (6)
Infection	22 (23)	19 (18)	13 (26)	11 (13)
Idiopathic pneumonia	4 (4)	4 (4)	2 (4)	6 (7)
Organ failure	17 (18)	17 (16)	8 (16)	10 (12)
Secondary cancer	0 (0)	1 (1)	0 (0)	0 (0)
Other causes	5 (5)	5 (5)	2 (4)	4 (5)
Unknown/data missing	2 (2)	13 (13)	4 (8)	10 (12)
Total	94 (100)	104 (100)	50 (100)	83 (100)

Data are presented as n (%).

UCBT indicates unrelated cord blood transplantation; and UBMT, unrelated bone marrow transplantation.

unrelated CBT did not separate these 2 diseases. Our report is the first to show the result of disease-specific analyses with a sufficient number of patients.

For AML patients, the recipients of CB were more likely to have advanced leukemia at the time of transplantation, as reported previously, suggesting that CB was used as an alternative stem cell source in the later phase of unrelated donor searches, especially in adults.[11,12,14] A larger proportion of CB recipients with ALL had the Philadelphia chromosome abnormality, which correlates with highly aggressive ALL and usually requires urgent transplantation, in which CB has an advantage over BM.[21]

Different outcomes of mortality were found between AML and ALL in a controlled comparison using multivariate analyses. Whereas significantly lower OS and LFS rates were observed in CB recipients with AML, rates of overall mortality and treatment failure were similar between CB and BM recipients with ALL. The relapse rate was not different between CBT and BMT in patients with both AML and ALL, which was consistent with previous reports.[11-13] In adult patients with ALL, a previous report showed no difference in the outcome of related compared with unrelated BM or peripheral blood transplantation in 1CR.[22] Favorable disease status at transplantation could be a more important factor affecting outcome rather than the type of stem cell source or donor type in patients with ALL. It is notable that TRM in HLA allele-matched unrelated BM recipients with AML was quite low in our study. This is probably associated with the low incidence of acute and chronic GVHD in the Japanese population, which is thought to be the result of genetic homogeneity.[23-26] Among patients with AML, although the difference was not statistically significant, a higher trend of TRM observed in CB recipients might be associated with higher overall and TRM rates in CB recipients. Reasons for higher TRM could include the graft source and delayed neutrophil recovery. Better supportive care is required after CBT for patients going through a prolonged neutropenic period. Development of better graft engineering or better conditioning regimens would help to decrease the TRM rate in CB recipients. Because relapse was the major cause of death in all groups, any attempt to decrease TRM should preserve the antileukemia effect to improve OS and LFS. Another reason for the higher TRM could be a higher risk patient population, higher risk for both disease status and comorbid conditions, requiring rapid transplantation. Searching for unrelated donors earlier and providing transplantation earlier in the disease course could help to decrease TRM in CB recipients.

Neutrophil and platelet recovery was slower in CB recipients with either AML or ALL, consistent with the results of previous reports.[11,12,27] Multiple studies have reported lower incidence of acute GVHD in CB recipients.[8-10,12,13] In our study, particularly in patients with ALL, the risk of developing grade 2 to 4 acute GVHD in CB recipients was lower compared with BM recipients, which was reported to be lower compared with the incidence reported from Western countries.[23-25] The risk of developing chronic GVHD was similar between CB and BM recipient with either disease, but the risk of developing extensive-type chronic GVHD was lower in CB recipients; the difference was significant in patients with AML. It is notable that there was no increase in the incidence of acute or chronic GVHD in CB recipients among patients with either AML or ALL, despite HLA disparity.

For differences in outcomes between AML and ALL, one possibility is a difference of treatment before conditioning therapy. Most AML patients received a more intense treatment for induction and consolidation therapy compared with that for ALL. There was no adjustment made for previous treatment, and this could be the reason for higher mortality in CBT, which requires a longer time for neutrophil recovery. Another possible cause of the difference in outcomes is the difference in conditioning regimens. Preparative regimens were similar between CB and BM recipients among ALL patients. However, in patients with AML, the proportion of standard regimens, such as cyclophosphamide and TBI or busulfan and cyclophosphamide, was smaller among CB recipients. These differences in the distribution of preparative regimens were also seen in a previous report.[11] Although the final model was adjusted for conditioning regimens, we cannot rule out the possibility of an effect that larger CB recipients received additional or different chemotherapeutic agents compared with BM recipients among patients with AML. Although the difference was small, the median age of CB recipients with AML was 4 years older than CB recipients with ALL (median age, 38 vs 34 years, $P = .021$), which might have affected the higher mortality rate among CB recipients with AML. It is also possible that some unknown biologic aspects have contributed to these differences, and this would require further evaluation in future studies.

Further subgroup analyses indicated that the superiority of HLA allele-matched BM versus CB for OS was mostly found in patients with AML showing 1CR at conditioning. However, because of the limited numbers of patients in these subgroup analyses and the possibility of an unidentified bias in stem cell source selection, our findings should be verified by further analysis in a larger population.

In conclusion, we found different outcomes between patients with AML and ALL, indicating the importance of disease-specific analyses in alternative donor studies. HLA-A, -B low-resolution, and -DRB1 high-resolution 0 to 2 mismatched single-unit CB is a favorable alternative stem cell source for patients without a suitable related or 8 of 8 matched unrelated BM donor. In the absence of a suitable donor, unrelated CBT should be planned promptly to transplant the patient while in a better disease status and better clinical condition. For patients with AML, decreasing mortality, especially in the early phase of transplantation, is required to improve the outcome for CB recipients.

Acknowledgments

The authors thank all the staff members of the collaborating institutes of the Japan Cord Blood Bank Network and Japan Marrow Donor Program for their assistance and cooperation and

Dr Takakazu Kawase for validating human leukocyte antigen data of the Japan Marrow Donor Program.

This work was supported by a Research Grant for Tissue Engineering (H17-014), a Research Grant for Allergic Disease and Immunology (H20-015), and a Research Grant for Cancer (H19-1) from the Japanese Ministry of Health, Labor, and Welfare.

Authorship

Contribution: Y.A. and R.S. designed the study and wrote the paper; Y.A. analyzed results and made the figures; S. Kato and Y.M. designed the research; T.-N.I., H.A., and M. Takanashi reviewed and cleaned the Japan Cord Blood Bank Network data and reviewed the results; S. Taniguchi, S. Takahashi, S. Kai, H.S., Y. Kouzai, M.K., and T.F. submitted and cleaned the data; and S.O., M. Tsuchida, K.K., Y.M., and Y. Kodera reviewed and cleaned the Japan Marrow Donor Program data and reviewed the results.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

A complete list of members from the Japan Marrow Donor Program and the Japan Cord Blood Bank Network can be found in the Supplemental Appendix (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

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References

1. Szydlo R, Goldman JM, Klein JP, et al. Results of allogeneic bone marrow transplants for leukemia using donors other than HLA-identical siblings. J Clin Oncol. 1997;15:1767-1777.
2. Petersdorf EW, Gooley TA, Anasetti C, et al. Optimizing outcome after unrelated marrow transplantation by comprehensive matching of HLA class I and II alleles in the donor and recipient. Blood. 1998;92:3515-3520.
3. Hansen JA, Gooley TA, Martin PJ, et al. Bone marrow transplants from unrelated donors for patients with chronic myeloid leukemia. N Engl J Med. 1998;338:962-968.
4. Wagner JE, Rosenthal J, Sweetman R, et al. Successful transplantation of HLA-matched and HLA-mismatched umbilical cord blood from unrelated donors: analysis of engraftment and acute graft-versus-host disease. Blood. 1996;88:795-802.
5. Kurtzberg J, Laughlin M, Graham ML, et al. Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. N Engl J Med. 1996;335:157-166.
6. Gluckman E, Rocha V, Boyer-Chammard A, et al. Outcome of cord-blood transplantation from related and unrelated donors: Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. N Engl J Med. 1997;337:373-381.
7. Rubinstein P, Carrier C, Scaradavou A, et al. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. N Engl J Med. 1998;339:1565-1577.
8. Rocha V, Wagner JE Jr, Sobocinski KA, et al. Graft-versus-host disease in children who have received a cord-blood or bone marrow transplant from an HLA-identical sibling: Eurocord and International Bone Marrow Transplant Registry Working Committee on Alternative Donor and Stem Cell Sources. N Engl J Med. 2000;342:1846-1854.
9. Rocha V, Cornish J, Sievers EL, et al. Comparison of outcomes of unrelated bone marrow and umbilical cord blood transplants in children with acute leukemia. Blood. 2001;97:2962-2971.
10. Eapen M, Rubinstein P, Zhang MJ, et al. Outcomes of transplantation of unrelated donor umbilical cord blood and bone marrow in children with acute leukaemia: a comparison study. Lancet. 2007;369:1947-1954.
11. Laughlin MJ, Eapen M, Rubinstein P, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. N Engl J Med. 2004;351:2265-2275.
12. Rocha V, Labopin M, Sanz G, et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. N Engl J Med. 2004;351:2276-2285.
13. Takahashi S, Iseki T, Ooi J, et al. Single-institute comparative analysis of unrelated bone marrow transplantation and cord blood transplantation for adult patients with hematologic malignancies. Blood. 2004;104:3813-3820.
14. Sanz MA. Cord-blood transplantation in patients with leukemia: a real alternative for adults. N Engl J Med. 2004;351:2328-2330.
15. Kodera Y, Morishima Y, Kato S, et al. Analysis of 500 bone marrow transplants from unrelated donors (UR-BMT) facilitated by the Japan Marrow Donor Program: confirmation of UR-BMT as a standard therapy for patients with leukemia and aplastic anemia. Bone Marrow Transplant. 1999;24:995-1003.
16. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. Bone Marrow Transplant. 1995;15:825-828.
17. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. Stat Med. 1999;18:695-706.
18. Gray RJ. A class of k-sample tests for comparing the cumulative incidence of a competing risk. Ann Stat. 1988;16:1141-1154.
19. Fine JP, Gray RJ. A proportional hazards model for subdistribution of a competing risk. J Am Stat Assoc. 1999;94:456-509.
20. Hsieh FY, Lavori PW. Sample-size calculations for the Cox proportional hazards regression model with nonbinary covariates. Control Clin Trials. 2000;21:552-560.
21. Barker JN, Krepski TP, DeFor TE, Davies SM, Wagner JE, Weisdorf DJ. Searching for unrelated donor hematopoietic stem cells: availability and speed of umbilical cord blood versus bone marrow. Biol Blood Marrow Transplant. 2002;8:257-260.
22. Kiehl MG, Kraut L, Schwerdtfeger R, et al. Outcome of allogeneic hematopoietic stem-cell transplantation in adult patients with acute lymphoblastic leukemia: no difference in related compared with unrelated transplant in first complete remission. J Clin Oncol. 2004;22:2816-2825.
23. Morishima Y, Morishita Y, Tanimoto M, et al. Low incidence of acute graft-versus-host disease by the administration of methotrexate and cyclosporine in Japanese leukemia patients after bone marrow transplantation from human leukocyte antigen compatible siblings; possible role of genetic homogeneity. The Nagoya Bone Marrow Transplantation Group. Blood. 1989;74:2252-2256.
24. Sasazuki T, Juji T, Morishima Y, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor: Japan Marrow Donor Program. N Engl J Med. 1998;339:1177-1185.
25. Morishima Y, Sasazuki T, Inoko H, et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. Blood. 2002;99:4200-4206.
26. Atsuta Y, Suzuki R, Yamamoto K, et al. Risk and prognostic factors for Japanese patients with chronic graft-versus-host disease after bone marrow transplantation. Bone Marrow Transplant. 2006;37:289-296.
27. Takahashi S, Ooi J, Tomonari A, et al. Comparative single-institute analysis of cord blood transplantation from unrelated donors with bone marrow or peripheral blood stem-cell transplants from related donors in adult patients with hematologic malignancies after myeloablative conditioning regimen. Blood. 2007;109:1322-1330.

blood

2009 113: 2851-2858
Prepublished online Nov 7, 2008;
doi:10.1182/blood-2008-08-171934

HLA mismatch combinations associated with decreased risk of relapse: implications for the molecular mechanism

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.

