whereas an elevated hepcidin level was a strong risk factor even after adjustment for other potential confounders. Furthermore, we observed consistent association of high hepcidin levels with high risk for developing bacterial infection when analyses were confined to either the low- or high-ferritin subgroups. These findings collectively suggest that hepcidin can be used as a better predictor of documented bacterial infections than serum ferritin levels. Moreover, various new techniques to quantify hepcidin-25, such as a competitive enzyme-linked immunoassay as well as mass spectrometry-based methods, have been recently developed [19,25,32,33]. Standardization of those methods will make it possible to use the serum hepcidin level as a biomarker in routine clinical practice.

Hepcidin was first isolated and characterized as an antimicrobial peptide in human blood [12]. In radial diffusion assays, synthetic hepcidin suppressed the growth of several strains of Gram-positive bacteria and some strains of Gram-negative bacteria, but not of *Escherichia coli* or *Pseudomonas fluorescens*. Our findings pertaining to the adverse association of high hepcidin levels with bacterial infection indicated that the bactericidal effect of hepcidin was either considerably limited in neutropenic settings such as HSCT or was ineffective on the bacterial organisms observed in our cohort. Moreover, we observed a significant adverse effect of hepcidin even after the adjustment for potential confounders, suggesting that hepcidin itself may play an unknown biologic role in susceptibility to bacterial infection, or it may represent an unknown surrogate marker for predicting bacterial infection. To answer this issue, the significance of pretransplant serum hepcidin levels needs to be evaluated in a more homogeneous group of patients having the same level of confounders.

We did not detect any adverse effect of high hepcidin levels on infection-related mortality or OS at 100 days after transplantation, although there was a marked difference in the incidence of bacterial infection. One possible explanation for this observation is that bacterial infection of the blood was well managed by prompt and appropriate treatment with antibiotics in our transplant centers. However, because the incidence of early death after HSCT is considerably low, the effect of bacterial infection on early mortality should be evaluated in larger cohort studies to gain enough statistical power for comparison. Alternatively, selective prophylactic administration of oral antibiotics such as fluoroquinolones to patients with a high risk of bacterial infection may be an effective approach; however, this approach will be effective only if most of the bacterial isolates at the transplant center are sufficiently sensitive to these prophylactic antibiotics. With regard to other endpoints, there was no association between high hepcidin levels and the incidence of CMV infection. The effect of hepcidin level on the incidence of fungal infection could not be evaluated because of the very low incidences of these conditions in our cohorts. These effects should also be evaluated in studies with a larger cohort in the future.

The present study, however, has some limitations. We cannot exclude the possibility of a pseudonegative result for bloodstream infection, because broad-spectrum antibiotics were administered to all neutropenic patients at the time of blood culture, regardless of the results of blood culture. In addition, the retrospective study design and heterogeneous background of diseases and transplantation procedures could also bias the results. Particularly, in the small cohort of 55 patients, the adjustment of HRs by confounders may be incomplete. In particular, the higher proportion of CB transplants and the high risk of diseases in the high-hepcidin group may cause bias, although we found consistently high HRs in the high-hepcidin group in various stratified categories. Therefore, larger studies are necessary to confirm our results.

In conclusion, our study revealed that the pretransplant serum hepcidin level was significantly associated with bacterial infection, particularly bloodstream infection, suggesting that quantification of serum hepcidin levels could be useful for predicting early bacterial complications. Prophylactic antibiotic therapy based on the local sensitivities of common bacterial isolates can be considered in the patients with high hepcidin levels who are undergoing allogeneic HSCT. Larger prospective studies are, however, warranted to confirm our findings.

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Conflict of interest: N.T. declares that he is the President of Medical Care Proteomics Biotechnology Co. Ltd. (Ishikawa-ken, Japan), a startup company, the stock of which is not publicly traded. The other authors declare that they have no conflicts of interest relevant to this paper.

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poorly phrased. There is no standard by which one can compare an odds ratio for CR to a hazard ratio for OS. Given how difficult it has been historically to improve upon the results with MP, and the conflicting results of the various MPT vs MP trials, the clear survival advantage seen with MP-Bortezomib in the VISTA trial is truly remarkable, and by far the most important take-home message. It is not appropriate to directly contrast the results of the E4A03 and VISTA studies (which I agree are both excellent). The contrast I tried to make in the editorial was in the reaction to these trials of stock analysts (whose obtrusive presence at the meeting has recently been noted[16]), who appear to place an excessively high value on improvements in CR. In my opinion this is not always appropriate, and a more balanced approach is warranted, with better surrogates (for example, molecular CR, suppression of cytogenetic abnormalities) for OS needed.