HLA mismatch combinations associated with decreased risk of relapse: implications for the molecular mechanism

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The finding that the risk of relapse in hematologic malignancy decreases after allogeneic hematopoietic stem cell transplantation (HSCT) has lead to the concept of a graft-versus-leukemia (GVL) effect. However, this beneficial effect is considered to be frequently offset by graft-versus-host disease (GVHD). Thus, improving HSCT outcomes by separating GVL from GVHD is a key clinical issue. This cohort study registered 4643 patients with hematologic malignancies who received transplants from unrelated donors. Six major human leukocyte antigen (HLA) loci were retrospectively genotyped. We identified 4 HLA-Cw and 6 HLA-DPB1 mismatch combinations responsible for a decreased risk of relapse; of these, 8 of 10 combinations were different from those responsible for severe acute GVHD, including all 6 of the HLA-DPB1 combinations. Pairs with these combinations of HLA-DPB1 were associated with a significantly better overall survival than were completely matched pairs. Moreover, several amino acid substitutions on specific positions responsible for a decreased risk of relapse were identified in HLA-Cw, but not in HLA-DPB1. These findings might be crucial to elucidating the mechanism of the decreased risk of relapse on the basis of HLA molecule. Donor selection made in consideration of these results might allow the separation of GVL from acute GVHD, especially in HLA-DPB1 mismatch combinations. (Blood. 2009;113:2851-2858)

Introduction

The use of allogeneic hematopoietic stem cell transplantation (HSCT), an established treatment for hematologic malignancies, is associated with several immunologic events with contrary effects in the recipient. In graft-versus-host disease (GVHD), for example, graft immune cells attack host organs, whereas in the graft-versus-leukemia (GVL) effect, they eradicate residual leukemia cells.[1-3] GVL is likely to function not only in hematologic malignancies but also in solid tumors, particularly breast cancer and renal cell carcinoma,[4-6] in which it is referred to as the graft-versus-tumor (GVT) effect. Because both GVL and GVHD are caused by either or both major and minor histocompatibility antigen mismatches between donor and recipient, the beneficial effect of allogeneic HSCT due to GVL is thought to be frequently offset by GVHD. Thus, improving HSCT outcome by separating GVL from GVHD is a key clinical issue. Importantly, however, while most such efforts have been in the area of minor histocompatibility antigen,[7] few researchers have approached this problem in terms of the major histocompatibility antigen.

We recently identified 16 human leukocyte antigen (HLA) mismatch combinations associated with a high risk of severe acute GVHD. Results showed that the overall number of these high-risk mismatches was strongly associated with the occurrence of severe acute GVHD and poor overall survival (OS).[8] We speculated that the intensity of GVL and acute GVHD in any particular mismatch might not necessarily be parallel, and that among HLA mismatch combinations not inducing severe acute GVHD, those that induce strong GVL might occur. In other words, the hypotheses of this study were that particular mismatch combinations allow the separation of GVL from acute GVHD and that specific amino acid substitutions in HLA molecules contribute to this mechanism.

As part of efforts to improve donor selection and allogeneic HSCT outcomes, we identified HLA mismatch combinations that resulted in a decreased risk of relapse in all 6 major HLA loci and compared them with mismatch combinations carrying a high risk of severe acute GVHD. Further, we investigated specific amino acid substitution positions in the HLA molecule responsible for a decreased risk of relapse.

Methods

Patients

This study was conducted using clinical data that were collected prospectively at transplant centers participating in the Japan Marrow Donor Program. Patients who received a first transplant of T cell–replete marrow for a hematologic malignancy from a serologically HLA-A, -B, and -DR antigen-matched unrelated donor between January 1993 and December 2005 through the Japan Marrow Donor Program (n = 4643) were registered. Eligible diagnoses included acute lymphoblastic leukemia (ALL); acute myeloid leukemia (AML), which included only de novo AML;

Submitted August 4, 2008; accepted October 31, 2008. Prepublished online as *Blood* First Edition paper, November 7, 2008; DOI 10.1182/blood-2008-08-171934.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Table 1. Patient characteristics

		A locus		B locus		C locus		DRB1 locus		DQB1 locus		DPB1 locus	
	Total	Match	Mismatch	Match	Mismatch	Match	Mismatch	Match	Mismatch	Match	Mismatch	Match	Mismatch
	4643	4018	625	4351	292	3308	1335	3718	925	3597	1046	1584	3059
Median age, y	31.5	31.8	29.6	31.7	28.3	31.8	30.9	31.7	30.9	31.7	30.8	31.8	31.4
Sex, donor/patient													
Male/male	1904	1673	231	1769	135	1387	517	1551	353	1492	412	678	1226
Male/female	923	789	134	874	49	650	273	734	189	704	219	299	624
Female/male	894	747	147	843	51	634	260	693	201	672	222	268	626
Female/female	922	809	113	865	57	637	285	740	182	729	193	339	583
Disease													
ALL	1464	1267	197	1372	92	1051	413	161	303	1132	332	452	1012
AML	1571	1360	211	1478	93	1114	457	1255	316	1224	347	574	997
CML	979	827	152	905	74	682	297	779	200	746	233	343	636
ML	564	507	57	536	28	43	146	468	96	49	118	192	372
MM	65	57	8	60	5	418	22	55	10	446	16	23	42
Risk of leukemia relapse*													
Standard risk	1684	1485	199	1588	96	1184	500	1375	309	1322	362	572	1112
High risk	1909	1607	302	1772	137	1365	544	1485	424	1451	458	642	1267
Disease other than leukemia	1050	926	124	991	59	759	291	858	192	824	226	370	680
GVHD prophylaxis													
Cyclosporine-based	2503	2159	344	2346	157	1802	701	2107	396	2030	473	881	1622
Tacrolimus-based	2140	1859	281	2005	135	1506	634	1611	529	1567	573	703	1437
ATG													
ATG	152	112	40	135	17	102	50	110	42	118	34	51	101
Non-ATG	4491	3906	585	4216	275	3206	1285	3608	883	3479	1012	1533	2958
Preconditioning													
TBI regimen	3687	3175	512	3445	242	2623	1064	2933	754	2834	853	1242	2445
Non-TBI regimen	956	843	113	906	50	685	271	785	171	763	193	342	614

ATG indicates antithymocyte globulin; and TBI, total body irradiation.

*Standard risk for leukemia relapse was defined as the status of the first complete remission of AML and ALL and the first chronic phase of CML at transplant, while high risk was defined as a more advanced status than standard risk in AML, ALL, and CML. Disease other than leukemia was defined as other than ALL, AML, and CML.

chronic myeloid leukemia (CML); malignant lymphoma (ML); and multiple myeloma (MM).

Patient characteristics are shown in Table 1. A final clinical survey of the patients was completed by December 2006. Informed consent was obtained from patients and donors in accordance with the Declaration of Helsinki, and approval for the study was obtained from the Institutional Review Board of Aichi Cancer Center and the Japan Marrow Donor Program.

HLA typing of patients and donors

Alleles at the HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 loci were identified by previously described methods in all 4643 pairs at the Japanese Red Cross Tokyo Metropolitan Blood Center.[8,9]

Matching of HLA allele between patient and donor

HLA allele mismatch among the donor-recipient pair was scored when the recipient's alleles were not shared by the donor (graft-versus-host vector) for all analyses.

Definition of relapse

Relapse was defined as the recurrence of malignancy as detected by the parameter by which the malignancy was first detected, namely marrow morphology; flow cytometry; cytogenetic studies, including fluorescence in situ hybridization; electrophoresis; immunofixation assays; polymerase chain reaction-based assays for disease markers; or imaging results. The day of relapse was defined as the day on which the respective clinical, hematologic, cytogenetic, or molecular relapse was recognized.

Definition of amino acid substitution

Amino acid sequences of HLA-Cw and -DPB1 molecules were obtained from the IMGT/HLA sequence database.[10] For example, Tyr99C-Phe99C indicated an amino acid substitution at position 99 in the HLA-C molecule in which the donor had tyrosine and the patient had phenylalanine. Substituted amino acids in HLA-Cw and -DPB1 are summarized in Tables S1 and S2 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Statistical analysis

OS rate was assessed using the Kaplan-Meier product limit method. To eliminate the effect of competing risk, the cumulative incidence of relapse was assessed using a previously described method.[11,12] The competing event for relapse was defined as death without relapse. Impact by the factor of interest was assessed using the log rank test. The impact of HLA allele mismatch combinations and the position and type of amino acid substitution (for example, alanine, arginine, and asparagine) in HLA molecules were evaluated using multivariable Cox regression analysis[13] for OS and the occurrence of acute GVHD, while the risk of relapse was evaluated using the multivariable proportional hazard modeling of subdistribution functions in competing risks.[14]

HLA mismatch combinations were evaluated for each locus separately. When the locus of interest was evaluated, we allowed the other loci to be mismatched, with the status of such mismatches adjusted for in the same way as other confounders. The HLA match and HLA one-allele mismatched in every locus were analyzed. For example, the A*0206-A*0201 mismatch combination meant that the donor had HLA-A*0206, the recipient had HLA-A*0201, while another HLA-A allele of the donor and recipient was identical. This mismatch was compared with the HLA-A allele match. Mismatch combinations that had 9 or fewer pairs were combined together as "other mismatch." The model was constructed with mismatch combinations, mismatch status in other loci (match, 1 allele mismatched, and 2 alleles mismatched, as an ordinal variable), and potential confounders. Confounders considered were sex (donor-recipient pair), patient age (linear), donor age (linear), transplant year, type of disease, risk of leukemia relapse (standard, high, and diseases other than leukemia), GVHD prophylaxis (cyclosporine [CSP] vs tacrolimus [FK]), ATG (vs no ATG), and

preconditioning (TBI vs non-TBI). These confounders were used in all analyses to maintain the comparability of results.

The impact of position and type of amino acid substitutions in HLA molecules was evaluated in pairs with oneallele mismatched in HLA-Cw and -DPB1 separately. The amino acid positions we analyzed were all positions at which an amino acid was substituted in the respective locus. We analyzed the impact of each amino acid substitution on each position separately. Multivariable models were constructed to include the position and type of amino acid substitution, mismatch status in other loci (match, 1 allele mismatched, and 2 alleles mismatched as an ordinal variable) and the confounders described above. A P value less than .05 was considered statistically significant. All statistical tests were 2-sided. All analyses were performed using STATA version 10.0 (StataCorp, College Station, TX) and R version 2.5.1 (The R Foundation for Statistical Computing, www.r-project.org).

Validation of statistical analysis

Statistical analyses were validated using the bootstrap resampling method.[15] Briefly, we estimated the measure of association with resampled data drawn repeatedly from the original data. Although approximately 100 to 200 bootstrapped samples are generally sufficient,[16] we used 1 000 bootstrap samples for all analysis validations. Further, we judged the results of analysis as statistically significant only when the results of both base analysis and analysis validation using bootstrap resampling were significant; cases in which the result of base analysis was significant but that of analysis validation using bootstrap resampling was not are indicated by an asterisk next to the P value of the base analysis.

Results

Impact of HLA allele mismatches in locus level on relapse

The number of mismatched alleles of HLA-Cw (1 allele mismatched: hazard ratio [HR], 0.68; 95% confidence interval [CI], 0.58-0.80; 2 alleles mismatched: HR, 0.43;95% CI, 0.24-0.75) and HLA-DPB1 (1 allele mismatched: HR, 0.80; 95% CI, 0.70-0.92; 2 alleles mismatched: HR, 0.62; 95% CI, 0.51-0.75) was strongly associated with a decreased risk of relapse. In contrast, no associations were seen for HLA-A (1 allele mismatched: HR, 1.00; 95% CI: 0.82-1.22; 2 alleles mismatched: HR, 0.79; 95% CI, 0.28-2.28), HLA-B (1 allele mismatched: HR, 1.06; 95% CI, 0.79-1.41; 2 alleles mismatched: not applicable), HLA-DRB1 (1 allele mismatched: HR, 0.93; 95% CI, 0.74-1.18; 2 alleles mismatched: HR, 1.18, 95% CI: 0.53-2.63) or HLA-DQB1 (1 allele mismatched: HR, 1.12; 95% CI, 0.90-1.40; 2 alleles mismatched: HR, 0.73; 95% CI, 0.35-1.52; Figure 1; Table 2).

Impact of HLA mismatch combinations on relapse

Four mismatch combinations in HLA-Cw and 6 in HLA-DPB1 were significantly associated with a decreased risk of relapse (Tables 3 and S3). In contrast, mismatch combinations in HLA-A, -B, -DRB1, and -DQB1 were not significantly associated with differences in risk of relapse (data not shown). The 10 HLA mismatch combinations associated with lower risks of relapse were Cw*0102-Cw*1402 (HR not estimated due to no event), Cw*0801-Cw*0102 (HR not estimated), Cw*1402-Cw*0304 (HR not estimated), Cw*1502-Cw*1402 (HR, 0.28; 95% CI, 0.09-0.88), DPB1*0402-DPB1*0201 (HR, 0.32, 95% CI, 0.12-0.87), DPB1*0501-DPB1*0201 (HR, 0.67; 95% CI:0.50-0.91), DPB1*0501-DPB1*0401 (HR, 0.36; 95% CI, 0.13-0.98), DPB1*0501-DPB1*0402 (HR, 0.55; 95% CI, 0.33-0.93), DPB1*0901-DPB1*0201 (HR, 0.37; 95% CI, 0.14-0.96), and DPB1*1301-DPB1*0201 (HR not estimated; Tables 3 and S3). All 10 HLA mismatch combinations were also significant on validation analysis using the bootstrap resampling method. We speculated that these mismatch combinations would mainly decrease the risk of relapse due to GVL, so we tentatively call them GVL mismatch combinations.

Evaluation of clinical importance of GVL mismatch combinations

We evaluated the clinical importance of GVL mismatch combinations in HLA-Cw and -DPB1. All analyses in this section were conducted in matched pairs other than the evaluated locus. In HLA-C mismatch, the small number of patients with GVL mismatch combinations (n = 13) in matched pairs at the allele level for HLA-A, -B, -DRB1, -DQB1, and -DPB1 prevented comprehensive analysis. We evaluated the GVL mismatch combinations of HLA-DPB1 in matched pairs for HLA-A, -B, -Cw, -DRB1, and -DQB1. Pairs with HLA-DPB1 mismatch were divided into 2 groups, those with a GVL mismatch combination and those with mismatch combinations other than GVL mismatch combinations. These were then compared with 12/12 matched pairs for association with severe acute GVHD, relapse, and OS (Table 4). The curve of the cumulative incidence of OS is shown in Figure 2. Multivariable analysis revealed that although OS was similar between the 12/12 matched pairs and the pairs with mismatch combinations other than GVL mismatch combinations, it was significantly improved in pairs with a GVL mismatch combination (Table 4). In terms of mortality due to relapse according to HLA-DPB1 matching status and whether the mismatch combinations were GVL mismatch combinations, the HLA-DPB1 matched group, HLA-DPB1 1 allele mismatched group, and GVL mismatch combination group showed an expected decreased mortality due to relapse (20.0%, 15.3%, and 10.5%, respectively). Further, mortality due to relapse in the GVL mismatch combination group was significantly lower than that in the HLA-DPB1 1 allele mismatched group (P = .049). We conducted the same analyses with stratification by leukemia type (ALL, AML, or CML) and found that the myeloid malignancies (AML and CML) had the same tendency (Table 4). In particular, in CML, GVL mismatch combinations in HLA-DPB1 were associated with a significantly reduced risk of relapse (HR, 0.14; 95% CI, 0.03-0.55) and significantly improved OS relapse (HR, 0.50; 95% CI, 0.25-0.98).

Impact of position and type of amino acid substitutions of HLA molecules on relapse

We surveyed all substituted positions in HLA-Cw and -DPB1 and found 159 specific amino acid substitutions at 55 positions in HLA-Cw and 55 specific amino acid substitutions at 19 positions in HLA-DPB1 (Tables S1,S2). Analysis revealed 3 specific amino acid substitutions responsible for a decreased risk of relapse in HLA-C, namely Ser9C-Tyr9C (HR, 0.53; 95% CI, 0.30-0.92), Phe99C-Tyr99C (HR, 0.52, 95% CI, 0.30-0.91), and Arg156C-Leu156C (HR, 0.59; 95% CI, 0.37-0.92). In contrast, no decrease in the risk of relapse was seen for substitutions in HLA-DPB1 (Table 5). However, Tyr9C-Ser9C and Tyr99C-Phe99C were strongly linked (see "Discussion"). These specific amino acid substitutions were all significant on validation analysis using the bootstrap resampling method.

Discussion

Improving outcomes in allogeneic HSCT for hematologic malignancies by separating GVL from GVHD is considered a key clinical

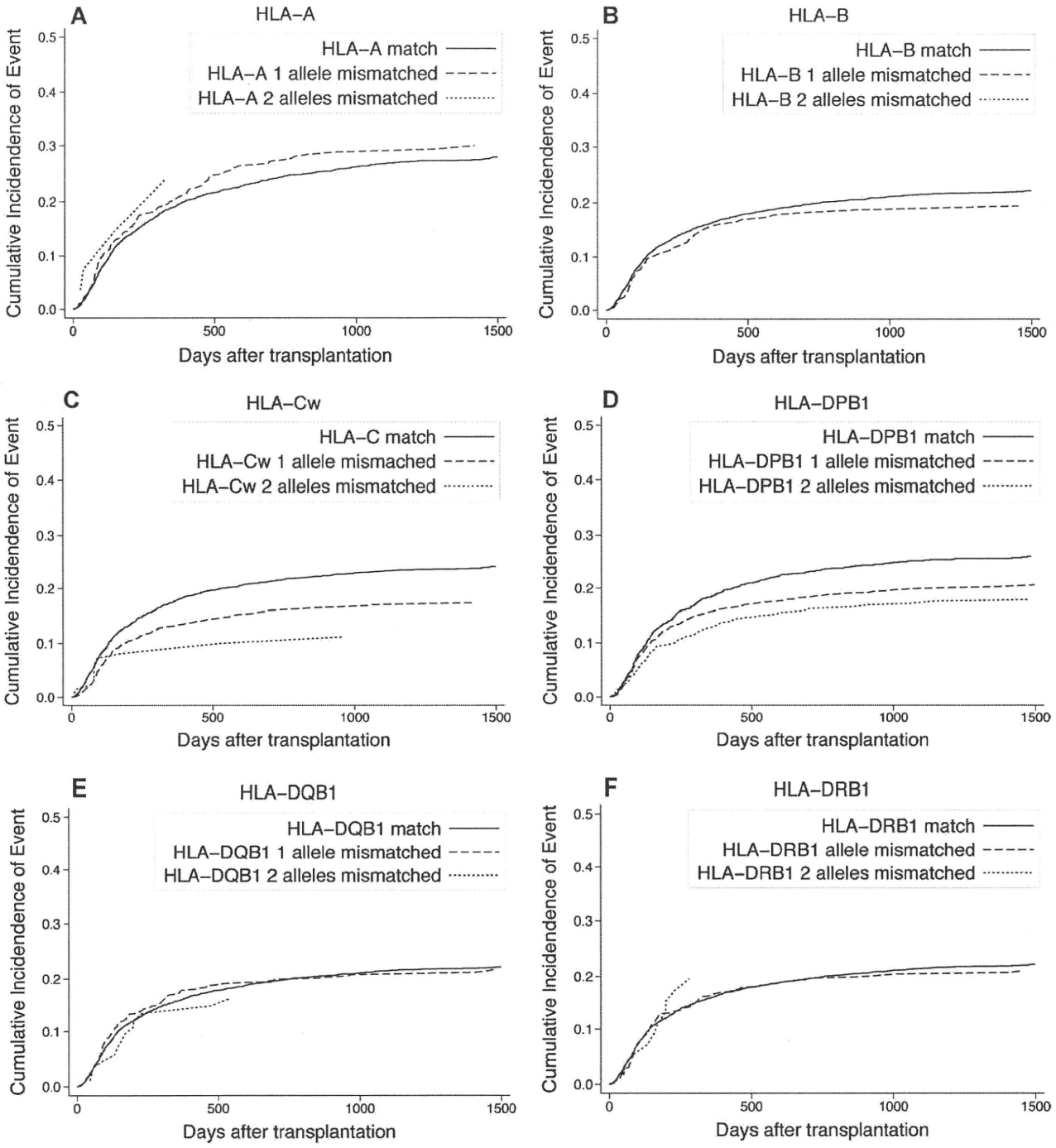

Figure 1. Impact of individual HLA locus mismatches on relapse. Cumulative incidence of relapse for each HLA locus. [—] indicates matched pairs in each locus; [----], 1-allele mismatched pairs in each locus; and [...], 2-allele mismatched pairs in each locus.

challenge. Here, our analysis demonstrated that several donor-recipient HLA mismatch combinations and specific amino acid substitutions in HLA molecules were associated with a decreased risk of relapse, and, in some cases, no significant increase in the risk of severe acute GVHD. These findings suggest that GVL might be separated from severe acute GVHD by selection of suitable HLA mismatch combinations.

We recently reported 16 significant high-risk HLA allele mismatch combinations for severe acute GVHD in 6 HLA loci, a number of which were highly associated with the occurrence of severe acute GVHD and worse OS.[8] Of note, a group of pairs with mismatches other than severe acute GVHD high-risk mismatches showed an incidence of severe acute GVHD and OS rates almost equal to those of 12/12 matched pairs. In the present study, we elucidated a total of 10 mismatch combinations that were significantly associated with a decreased risk of relapse, which we termed GVL mismatch combinations. Of course, it is possible that some mismatch combinations not classified as GVL mismatch combinations might actually induce strong GVL. Misclassification might have occurred as a result of insufficient statistical power due to the relatively small number of patients in the subcategories. Among these mismatch combinations, 2 of 4 in HLA-Cw were identical to the severe acute GVHD high-risk combinations; a third had a marginal effect on the occurrence of severe acute GVHD, while the

Table 2. Impact of HLA mismatches in allele level on relapse

	All diseases		
	n	HR (95% CI)	P
HLA-A matched	4018	1.00 (ref)	
HLA-A 1 allele mismatched	597	1.00 (0.82-1.22)	.99
HLA-A 2 alleles mismatched	28	0.79 (0.28-2.28)	.67
HLA-B matched	4351	1.00 (ref)	
HLA-B 1 allele mismatched	288	1.06 (0.79-1.41)	.7
HLA-B 2 alleles mismatched*	4	ND	ND
HLA-C matched	3308	1.00 (ref)	
HLA-C 1 allele mismatched	1212	0.68 (0.58-0.80)	<.001
HLA-C 2 alleles mismatched	123	0.43 (0.24-0.75)	.003
HLA-DRB1 matched	3718	1.00 (ref)	
HLA-DRB1 1 allele mismatched	866	0.93 (0.74-1.18)	.56
HLA-DRB1 2 alleles mismatched	59	1.18 (0.53-2.63)	.68
HLA-DQB1 matched	3597	1.00 (ref)	
HLA-DQB1 1 allele mismatched	958	1.12 (0.90-1.40)	.30
HLA-DQB1 2 alleles mismatched	88	0.73 (0.35-1.52)	.40
HLA-DPB1 matched	1584	1.00 (ref)	
HLA-DPB1 1 allele mismatched	2190	0.80 (0.70-0.92)	.002
HLA-DPB1 2 alleles mismatched	869	0.62 (0.51-0.75)	<.001

Each group was compared with the matched group in each locus after adjusting for other matching status of HLA, sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, high, and diseases other than leukemia), GVHD prophylaxis (CSP vs FK), ATG vs no ATG), and preconditioning (TBI vs non-TBI).

ref indicates reference; and ND, not determined.

*Comprehensive analysis could not be performed due to the small number of cases.

fourth combination was different from acute GVHD high-risk mismatch combinations. In contrast, all 6 mismatch combinations in HLA-DPB1 were different from acute GVHD high-risk mismatch combinations (Table 3). As expected, HLA-A, -B, -Cw, -DRB1, and -DQB1 matched pairs with GVL mismatch combinations of HLA-DPB1 were associated with significantly better OS than 12/12 matched pairs (Table 4; Figure 2), indicating that the beneficial antitumor effect of GVL mismatch combinations in HLA-DPB1 would not be offset by the effect of severe acute GVHD. We speculate that conformational changes of HLA molecules in each mismatch combination control the intensity of the acute GVHD and GVL effect, as described later in "Discussion" and in our previous report[8]; namely, conformational changes of HLA molecules in GVL mismatch combinations in HLA-DPB1 induce strong GVL with mild or no acute GVHD. These findings suggest that HLA mismatch selection according to these results might improve HSCT outcomes over those obtained with a complete match. The same tendency was seen for AML and CML, whereas the effect of GVL mismatch combination in the HLA-DPB1 allele in ALL patients would be weaker than in the other leukemia types (Table 4). Comprehensive analyses for ML and MM could not be done because of the small number in each group. Thus, the effects of GVL mismatch combination vary according to disease type and may also change according to other factors, including particular cytogenetic abnormalities.

Recent research has shown that HLA-Cw and -DPB1 mismatch at the allele level is strongly associated with a decreased risk of relapse.[17,18] These findings were confirmed in the present large cohort. In addition, the present study also clarified that the mismatching of 2 alleles in either the HLA-Cw or -DPB1 locus had a stronger association with decreased risk than respective mismatching of one allele. Moreover, no association whatsoever was seen for

Table 3. GVL mismatch combinations

Mismatch combination, donor-recipient	n	HR (95% CI)	P
Cw*0102-Cw*1402*†	13	ND	ND
Cw*0801-Cw*0102*†	10	ND	ND
Cw*1402-Cw*0304†	20	ND	ND
Cw*1502-Cw*1402	43	0.28 (0.09-0.88)	.030
DPB1*0402-DPB1*0201*	54	0.32 (0.12-0.87)	.026
DPB1*0501-DPB1*0201*	301	0.67 (0.50-0.91)	.009
DPB1*0501-DPB1*0401*	48	0.36 (0.13-0.98)	.046
DPB1*0501-DPB1*0402*	112	0.55 (0.33-0.93)	.026
DPB1*0901-DPB1*0201*	43	0.37 (0.14-0.96)	.042
DPB1*1301-DPB1*0201*†	20	ND	ND

As an example of the mismatch combination analysis, the Cw*0102-Cw*1402 mismatch combination meant that the donor has HLA-Cw*0102, the recipient has HLA-Cw*1402 and another HLA-Cw allele of each donor and recipient was identical. Each mismatch pair in HLA-Cw was compared with the HLA-Cw allele match, and each mismatch pair in HLA-DPB1 was compared with the HLA-DPB1 allele match. All indicated results were concurrently significant in both the base analysis and validation analysis using bootstrap resampling.

ND indicates not determined.

*Mismatch combinations that were not significantly associated with a higher occurrence of severe acute GVHD in our previous study.[8] However, the Cw*0102-Cw*1402 mismatch combination has a marginal effect on the occurrence of severe acute GVHD; that is, Cw*0102-Cw*1402 was significantly associated with a higher occurrence of severe acute GVHD in base analysis, but not in validation analysis.

†HR was not estimated due to the lack of an event in this group.

Table 4. Clinical importance of GVL mismatch combinations in HLA-DPB1 mismatch

		Acute GVHD		Relapse		OS*	
All diseases	**n**	**HR (95% CI)**		**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
HLA-DPB1 matched	864	1.00 (ref)		1.00 (ref)		1.00 (ref)	
HLA-DPB1 1 allele mismatched	808	1.34 (1.03-1.74)	.028	0.83 (0.68-1.01)	0068	0.96 (0.83-1.12)	.62
GVL mismatch combination	258	1.18 (0.81-1.73)	.375	0.47 (0.33-0.67)	<.001	0.75 (0.59-0.94)	.012
ALL							
HLA-DPB1 matched	250	1.00 (ref)		1.00 (ref)		1.00 (ref)	
HLA-DPB1 1 allele mismatched	263	1.56 (0.96-2.54)	.067	0.85 (0.6-1.19)	.33	1.10 (0.85-1.43)	.48
GVL mismatch combination	80	1.27 (0.63-2.57)	.5	0.75 (0.45-1.26)	.28	0.95 (0.65-1.39)	.8
AML							
HLA-DPB1 matched	308	1.00 (ref)		1.00 (ref)		1.00 (ref)	
HLA-DPB1 1 allele mismatched	264	1.47 (0.9-2.39)	.13	0.83 (0.61-1.14)	.26	0.95 (0.74-1.23)	.72
GVL mismatch combination	89	1.25 (0.62-2.5)	.54	0.44 (0.24-0.78)	.006	0.71 (0.48-1.06)	.1
CML							
HLA-DPB1 matched	176	1.00 (ref)		1.00 (ref)		1.00 (ref)	
HLA-DPB1 1 allele mismatched	162	1.25 (0.74-2.14)	.41	0.69 (0.40-1.20)	.19	0.93 (0.65-1.33)	.69
GVL mismatch combination	54	1.13 (0.51-2.47)	.66	0.14 (0.03-0.55)	.005	0.50 (0.25-0.98)	.041

Each group was compared with the HLA-DPB1 matched group. Confounders considered were sex (donor-recipient pairs), patient age (linear), donor age (linear), type of disease, risk of leukemia relapse (standard, high, and diseases other than leukemia), GVHD prophylaxis (CSP vs FK), ATG vs no ATG), and preconditioning (TBI vs non-TBI).
ref indicates reference.
*The HR indicates the likelihood that OS will be shorter (if HR > 1) or longer (HR < 1) than when the HLA type matches (ie, the Ref condition).

HLA-A, -B, -DRB1, or -DQB1 (Figure 1; Table 2). Furthermore, all 10 GVL mismatch combinations were elucidated from mismatch combinations of HLA-Cw and HLA-DPB1 (Tables 3 and S3), although we also analyzed HLA-A, -B, -DRB1, and -DQB1. These findings indicate that GVL after allogeneic HSCT is mainly induced by HLA-Cw and -DPB1, not HLA-A, -B, -DRB1 or -DQB1, although the role of each HLA locus might vary with the type of disease.[18] There are 3 possible explanations for this. First, the relative expression of HLA-Cw and -DPB1 on malignant cells may be higher than that on normal hematopoietic cells; second, HLA-Cw and -DPB1 may be preferentially expressed on malignant stem cells; and third, surface expression of a few key molecules—such as major histocompatibility complex (MHC), adhesion, and costimulatory molecules—on malignant cells may determine the effect of each HLA locus on GVL.[19-21] In other words, some molecules might stimulate GVL of HLA-Cw or -DPB1, and other molecules might block GVL of other than HLA-Cw and -DPB1. Further investigation of this question is warranted.

In this study, 3 specific amino acid substitutions responsible for GVL at positions 9, 99, and 156 were identified in HLA-Cw, of which only 2, Ser9C-Tyr9C and Phe99C-Tyr99C, were strongly linked in our sample. We were therefore unable to determine which substitutions are the main contributors to the effect of interest (Table 5). These amino acid positions, 9, 99, and 156, were identical to those we elucidated in our previous study as responsible for severe acute GVHD.[8] These findings suggest that these 3 amino acid positions are important determinants of alloreactivity. Although position 156 of the HLA molecule has been shown to modify T-cell alloreactivity in vitro in HLA-A2,[22-24] -B35,[25] and -B44,[26] to our knowledge, the present study is the first to identify positions 9 and 99. On the other hand, substituted amino acids were not necessarily identical. In Ser9C-Tyr9C and Phe99C-Tyr99C substitutions, for example, the substituted amino acid position was identical with that responsible for severe acute GVHD, whereas the substituted amino acids were inverse between donor and recipient, even though both substituted position and amino acids were identical in the Arg156C-Leu156C substitution. These findings suggest that Ser9C-Tyr9C and Phe99C-Tyr99C might play an important role in separating GVL from acute GVHD in HLA-Cw mismatch, although the mechanism requires further molecular clarification.

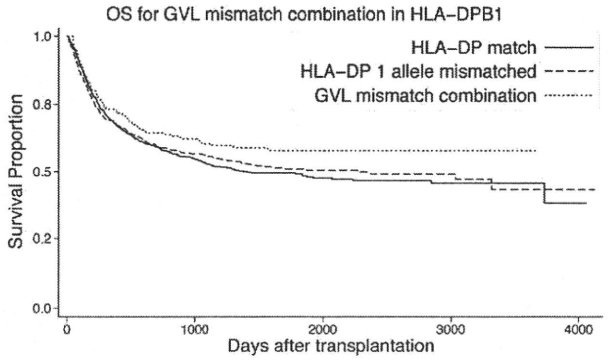

Figure 2. Clinical importance of GVL mismatch combinations in HLA-DP mismatch. Kaplan-Meier estimates of survival according to HLA-DPB1 mismatch status. The solid line indicates HLA-DPB1 matched pairs; the short broken line, HLA-DPB1 1 allele mismatched but not GVL mismatch combinations; and the dotted line, HLA-DPB1 1 allele mismatched (GVL mismatch combinations). All groups are HLA-A, -B, -C, -DRB1, and -DQB1 matched pairs.

Table 5. Impact of position and type of amino acid substitution of HLA molecules on relapse

Position and amino acid substitution in HLA-C (donor-recipient)	n	HR (95% CI)	*P*
Ser9C-Tyr9C	152	0.53 (0.30-0.92)	.024
Phe99C-Tyr99C	153	0.52 (0.30-0.91)	.022
Arg156C-Leu156C*	225	0.59 (0.37-0.92)	.020

The impact of position and type of amino acid substitution in HLA molecules was evaluated in pairs with HLA one-locus mismatch in HLA-C and -DPB1 separately. For example, Tyr9C-Ser9C indicated amino acid substitutions of position 9 in the HLA-C molecule in which the donor had tyrosine and the patient serine. The impact of position and kind of amino acid substitution in each HLA molecule was evaluated in pairs with HLA one locus mismatch in each HLA locus separately. Pairs that substituted a specific amino acid at each position were compared with amino acid matched pairs at that position.

No significant amino acid substitutions were found in HLA-DPB1.

All indicated results were concurrently significant in both base analysis and validation analysis using bootstrap resampling.

The 2 specific amino acid substitutions Tyr9C-Ser9C and Tyr99C-Phe99C were strongly linked in our sample.

*An amino acid substitution that was significantly associated with a higher occurrence of severe acute GVHD in our previous study.[8]

With regard to specific amino acid substitutions of HLA-DPB1, we found no significant association among these with a decreased risk of relapse. Shaw et al[27] reported that mismatches at position 57 and 65 in the HLA-DPB1 molecule were associated with transplant complications, but not with GVHD or relapse, which is consistent with our present data. We speculate that, compared with MHC class I, the conformational diversity of MHC class II and peptide complex hampers the identification of strict rules of association between specific amino acid substitutions in MHC class II molecules and the occurrence of alloreaction such as GVHD and GVL. In HLA class I, binding peptides are held by their ends, whereas peptides bind to HLA class II by attachment in the middle, allowing them to vary greatly in length.[28]

Given that this analysis was conducted using a Japanese cohort of patients who received transplants through the Japan Marrow Donor Program, the applicability of our data to other ethnic groups warrants discussion. We speculate that the effect of alloreaction is a reflection and summation of HLA allele mismatch combinations. Discrepancies in the effect of HLA locus on alloreactions between ethnically diverse transplantation might be explained by the proportions of each HLA mismatch combination in each HLA locus. In HLA-DPB1, on the other hand, the allele variations between white and Japanese populations are relatively close, hence our findings in HLA-DPB1 might also be useful for white populations. Regarding HLA-Cw and killer immunoglobulin-like receptor (KIR) incompatibility, we previously reported adverse effects in unrelated T cell–replete HSCT through the Japan Marrow Donor Program,[18] although Ruggieri et al[29] demonstrated that beneficial effects were shown in T-cell depleted haploidentical transplantation. We speculated that in vivo and/or in vitro T-cell depletion could account for this discrepancy.[30] Therefore, results for mismatch combinations in HLA-Cw obtained in other populations treated in other settings may differ from our results. Nevertheless, clarification of these questions would require the same study in other ethnic populations.

Given the general acceptance that GVL is more closely correlated with chronic GVHD than acute GVHD,[3] separating GVL from chronic GVHD may be more difficult than separating it from acute GVHD. On this basis, our results suggest that GVL could be separated from acute GVHD in HSCT from a specific HLA partially mismatched donor. Clarification of whether GVL can also be separated from chronic GVHD requires further study.

In conclusion, we identified 4 HLA-C and 6 HLA-DPB1 mismatch combinations that decrease the risk of relapse in patients after HSCT. Eight of 10 GVL combinations were different from those responsible for severe acute GVHD. In particular, all 6 GVL combinations in HLA-DPB1 were different. Further, pairs with these GVL combinations of HLA-DPB1 were associated with significantly better OS than completely matched pairs. These findings suggest that donor selection according to these results could separate the occurrence of GVL from acute GVHD, especially in HLA-DPB1. Further, amino acid substitutions on specific positions responsible for this decreased risk of relapse were also elucidated in HLA-C, but not in HLA-DPB1. Our finding that specific amino acid substitutions decrease the risk of relapse might be key to revealing the mechanism of the decreased risk of relapse due to GVL with regard to the HLA molecule.

Acknowledgments

We thank the staff members of the transplant centers, donor centers, and the Japan Marrow Donor Program office for their generous cooperation; Ms Ryoko Yamauchi for the data management; and Drs Toshitada Takahashi and Setsuko Kawase for their expert technical assistance.

This study was supported in part by Health and Labor Science Research Grant (Research on Allergic disease and Immunology) from the Ministry of Health, Labor, and Welfare (Tokyo, Japan) and the Grant-in-Aid for Cancer Research (19-1) from the Ministry of Health, Labor, and Welfare (Tokyo, Japan).

Authorship

Contribution: T.K., Y.M., T.S., S.O., and Y.K. participated in the conception of this study; K.K., H.I., and H.S. participated in the assessment of histocompatibility; Y.M. and S.K. participated in the execution of transplantation; T.K. and K.M. participated in the statistical data analysis; T.K. and Y.M. wrote the paper; and all authors checked the final version of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

1. Weiden PL, Flournoy N, Thomas ED, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. N Engl J Med. 1979;300:1068-1073.
2. Gale RP, Horowitz MM. Graft-versus-leukemia in bone marrow transplantation: the Advisory Committee of the International Bone Marrow Transplant Registry. Bone Marrow Transplant. 1990; 6(suppl 1):94-97.
3. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood. 1990;75:555-562.
4. Carella AM, Beltrami G, Corsetti MT, et al. Reduced intensity conditioning for allograft after cytoreductive autograft in metastatic breast cancer. Lancet. 2005;366:318-320.
5. Bregni M, Dodero A, Peccatori J, et al. Nonmyeloablative conditioning followed by hematopoietic cell allografting and donor lymphocyte infusions for patients with metastatic renal and breast cancer. Blood. 2002;99:4234-4236.
6. Ueno NT, Cheng YC, Rondon G, et al. Rapid induction of complete donor chimerism by the use of a reduced-intensity conditioning regimen composed of fludarabine and melphalan in allogeneic stem cell transplantation for metastatic solid tumors. Blood. 2003;102:3829-3836.
7. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. Nat Rev Cancer. 2004;4:371-380.
8. Kawase T, Morishima Y, Matsuo K, et al. High-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease and implication for its molecular mechanism. Blood. 2007;110:2235-2241.
9. Sasazuki T, Juji T, Morishima Y, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor: Japan Marrow Donor Program. N Engl J Med. 1998;339:1177-1185.
10. European Molecular Biology Laboratory–European Bioinformatics Institute. IMGT/HLA database. http://www.ebi.ac.uk/imgt/hla/. Accessed May 1, 2008.
11. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. Stat Med. 1999;18:695-706.
12. Coviello V, Boffess M. Cumulative incidence estimation in the presence of compiting risks. Stata J. 2004;4:103-112.
13. Cox DR. Regression models and life-tables. J R Stat Soc Ser B. 1972;34:187-220.
14. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc. 1999;94:496-497.
15. Efron B. Bootstrap methods: another look at the jackknife. Ann Stat. 1979;7:1-26.

16. Manly BFJ. Randomization, Bootstrap and Monte Carlo Methods in Biology. London, United Kingdom: Chapman and Hall; 1997.
17. Shaw BE, Gooley TA, Malkki M, et al. The importance of HLA-DPB1 in unrelated donor hematopoietic cell transplantation. Blood. 2007;110: 4560-4566.
18. Morishima Y, Yabe T, Matsuo K, et al. Effects of HLA allele and killer immunoglobulin-like receptor ligand matching on clinical outcome in leukemia patients undergoing transplantation with T-cell-replete marrow from an unrelated donor. Biol Blood Marrow Transplant. 2007;13:315-328.
19. Weichold FF, Jiang YZ, Dunn DE, et al. Regulation of a graft-versus-leukemia effect by major histocompatibility complex class II molecules on leukemia cells: HLA-DR1 expression renders K562 cell tumors resistant to adoptively transferred lymphocytes in severe combined immunodeficiency mice/nonobese diabetic mice. Blood. 1997;90:4553-4558.
20. Gribben JG, Guinan EC, Boussiotis VA, et al. Complete blockade of B7 family-mediated costimulation is necessary to induce human alloantigen-specific anergy: a method to ameliorate graft-versus-host disease and extend the donor pool. Blood. 1996;87:4887-4893.
21. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Gray GS, Vallera DA. Coblockade of the LFA1: ICAM and CD28/CTLA4:B7 pathways is a highly effective means of preventing acute lethal graft-versus-host disease induced by fully major histocompatibility complex-disparate donor grafts. Blood. 1995;85:2607-2618.
22. Hogan KT, Clayberger C, Bemhard EJ, et al. Identification by site-directed mutagenesis of amino acid residues contributing to serologic and CTL-defined epitope differences between HLA-A2.1 and HLA-A2.3. J Immunol. 1988;141:2519-2525.
23. Mattson DH, Shimojo N, Cowan EP, et al. Differential effects of amino acid substitutions in the beta-sheet floor and alpha-2 helix of HLA-A2 on recognition by alloreactive viral peptide-specific cytotoxic T lymphocytes. J Immunol. 1989;143: 1101-1107.
24. Shimojo N, Cowan EP, Engelhard VH, Maloy WL, Coligan JE, Biddison WE. A single amino acid substitution in HLA-A2 can alter the selection of the cytotoxic T lymphocyte repertoire that responds to influenza virus matrix peptide 55-73. J Immunol. 1989;143:558-564.
25. Tynan FE, Elhassen D, Purcell AW, et al. The immunogenicity of a viral cytotoxic T cell epitope is controlled by its MHC-bound conformation. J Exp Med. 2005;202:1249-1260.
26. Macdonald WA, Purcell AW, Mifsud NA, et al. A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. J Exp Med. 2003;198:679-691.
27. Shaw BE, Marsh SGE, Mayor NP, Madrigal JA. Matching status at amino acid positions 57 and 65 of the HLA-DPB1 beta chain determines outcome in recipients of unrelated donor haematopoietic stem cell transplants [abstract]. Blood. 2004;104:Abstract 827.
28. Steven GE, Peter P, Linda DB. The HLA Facts Book. London, United Kingdom: Academic Press; 2000.
29. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295:2097-2100.
30. Yabe T, Matsuo K, Hirayasu K, et al. Donor killer immunoglobulin-like receptor (KIR) genotype-patient cognate KIR ligand combination and anti-thymocyte globulin preadministration are critical factors in outcome of HLA-C-KIR ligand-mismatched T cell-replete unrelated bone marrow transplantation. Biol Blood Marrow Transplant. 2008;14:75-87.

THE JOURNAL OF IMMUNOLOGY

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J Immunol 2009;183;5991-5998; Prepublished online 7 October 2009;
doi:10.4049/jimmunol.0804310
http://www.jimmunol.org/content/183/9/5991

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

CTL Clones Isolated from an HLA-Cw-Mismatched Bone Marrow Transplant Recipient with Acute Graft-Versus-Host Disease[1]

Kyoko Sugimoto, Makoto Murata,[2] Seitaro Terakura, and Tomoki Naoe

HLA-Cw disparity in a donor increases the risk of acute graft-vs-host disease (GVHD) after bone marrow transplantation. Acute GVHD is mediated by donor CTLs. However, mismatched HLA-Cw-specific CTLs generated in posttransplant recipients who developed acute GVHD have not been characterized in detail. In this study, CTL clones isolated from a recipient at the onset of acute GVHD who was transplanted from an HLA-A, -B, and -DRB1-matched, HLA-Cw-mismatched (recipient, Cw*0303/Cw*0702; donor, Cw*0801/Cw*0702), unrelated donor were characterized. The seven isolated CTLs, including $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T lymphocytes, lysed recipient cells, *HLA-Cw*0303*-transfected 721.211 cells, and *HLA-Cw*0303*-transfected donor cells, but not untransfected 721.211 cells or donor cells. Thus, all CTLs recognized the mismatched Cw*0303 molecule as an alloantigen. The sequences of Cw*0303 and Cw*0801 differ by 16 aas. Stimulation of CTLs by COS cells transfected with *Cw*0303* cDNA constructs demonstrated that *Cw*0303* mutants in which individual amino acids constituting peptide-binding pockets were substituted with the corresponding Cw*0801 amino acids significantly decreased IFN-γ production by all CTLs, whereas *Cw*0303* mutants bearing Cw*0801 amino acids outside the positions constituting peptide-binding pockets stimulated all CTLs to the same degree as the wild-type *Cw*0303* construct. These data suggest that all CTLs recognized the Cw molecule in a peptide-dependent manner. ELISPOT revealed that Cw*0303-reactive T cells accounted for one-half of the total of alloreactive T cells in the blood during GVHD. Taken together, non-self Cw-specific CTL clones with a variety of phenotypes and peptide specificities can be generated in posttransplant recipients with acute GVHD. *The Journal of Immunology,* 2009, 183: 5991–5998.

Mismatches of HLA confer an enhanced risk of acute graft-vs-host disease (GVHD)[3] after allogeneic hematopoietic stem cell transplantation (HSCT) (1). HLA-Cw disparity in an unrelated bone marrow donor, which can occur even in recipient/donor pairs that are matched for HLA-A, -B, and -DRB1 alleles, increases the risk of severe acute GVHD (2–4). Acute GVHD is mediated by donor CTLs recognizing non-self HLA molecules or MHCs (5–7). CTLs specific for non-self HLA-Cw molecules have been successfully generated using the technique of in vitro mixture of PBMCs obtained from two unrelated individuals or a mixture of PBMCs and cell lines expressing the HLA-Cw molecule on their cell surface (8–12). However, the immunogenicity of original HLA-Cw Ags is considered to be low due to their low level of cell surface expression (13, 14), except HeLa cells expressing HLA-Cw transcripts at levels nearly equal to those of HLA-A and B (15). In fact, HLA-Cw-specific CTLs, especially CTL clones that are naturally generated in posttransplant recipients who develop acute GVHD, have not been characterized in detail.

In this study, CTL clones isolated from a recipient at the onset of severe acute GVHD who had received bone marrow from an HLA-A, -B, and -DRB1 allele-matched and HLA-Cw allele-mismatched unrelated donor were characterized. All seven isolated CTLs, including $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ CTL clones, recognized the mismatched HLA-Cw*0303 molecule as an alloantigen, whereas no CTLs recognized MHCs. We further demonstrated that all CTLs recognized the Cw*0303 molecule in a peptide-dependent manner. Thus, it appears that non-self Cw-specific CTL clones with a variety of phenotypes and peptide specificities can arise in post-HSCT recipients with acute GVHD.

Materials and Methods

Study patient

A 31-year-old woman with acute lymphoblastic leukemia (ALL) received bone marrow transplantation from an HLA-A, -B, and -DRB1 allele-matched (A*0206/A*2402, B*0702/B*4801, DRB1*0101/DRB1*0901), HLA-Cw allele-mismatched (recipient, Cw*0303/Cw*0702; donor, Cw*0801/Cw*0702), killer Ig-like receptor ligand-matched, unrelated female donor. The preparative regimen consisted of 120 mg/kg cyclophosphamide and 12 Gy total body irradiation. GVHD prophylaxis consisted of 0.03 mg/kg tacrolimus and short-term methotrexate. A neutrophil count $>0.5 \times 10^9$/L and a platelet count $>20 \times 10^9$/L were achieved on days 14 and 22, respectively. The patient developed grade II acute GVHD involving the skin (stage 3), evaluated according to previously published criteria (16). The GVHD resolved gradually without addition of other immunosuppressive agents. The patient continued to be in complete remission 4 years after transplantation.

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Received for publication December 23, 2008. Accepted for publication August 20, 2009.

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[1] This study was supported in part by Health and Labor Science Research Grant (Research on Allergic Disease and Immunology); Grant-in-Aid for Cancer Research 20–14 from the Ministry of Health, Labour and Welfare of Japan; and Grant-in-Aid for Scientific Research 20591149 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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[3] Abbreviations used in this paper: GVHD, graft-vs-host disease; HSCT, hematopoietic stem cell transplantation; ALL, acute lymphoblastic leukemia; B-LCL, EBV-transformed lymphoblastoid cell.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0804310

TABLE I. *Characteristics of isolated CTLs*

CTL	Lysis by CTL (%)[a]		Surface Marker[b]		
	Recipient-LCL	Donor-LCL	CD3	CD4	CD8
60D6	75	1	+	−	+
60F1	85	2	+	−	+
64A11	84	1	+	−	+
11F1	73	0	+	−	+
52F11	72	3	+	+	−
42C5	74	2	+	+	+/−
46D4	73	1	+	+	+/−

[a] E:T ratio was 10:1.

[b] Positive, dull positive, and negative expressions of each surface marker are indicated by +, +/−, and −, respectively.

Cell culture

CTL clones were isolated from a blood sample as described previously (17). Briefly, PBMCs obtained from the recipient at the onset of acute GVHD were stimulated in vitro with aliquots of gamma-irradiated PBMCs that had been obtained from the recipient pretransplant and cryopreserved. After three weekly stimulations, the CTL clones were isolated from the polyclonal T cell culture by limiting dilution. The CTLs were expanded by stimulation every 14 days with 30 ng/ml OKT3 mAb (Janssen Pharmaceutical), using unrelated allogeneic gamma-irradiated (25 Gy) PBMCs and gamma-irradiated (75 Gy) EBV-transformed lymphoblastoid cells (B-LCL) as feeder cells. The culture medium consisted of RPMI-1640-HEPES (Sigma-Aldrich) containing 10% pooled, heat-inactivated human serum and recombinant human IL-2 (R&D Systems). The T cells were used in assays 14 days after stimulation or 1 day after thawing a frozen aliquot. PBMCs containing >90% ALL cells were obtained from Cw*0303-positive (n = 3) and Cw*0303-negative (n = 3) unrelated patients. Primary dermal fibroblast lines were established from skin biopsy specimens as described (18). All blood or tissue samples were collected after written informed consent was obtained. B-LCLs and 721.221 cells were maintained in RPMI-1640-HEPES with 10% FBS. COS cells and fibroblast lines were maintained in DMEM (Sigma-Aldrich) with 10% FBS.

Chromium release assay

B-LCLs, 721.221, PBMCs, and fibroblasts were used as target cells in the cytotoxicity assay. B-LCLs, 721.221 cells, and PBMCs were labeled for 2 h, and fibroblasts were labeled overnight with ^{51}Cr. The ^{51}Cr-labeled target cells were washed twice, dispensed at 2×10^3cells/well into triplicate cultures in 96-well plates, and incubated for 4 h at 37°C with CTL clones at various E:T ratios. Percent-specific lysis was calculated as [(experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)] × 100.

Flow cytometric analysis of CTL clones

CTL clones were analyzed using three-color flow cytometry for expression of CD3, CD4, and CD8 using PE-cyanin 5.1-conjugated anti-CD3 (Beckman Coulter), PE-conjugated anti-CD4 (BD Biosciences), and FITC-conjugated anti-CD8 (BD Biosciences) Abs. The TCR Vβ repertoire was determined by flow cytometry using an IOTest β Mark TCR Vβ Repertoire Kit (Beckman Coulter). A BD FACSAria (BD Biosciences) was used to perform the analysis.

Determination of TCR nucleotide sequences

The nucleotide sequences of the uniquely rearranged TCR Vβ chain gene of each CTL clone were determined by direct DNA sequencing of the amplified PCR product of TCR (19). Briefly, total RNA was isolated from each CTL clone using the QIAamp RNA Blood Mini Kit (Qiagen) and converted into cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed on cDNA using each TCR Vβ-specific primer set: Vβ1, 5′-CTTGCACTCTGAACTAAACC-3′ and 5′-CTCAAACACAGCGACCTC-3′ (common antisense primer); Vβ13.2, 5′-AAGATGGCCCTCGGGCTCCTGTGTGG-3′ and common antisense primer; Vβ14, 5′-ATAAGGGAGATGTTCCTGAA-3′ and common antisense primer. The PCR products were purified and directly sequenced with the same primers and the BigDye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems) using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

HLA-Cw cDNA constructs

Total RNA was isolated from the recipient and donor B-LCLs and converted into cDNA. Constructs containing the full-length *HLA-Cw* gene were generated from the cDNA by PCR and cloned into the pEAK10 expression vector (Edge BioSystems). Sense and antisense primers contained the recognition sequence for *Hin*dIII and *Not*I, respectively, to facilitate cloning of the PCR product into pEAK10. The sequences used were 5′-TATAAAGCTTTTCTCCCCAGACGCCGAGA-3′ (sense) and 5′-ATATGCGGCCGCGTCTCAGGCTTTACAAGCGA-3′ (antisense). *Cw*0303* mutants in which individual amino acids were substituted with the corresponding amino acid in Cw*0801 were produced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Transfection of B-LCL and 721.211 cells with HLA cDNA

Donor B-LCL and 721.211 cells (5×10^6) were transfected by electroporation (220 V, 500 μFD) in 200 μl of potassium-PBS with 15 μg of the pEAK10 plasmid encoding *HLA-Cw*0303* cDNA and selected with 0.8 μg/ml puromycin (Edge BioSystems), beginning 48 h after transfection. Three days after selection, they were used as a target in the cytotoxicity assay. The expression of HLA-Cw3 on transfected 721.211 cells was tested by flow cytometric analysis using the anti-class I mAb W6/32 (BioLegend).

Transfection of COS cells and CTL stimulation assay

COS cells (5×10^3) were plated in individual wells of 96-well flat-bottom plates, cultured for 24 h, and then transfected with 100 ng of the pEAK10 plasmid encoding *HLA-Cw*0303*, *HLA-Cw*0801*, or 16 types of *HLA-Cw*0303* mutant cDNA using the FuGENE 6 Transfection Reagent (Roche). Then, 2×10^4 CTL clones were added to each well of COS cells 48 h after transfection, and after a further 24 h of coculture at 37°C, IFN-γ production was measured in the supernatant using ELISA (Endogen). The expressions of HLA-Cw*0303 or 16 types of HLA-Cw*0303 mutant on COS transfectants were tested by flow cytometric analysis using the anti-class I mAb YTH862.2 (Santa Cruz Biotechnology).

TABLE II. *Clonotypes of isolated CTLs*

CTL	TCR Vβ	Nucleotide and Deduced Amino Acid Sequences of the CDR3 Region
60D6	Vβ14	GCC AGC AGT TTT GGG ACA GGG GAC TAC GAG CAG TAC A S S F G T G D Y E Q Y
60F1	Vβ13.2	GCC AGC AGT TAC CCC CCT CAG CGG GAG CAA TGG GAG ACC CAG TAC TTC A S S Y P P Q R E Q W E T Q Y F
64A11	Vβ1	GCC AGC AGC CCC GAT GGA CTA GAA CGG GAT GAG CAG TTC A S S P D G L E G D E Q F
11F1	ND[a]	
52F11	Vβ1	GCC AGC AGC CCC GAT GGA CTA GAA CGG GAT GAG CAG TTC A S S P D G L E G D E Q F
42C5	Vβ1	GCC AGC AGC CCC GAT GGA CTA GAA CGG GAT GAG CAG TTC A S S P D G L E G D E Q F
46D4	Vβ13.2	GCC AGC AGT TTC GGA CAG GGG GCT TAC GAG CAG TAC A S S F G Q G A Y E Q Y

[a] ND, not detected.

ELISPOT

T cells were isolated from PBMCs by negative depletion using the Pan T Cell Isolation Kit II (Miltenyi Biotec) and used as responder T cells. Responder T cells at a concentration of 2×10^5 per well were plated in individual wells of the 96-well MultiScreen-IP filter plates (Millipore) coated with anti-human IFN-γ Ab (5 μg/ml; Mabtech) and tested in triplicate against a total of 2×10^5 stimulator cells: recipient B-LCL; donor B-LCL; and *HLA-Cw*0303*-transfected donor B-LCL. The plates were incubated for 24 h at 37°C, washed, and incubated with biotinylated anti-human IFN-γ Ab (1 μg/ml; Mabtech) for 2 h at room temperature. After addition of streptavidin (Fitzgerald Industries International) to the wells, the plates were developed with 3-amino-9-ethylcarbazol substrate kit (Vector Laboratories). Spots were counted using a microscope, and mean numbers were calculated from triplicate wells after subtraction of the number of spots obtained with medium alone.

Results

Isolation of CTLs

Seven CTLs were isolated from the peripheral blood of the recipient just after the onset of grade II acute GVHD involving the skin. All seven isolated CTLs lysed recipient B-LCL but failed to lyse donor B-LCL (Table I). Flow cytometric analysis revealed that four CTLs were $CD8^+$, one was $CD4^+$, and two were $CD4^+$ $CD8^{+/-}$. We compared the sequences of the *HLA-Cw* cDNA of each CTL clone with the canonical sequences of the recipient *Cw*0303*, the donor *Cw*0801*, and the recipient and donor *Cw*0702* obtained from the GenBank DNA sequence database. All clones had *Cw*0801* and *Cw*0702* (data not shown), demonstrating that all isolated clones originated from the donor.

Clonotyping of isolated CTLs

The TCR Vβ usage of each CTL was studied, and the nucleotide sequences of the amplified PCR products of the *TCR Vβ* gene were determined (Table II). The $CD8^+$ 60D6 CTL used Vβ14. The $CD8^+$ 60F1 and $CD4^+CD8^{+/-}$ 46D4 CTLs used the same Vβ13.2, but neither nucleotide sequences nor amino acid sequences in the CDR3 regions were identical in these two CTLs, indicating that these CTLs were different clones. Three of the CTLs, including $CD8^+$ CTL 64A11, $CD4^+$ CTL 52F11, and $CD4^+CD8^{+/-}$ CTL 42C5, had the same nucleotide sequences in the CDR3 regions of their TCR Vβ1, suggesting that these three CTLs with variable phenotypes originated from a single clone. The TCR Vβ usage of 11F1 could not be determined because this clone did not react with any of the anti-TCR Vβ Abs used in flow cytometric analysis. Thus, based on these data, the seven isolated CTLs appear to have been derived from five independent clones.

All CTL clones recognized the mismatched HLA-Cw molecule but not minor histocompatibility Ags

To identify the genes encoding the Ag recognized by the isolated CTLs, the $CD8^+$ clones, 60D6 and 60F1, were initially studied. Both clones lysed all 15 B-LCL lines from unrelated individuals who shared HLA-Cw*0303 with the recipient, but not all B-LCL lines from 17 unrelated individuals who shared class I HLA molecules other than HLA-Cw*0303 (data not shown). Thus, both CTL clones recognized the HLA-Cw*0303 molecule as an alloantigen.

Next, 721.221 cells, which lack HLA class I expression on their cell surface, were transfected with a full-length *HLA-Cw*0303* cDNA construct and used as a target in the cytotoxicity assay. Both 60D6 and 60F1 clones clearly lysed *HLA-Cw*0303*-transfected 721.221 cells, but not untransfected 721.221 cells (Fig. 1, *A* and *B*). Then, whether some of the other CTL clones recognized the HLA-Cw*0303 molecule was determined. Unexpectedly, all of the other CTLs, including $CD4^+$ clones, lysed *HLA-Cw*0303*-transfected 721.221 cells (Fig. 1, *C–G*). Thus, all isolated CTLs with variable phenotypes recognized the mismatched HLA-Cw*0303 molecule as an alloantigen.

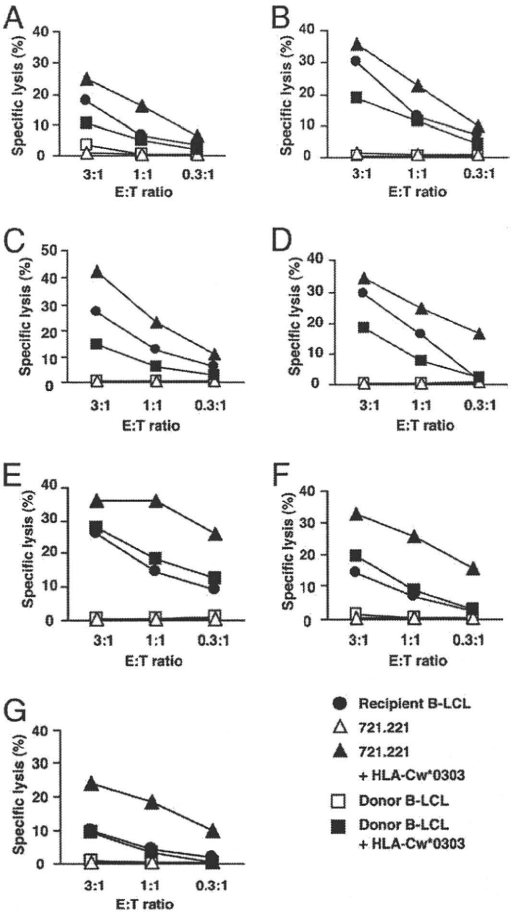

FIGURE 1. Cytotoxicities of CTLs against donor B-LCL and 721.221 cells transfected with *HLA-Cw*0303*. Donor B-LCL and 721.221 cells were transfected with a plasmid encoding *HLA-Cw*0303* cDNA and used as targets for CTL clones 60D6 (*A*), 60F1 (*B*), 64A11 (*C*), 11F1 (*D*), 52F11 (*E*), 42C5 (*F*), and 46D4 (*G*) in a cytotoxicity assay. Selection of the 721.221 cells transfected with *HLA-Cw*0303* cDNA in puromycin provided a population of ~60% W6/32$^+$ cells. The origin of 721.221 cells, LCL 721 cells, express HLA-A1, -A2, -B5, and -B8, and we confirmed that *Cw*0303*-transfected 721.221 cells were negative for all of A1, A2, B5, and B8. Because a commercial Cw3-specific Ab and information for Cw alleles of LCL 721 cells are not available, we could not perform an inhibitory study and exclude the possibility that two genes of undetermined original *HLA-Cw* alleles are transcribed or translated into protein in the *Cw*0303*-transfected 721.221 cells. An Ab reacting with Cw3, but not A2, A24, B7, B48, Cw7, and Cw8, which were expressed on untransfected donor LCL, was also not available. The lysis of recipient B-LCL, untransfected 721.221 cells, *HLA-Cw*0303*-transfected 721.221 cells, untransfected donor B-LCL, and *HLA-Cw*0303*-transfected-donor B-LCL is shown as the mean of triplicate cultures at various E:T ratios.

Donor B-LCL was then transfected with an *HLA-Cw*0303* cDNA construct and examined in the cytotoxicity assay. All CTL clones lysed *HLA-Cw*0303*-transfected donor B-LCL (Fig. 1). Thus, none of the isolated CTLs recognized the recipient's minor histocompatibility Ags.

*T cell recognition of the HLA-Cw*0303 molecule with a variety of peptide specificities*

Various forms of direct T cell recognition of the allogeneic MHC, ranging from peptide independent to peptide dependent, have been

TABLE III. *Summary of IFN-γ production of CTLs stimulated by Cw*0303 mutants*

	Substituted Amino Acid				IFN-γ Production of CTLs						
*Cw*0303* Mutant	Position	Cw*0303 →	Cw*0801	Pocket(s)[a]	60D6	60F1	64A11	11F1	52F11	42C5	46D4
Cw*0303–1	1	Gly	Cys	–							
Cw*0303–21	21	His	Arg	–							
Cw*0303–35	35	Arg	Gln	B				↓[b]			
Cw*0303–91	91	Arg	Gly	–							
Cw*0303–94	94	Ile	Thr	–							
Cw*0303–95	95	Ile	Leu	–							
Cw*0303–103	103	Val	Leu	–							
Cw*0303–114	114	Asp	Asn	D, E			↓		↓	↓	
Cw*0303–116	116	Tyr	Phe	F	↓	↓	↓	↓	↓	↓	
Cw*0303–152	152	Glu	Thr	E	↓	↓	↓	↓	↓	↓	↓
Cw*0303–163	163	Leu	Thr	A		↓					
Cw*0303–173	173	Lys	Glu	–							
Cw*0303–177	177	Glu	Lys	–							
Cw*0303–219	219	Trp	Arg	–							
Cw*0303–275	275	Glu	Gly	–							
Cw*0303–304	304	Val	Met	–							

[a] Peptide-binding pocket(s) constituted by each amino acid position.
[b] Significant decrease ($P < 0.05$) of IFN-γ production of CTLs stimulated by *Cw*0303* mutants compared with the wild-type *Cw*0303*.

demonstrated (20). If allorecognition of isolated CTLs is dependent on peptides bound to HLA molecules, changing HLA-Cw*0303 molecules by amino acid substitutions at residues constituting peptide-binding pockets should affect the presentation of peptides and allorecognition of CTLs. The sequences of the recipient Cw*0303 and the donor Cw*0801 differ by 16 aas. Sixteen variants of *Cw*0303* mutants, in which individual amino acids were substituted with the corresponding amino acid in Cw*0801 (Table III), were generated. COS cells were then transfected with each mutant and examined using the CTL stimulation assay. The expression level of HLA class I on the surface of COS cells transfected with all 16 types of *HLA-Cw*0303* mutant cDNA was almost the same as that with wild-type *Cw*0303* cDNA.

IFN-γ production of the 60D6 CTL was significantly decreased when stimulated by *Cw*0303-116* and *Cw*0303-152* mutants, in which amino acids at position 116 (tyrosine) and 152 (glutamic acid) were substituted with phenylalanine and threonine, respectively, compared with the wild-type *Cw*0303* construct (Fig. 2*A*). Amino acids at positions 116 and 152 constitute peptide-binding pockets F and E, respectively, of the HLA class I molecule (Table III and Refs. 21 and 22). IFN-γ production of the 60F1, 11F1, and 46D4 CTLs was significantly decreased when stimulated by *Cw*0303* mutants in which amino acids at positions 116/152/163 for 60F1, 35/116/152 for 11F1, and 152 for 46D4 were substituted with the corresponding Cw*0801 amino acids, compared with the wild-type *Cw*0303* construct (Fig. 2, *B*, *D*, and *G*). All of these amino acid positions also constitute peptide-binding pockets (Table III). The 64A11, 52F11, and 42C5 CTLs, which presumably originated from a single clone, demonstrated the same pattern; IFN-γ production was significantly decreased when stimulated by *Cw*0303-114*, *-116*, or *-152* mutants constituting peptide-binding pockets (Fig. 2, *C*, *E*, and *F*, and Table III). Finally, the *Cw*0303* mutants bearing Cw*0801 amino acids outside the positions constituting peptide-binding pockets stimulated all CTLs to the same degree as the wild-type *Cw*0303* construct (Fig. 2). These data suggest that all CTLs recognized the Cw molecule in a peptide-dependent manner. In addition, the fact that the seven isolated CTLs consisting of five CTL clones had five different recognition patterns for the mutated HLA-Cw*0303 molecules (Table III) suggests that CTLs for a variety of peptide/non-self Cw molecule complexes are expanded in post-HSCT recipients with acute GVHD.

*Correlation between HLA-Cw*0303-reactive T cells and acute GVHD and graft-vs-leukemia effect*

We determined the frequencies of Cw*0303-reactive T cells in the posttransplant recipient blood samples using the IFN-γ ELISPOT (Fig. 3*A*). Cw*0303-reactive T cells accounted for about one-half of the total of alloreactive T cells on day 28 after transplant, when skin acute GVHD developed, and on day 104 after transplant, when acute GVHD subsided under immunosuppressant use, but no IFN-γ-producing Cw*0303-reactive T cells were detected on day 153 after transplant, when skin acute GVHD completely disappeared with a minimal immunosuppressant (Fig. 3*B*). Additionally, all isolated CTLs lysed recipient-derived dermal fibroblasts as well as recipient PBMCs, but not Cw*0303-negative fibroblasts (Fig. 4). These data are consistent with the participation of Cw*0303-reactive T cells in the development of skin acute GVHD in this patient.

We could not examine the cytotoxicity of CTL clones against autologous leukemic cells because the patient's ALL cells were not cryopreserved. Instead, we determined whether each CTL clone lyses PBMCs containing >90% ALL cells obtained from Cw*0303-positive ($n = 3$) and Cw*0303-negative ($n = 3$) unrelated patients. All clones lysed all Cw*0303-positive ALL cells, but failed to lyse all Cw*0303-negative ALL cells (Fig. 5). These data suggest that the expression level of HLA-Cw molecules on the surface of at least some ALL cells is enough to be recognized by CTLs, and that the isolated Cw*0303-specific CTL clones might be involved in the graft-vs-leukemia effect as well as acute GVHD.

Discussion

In this study, several CTL clones that lysed recipient cells but not donor cells were isolated from a recipient at the onset of acute GVHD who had received bone marrow from an HLA-A, -B, and -DRB1-matched, HLA-Cw-mismatched, killer Ig-like receptor ligand-matched, unrelated donor. All isolated CTL clones recognized the mismatched HLA-Cw molecule as an alloantigen, whereas no clone recognized minor histocompatibility Ags. A question left unresolved was whether this finding is unique to this single patient or can be duplicated from additional Cw-mismatched patients. In addition, we cannot exclude the possibility that this finding could be affected by our in vitro culture conditions.

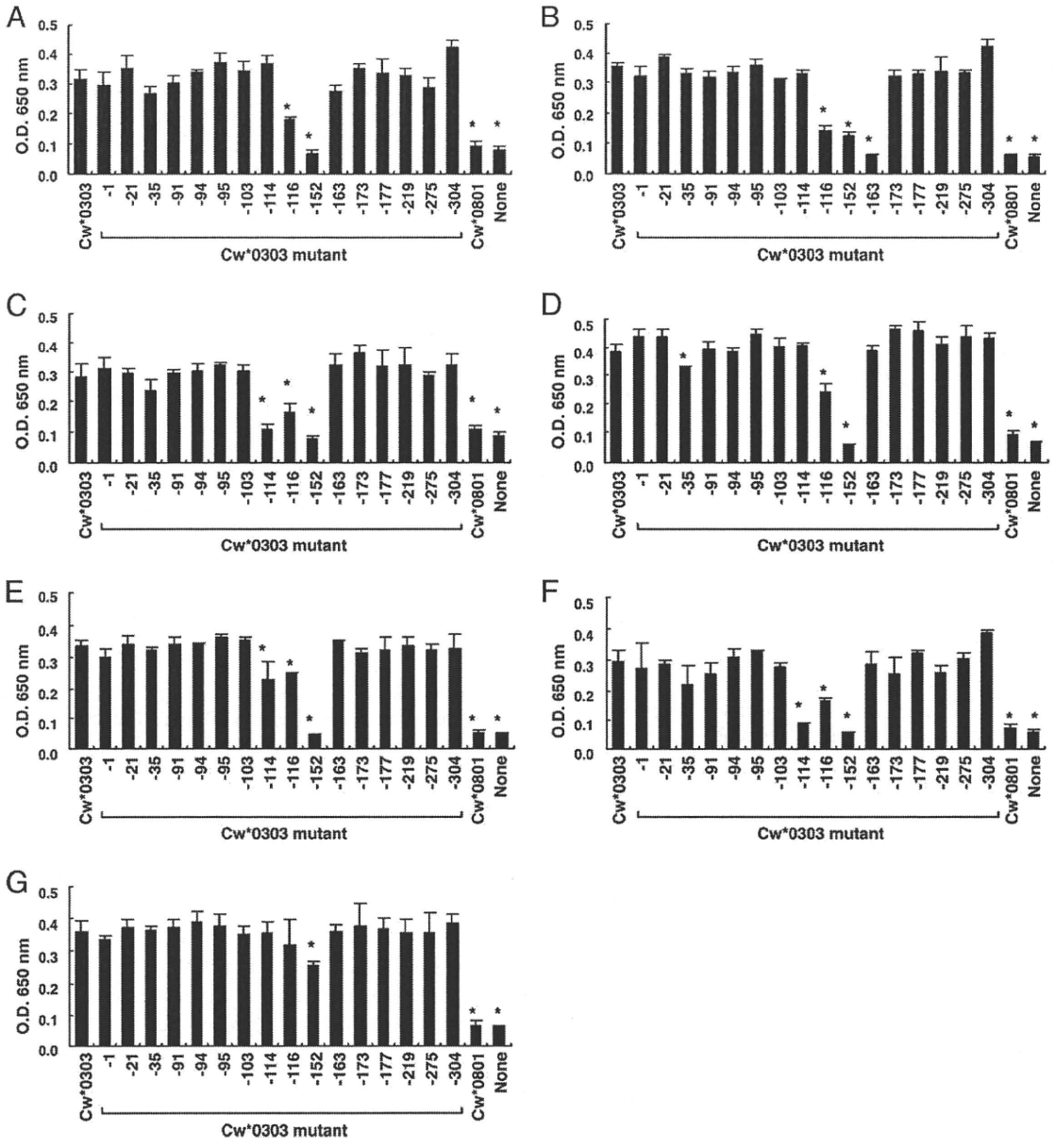

FIGURE 2. Decreased IFN-γ production by CTLs when cocultured with COS cells transfected with *HLA-Cw*0303* mutants in which an amino acid constituting a peptide-binding pocket is substituted with the corresponding amino acid in Cw*0801. COS cells were transfected with a plasmid encoding wild-type *Cw*0303* cDNA, *Cw*0801* cDNA, or *Cw*0303* mutants in which individual amino acids are substituted with the corresponding Cw*0801 amino acids. COS cells transfected with wild-type *Cw*0303* cDNA, 16 types of *Cw*0303* mutant cDNA, and wild-type *Cw*0801* cDNA contained ~70% anti-HLA-class I mAb YTH862.2$^+$ cells, whereas nontransfected COS cells contained ~0% YTH862.2$^+$ cells. COS transfectants were cocultured with CTL clones 60D6 (*A*), 60F1 (*B*), 64A11 (*C*), 11F1 (*D*), 52F11 (*E*), 42C5 (*F*), and 46D4 (*G*), and IFN-γ production was measured in the supernatant using an ELISA. Data are the means of triplicate determinations. *, Significant difference ($p < 0.05$: Student's t test) in the IFN-γ production stimulated by *Cw*0303* mutants compared with wild-type *Cw*0303*.

Previous statistical studies have shown that HLA-Cw matching plays an important role in the outcome of allogeneic HSCT. Analyses of transplants performed under the auspices of the Japan Marrow Donor Program identified that HLA-Cw incompatibilities were significantly associated with a higher incidence of acute GVHD and lower leukemia relapse (2, 4, 23). Analyses of transplants through the National Marrow Donor Program also demonstrated that HLA-Cw mismatch adversely affected engraftment, acute GVHD, and mortality after transplants (3, 24). Another study from the European Blood and Marrow Transplant Group showed an increased mortality trend in patients who received bone marrow transplants from HLA-Cw-mismatched unrelated donors (25). Alternatively, the impact of HLA-Cw mismatches on T cell responses in post-HSCT recipients remains largely unexplored. A few studies have shown that the frequency of CTL precursors is closely correlated with the occurrence of acute GVHD (26–29), and no data are available on the characteristics of individual T cell clones generated in patients who actually received an allogeneic HSCT from an HLA-Cw-mismatched donor and developed acute GVHD.

Isolated CTL clones included CD8$^+$, CD4$^+$, and CD4$^+$CD8$^+$ cells. In contrast to precursor T cells in the thymic cortex that simultaneously coexpress CD4 and CD8 molecules, mature peripheral blood T cells released in the circulation following a selection process generally express either CD4 or CD8. However, double-positive T cells expressing both CD4 and CD8 have been

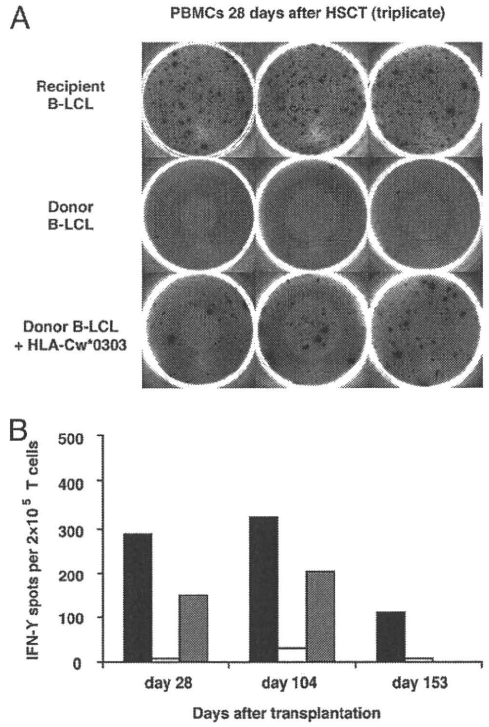

FIGURE 3. Detection of HLA-Cw*0303-specific CTLs in recipient PBMCs after transplantation. *A*, Representative ELISPOT wells show triplicate results of PBMCs at 28 days after HSCT stimulated by recipient B-LCL, donor B-LCL, and *HLA-Cw*0303*-transfected donor B-LCL. *B*, The frequency of CTLs in PBMCs that recognized HLA-Cw*0303 at different time points (28, 104, and 153 days after HSCT) was measured by IFN-γ ELISPOT analysis. The frequency of IFN-γ-producing cells is shown against recipient B-LCL (■), donor B-LCL (□), and *HLA-Cw*0303*-transfected donor B-LCL (▨). Data are the means of triplicate determinations.

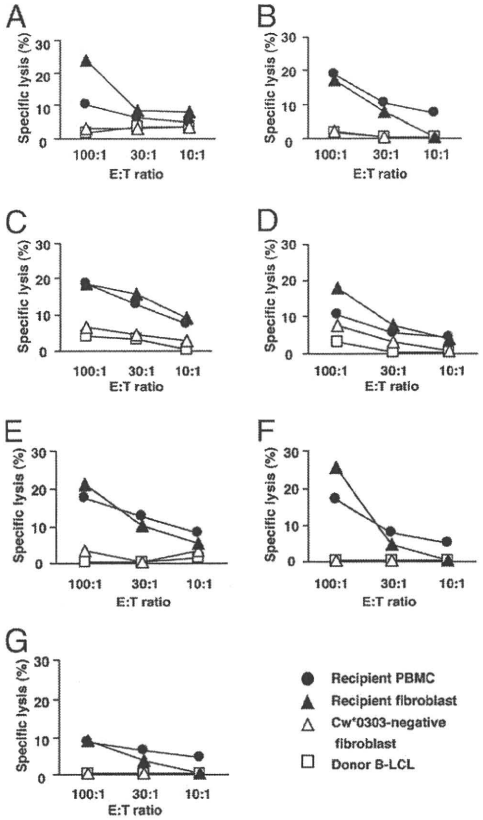

FIGURE 4. Cytotoxicities of CTLs against recipient-derived dermal fibroblasts for CTL clones 60D6 (*A*), 60F1 (*B*), 64A11 (*C*), 11F1 (*D*), 52F11 (*E*), 42C5 (*F*), and 46D4 (*G*) in a cytotoxicity assay. The lysis of recipient PBMC, recipient-derived dermal fibroblasts, HLA-Cw*0303-negative unrelated patient-derived dermal fibroblasts, and donor B-LCL is shown as the mean of triplicate cultures at various E:T ratios.

described in several pathological conditions, including autoimmune, neoplastic, and chronic inflammatory disorders, as well as in normal individuals (30, 31). These data suggest the existence of non-self HLA-specific, $CD4^+CD8^+$ CTLs in the peripheral blood of an allogeneic bone marrow transplant recipient. Isolated $CD4^+CD8^+$ CTL 42C5 shared TCR Vβ with $CD8^+$ CTL 64A11 and $CD4^+$ CTL 52F11, although we cannot rule out the possibility that this finding could be the result of in vitro culture conditions. Double-positive T cells in the peripheral blood may be terminally differentiated from $CD4^+$ or $CD8^+$ T cells, or, alternatively, released from the thymus as mature cells (31). The thymus of HSCT recipients is severely damaged by preconditioning regimens consisting of high-dose anticancer drugs and/or irradiation. Whether circulating double-positive T cells in post-HSCT recipients at the onset of acute GVHD are terminally differentiated from single-positive T cells or directly derived from the thymus, and whether the higher numbers of double-positive T cells in target organs correlate with a poor GVHD prognosis are of considerable interest. Also, further studies to isolate CTL clones from additional HLA-mismatched HSCT recipients could provide the frequency information relating to the distribution of $CD8^+$, $CD4^+$, and $CD4^+CD8^+$ CTLs in these patients.

All CTLs recognized the Cw molecule in a peptide-dependent manner. To assess the importance of peptides presented by non-self HLA-Cw molecules in allorecognition of isolated CTLs, *HLA-Cw*0303* mutants were constructed, and the residue that affects recognition of CTLs was examined. IFN-γ production was decreased when stimulated by *Cw*0303* mutants in which an amino acid constituting a peptide-binding pocket was substituted with a Cw*0801 amino acid, whereas the *Cw*0303* mutants bearing Cw*0801 amino acids outside the positions constituting peptide-binding pockets stimulated all CTLs to the same degree as the wild-type *Cw*0303* construct. These data demonstrate that peptides bound to HLA molecules play an important role in the recognition of these CTLs. These mutations appear to alter the affinity of peptides with the HLA-Cw*0303 molecule, resulting in presentation of different peptide repertoires or no peptides and subsequent loss of CTL recognition. Another possibility is that the mutations may change the conformation of the peptide-HLA molecule complex so that T cell receptors can no longer effectively recognize them. Although the peptides recognized by isolated CTLs are uncertain, each CTL clone showed a different recognition pattern for the mutated HLA-Cw*0303 molecules. Thus, it is reasonable to conclude that polyclonal CTLs for a variety of peptides presented by non-self HLA-Cw molecules are expanded in post-HSCT recipients from Cw-mismatched donors, although the possibility that some T cell clones are dominant cannot be excluded (32). Further efforts to identify the peptides recognized by isolated CTLs should help to elucidate the mechanisms of immunoreactions, such as acute GVHD in HLA-Cw*0303-mismatched HSCT.

A recent statistical analysis showed a significant association between some specific amino acid substitutions of recipient and donor HLA class I molecules and the occurrence of acute GVHD after unrelated bone marrow transplantation (33). Substitutions of amino acids at positions 9 and 116 in the HLA-A molecule and positions 9, 77, 80, 99, 116, and 156 in the HLA-Cw molecule

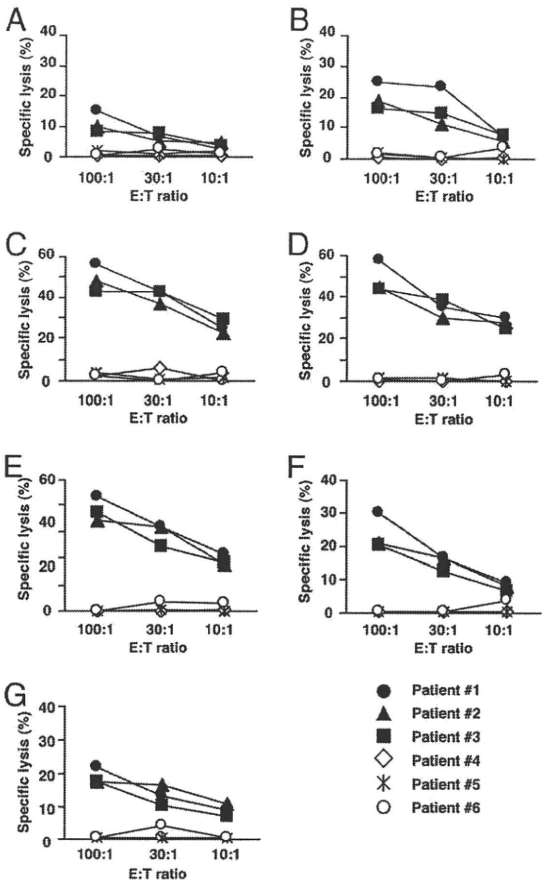

FIGURE 5. Cytotoxicities of CTLs against ALL cells for CTL clones 60D6 (*A*), 60F1 (*B*), 64A11 (*C*), 11F1 (*D*), 52F11 (*E*), 42C5 (*F*), and 46D4 (*G*) in a cytotoxicity assay. PBMCs containing >90% ALL cells were obtained from Cw*0303-positive (n = 3; patients 1–3) and Cw*0303-negative (n = 3; patients 4–6) unrelated patients and used as a target in the cytotoxicity assay. Data are means of triplicate cultures at various E:T ratios.

between recipient and donor were identified as risk factors for severe acute GVHD. Because amino acids at positions 77 and 80 in the HLA-Cw molecule are epitopes for killer Ig-like receptor (34), these substitutions may be associated with alloreactivity of donor NK cells. Amino acids at other positions, 9, 99, 116, and 156, in the HLA-class I molecule constitute peptide-binding pockets. Therefore, higher T cell responses may explain the increased occurrence of acute GVHD in recipients who have these amino acid substitutions. However, no immunobiological evidence to support this hypothesis exists. The present study clearly demonstrated that recognition of Cw*0303-specific CTLs generated in the recipient with acute GVHD was affected by substitutions of amino acids constituting peptide-binding pockets in the Cw*0303 molecule; in particular, recognition of most CTL clones was affected by substitution of an amino acid at position 116.

In conclusion, T cells recognizing recipient Cw molecules in a peptide-dependent manner are important in the alloreaction that occurs in post-HSCT recipients from HLA-Cw-mismatched donors.

Acknowledgments

We thank Chika Wakamatsu and Yuka Nomura for their technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Petersdorf, E. W. 2004. HLA matching in allogeneic stem cell transplantation. *Curr. Opin. Hematol.* 11: 386–391.
2. Morishima, Y., T. Sasazuki, H. Inoko, T. Juji, T. Akaza, K. Yamamoto, Y. Ishikawa, S. Kato, H. Sao, H. Sakamaki, et al. 2002. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. *Blood* 99: 4200–4206.
3. Flomenberg, N., L. A. Baxter-Lowe, D. Confer, M. Fernandez-Vina, A. Filipovich, M. Horowitz, C. Hurley, C. Kollman, C. Anasetti, H. Noreen, et al. 2004. Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. *Blood* 104: 1923–1930.
4. Morishima, Y., T. Yabe, K. Matsuo, K. Kashiwase, H. Inoko, H. Saji, K. Yamamoto, E. Maruya, Y. Akatsuka, M. Onizuka, H. Sakamaki, et al. 2007. Japan Marrow Donor Program: effects of HLA allele and killer immunoglobulin-like receptor ligand matching on clinical outcome in leukemia patients undergoing transplantation with T-cell-replete marrow from an unrelated donor. *Biol. Blood Marrow Transplant.* 13: 315–428.
5. Kaminski, E. R. 1989. How important is histocompatibility in bone marrow transplantation? *Bone Marrow Transplant.* 4: 439–444.
6. Goulmy, E. 1997. Human minor histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy. *Immunol. Rev.* 157: 125–140.
7. Riddell, S. R., C. Berger, M. Murata, S. Randolph, and E. H. Warren. 2003. The graft versus leukemia response after allogeneic hematopoietic stem cell transplantation. *Blood Rev.* 17: 153–162.
8. Kariyone, A., M. Tanabe, T. Juji, K. Kano, and M. Takiguchi. 1990. Functional expression of HLA-C blank antigens on human blood lymphocytes. *J. Immunol.* 145: 3714–3718.
9. Rufer, N., B. S. Breur-Vriesendorp, J. M. Tiercy, A. S. Slavcev, N. M. Lardy, P. Francis, R. Kressig, D. E. Speiser, C. Helg, B. Chapuis, et al. 1993. High-resolution histocompatibility testing of a group of sixteen B44-positive, ABDR serologically matched unrelated donor-recipient pairs: analysis of serologically undisclosed incompatibilities by cellular techniques, isoelectrofocusing, and HLA oligotyping. *Hum. Immunol.* 38: 235–239.
10. Barnardo, M. C., N. J. Davey, M. Bunce, P. A. Brookes, R. I. Lechler, K. I. Welsh, and J. R. Batchelor. 1996. A correlation between HLA-C matching and donor antirecipient CTL precursor frequency in bone marrow transplantation. *Transplantation* 61: 1420–1423.
11. Grundschober, C., N. Rufer, A. Sanchez-Mazas, A. Madrigal, M. Jeannet, E. Roosnek, and J. M. Tiercy. 1997. Molecular characterization of HLA-C incompatibilities in HLA-ABDR-matched unrelated bone marrow donor-recipient pairs: sequence of two new Cw alleles (*Cw*02023* and *Cw*0707*) and recognition by cytotoxic T lymphocytes. *Tissue Antigens* 49: 612–623.
12. Yazaki, M., T. Takahashi, K. Mizutani, Y. Ito, H. Wakiguchi, M. Inoue, K. Kawa, K. Kato, T. Kato, H. Saito, and H. Togari. 2002. Human leucocyte antigen-Cw-specific cytotoxic T lymphocytes generated from naive cord blood used for cord blood stem cell transplantation. *Br. J. Haematol.* 117: 893–898.
13. McCutcheon, J. A., J. Gumperz, K. D. Smith, C. T. Lutz, and P. Parham. 1995. Low HLA-C expression at cell surfaces correlates with increased turnover of heavy chain mRNA. *J. Exp. Med.* 181: 2085–2095.
14. Neisig, A., C. J. Melief, and J. Neefjes. 1998. Reduced cell surface expression of HLA-C molecules correlates with restricted peptide binding and stable TAP interaction. *J. Immunol.* 160: 171–179.
15. David, R. J. 2003. Locus-specific constitutive and cytokine-induced HLA class I gene expression. *J. Immunol.* 170: 1894–1902.
16. Przepiorka, D., D. Weisdorf, P. Martin, H. G. Klingemann, P. Beatty, J. Hows, and E. D. Thomas. 1995. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant.* 15: 825–828.
17. Murata, M., E. H. Warren, and S. R. Riddell. 2003. A human minor histocompatibility antigen resulting from differential expression due to a gene deletion. *J. Exp. Med.* 197: 1279–1289.
18. Riddell, R. S., M. Rabin, A. P. Geballe, W. J. Britt, and P. D. Greenberg. 1991. Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression. *J. Immunol.* 146: 2795–2804.
19. Narimatsu, H., M. Murata, S. Terakura, K. Sugimoto, and T. Naoe. 2008. Potential role of a mismatched HLA-specific CTL clone developed pre-transplant in graft rejection following cord blood transplantation. *Biol. Blood Marrow Transplant.* 14: 397–402.
20. Whitelegg, A., and L. D. Barber. 2004. The structural basis of T-cell allorecognition. *Tissue Antigens* 63: 101–108.
21. Steven, G. E., P. Peter, and D. B. Linda. 2000. *The HLA Facts Book.* London: Academic Press.
22. Zhao, B., A. E. Png, E. C. Ren, P. R. Kolatkar, V. S. Mathura, M. K. Sakharkar, and P. Kangueane. 2003. Compression of functional space in HLA-A sequence diversity. *Hum. Immunol.* 64: 718–728.
23. Sasazuki, T., T. Juji, Y. Morishima, N. Kinukawa, H. Kashiwabara, H. Inoko, T. Yoshida, A. Kimura, T. Akaza, N. Kamikawaji, Y. Kodera, and F. Takaku. 1998. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor: Japan Marrow Donor Program. *N. Engl. J. Med.* 339: 1177–1185.
24. Lee, S. J., J. Klein, M. Haagenson, L. A. Baxter-Lowe, D. L. Confer, M. Eapen, M. Fernandez-Vina, N. Flomenberg, M. Horowitz, C. K. Hurley, et al. 2007.