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Molecular detection of *AML1-MTG8*-positive cells in peripheral blood from a patient with isolated extramedullary relapse of t(8;21) acute myeloid leukemia

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A few studies have reported that *AML1-MTG8* expression levels in bone marrow (BM) are 1- to 3-log higher than those in peripheral blood (PB) when detected by quantitative PCR methods in acute myeloid leukemia (AML) with the t(8;21) translocation.[1–3] However, the relationship between BM and PB is retained at any time during the clinical course is unknown. Here we present a patient with t(8;21) AML who demonstrated isolated ovarian relapse after allogeneic BM transplantation (BMT). *AML1-MTG8* chimeric transcripts could be repeatedly detected in both BM and PB during the clinical course. Moreover, the *AML1-MTG8* expression levels detected by real-time quantitative (RQ)-PCR methods in PB were higher than those in BM before and at the time of the extramedullary relapse (EMR). Thus, we propose that the presence of EMR is responsible for repeated detection of minimal residual disease (MRD) and discuss the clinical significance of different *AML1-MTG8* expression levels between BM and PB for the diagnosis of isolated EMR.

A 22-year-old woman was diagnosed with AML (French-American-British (FAB) subtype M2) with thoracic vertebrae involvement in March 1998. Cytogenetic evaluation revealed the t(8;21)(q22;q22) chromosomal translocation. She achieved complete remission (CR) with induction chemotherapy and

radiotherapy; however, in December 1998 she had a BM relapse, involving the thoracic vertebrae and the spine. Salvage chemotherapy reduced the total number of leukemic blasts in BM to below 5%. In March 1999, she underwent BMT from her HLA-2-antigen-mismatched/haploidentical sister at Osaka University Hospital. A total of 3.0×10^8 per kg unmanipulated nucleated cells were infused. The transplant protocol consisted of a high dose of cytarabine and cyclophosphamide and total body irradiation (12 Gy), followed by a short course of methotrexate, tacrolimus and methylprednisolone (2 mg/kg) for graft-versus-host disease (GVHD) prophylaxis. The patient achieved an absolute neutrophil count above 0.5×10^9 per liter on day 20. The last platelet transfusion was performed on day 125. CR and complete donor chimerism was confirmed by a BM examination on day 24. No acute GVHD developed.

We sequentially measured *AML1-MTG8* expression levels using RQ-PCR methods during the clinical course as previously described.[4] *AML1-MTG8* levels in BM stayed below 1.0×10^{-5} after BMT, but increased to 3.8×10^{-5} on day 96. Thereafter, *AML1-MTG8* levels in BM and PB were monitored biweekly and weekly, respectively. Whereas *AML1-MTG8* levels in BM and PB showed parallel movement, those in PB were constantly over 2.0×10^{-4} and were higher than those in BM. Because a BM examination still revealed CR, we suspected a regrowth of leukemia cells in the patient and performed a systemic examination. Computed tomography scans of the pelvis revealed little ascites in the patient on day 109, but an enlargement of the left ovary with a diameter of 5 cm with moderate ascites on day 158 (Figure 1). The ovarian tumor was diagnosed as EMR due to the contamination of t(8;21)-positive leukemia cells in ascites by culdocentesis.

As tacrolimus and prednisolone were tapered rather rapidly for induction of a graft-versus-leukemia effect, skin GVHD developed on day 170. Following increase in tacrolimus and prednisolone, the skin rash disappeared in about a week, followed by shrinkage of the tumor to a diameter of 2.5 cm and disappearance of the ascites on day 185. *AML1-MTG8* levels in both BM and PB decreased to below 1.0×10^{-5} on day 187; however, the levels increased again with some fluctuations. Magnetic resonance imaging of the pelvis on day 262 showed enlargement of the left ovarian tumor again. Because BM was still present in CR, bilateral salpingo-oophorectomy was performed on day 319. The bilateral ovaries appeared to be involved, making complete resection impossible due to tight adhesion with surrounding tissues. *AML1-MTG8* levels were highest in the BM greater than those in PB, when morphologic BM relapse occurred on day 355 (Figure 1). Despite chemotherapy and donor lymphocyte infusion, the patient died of renal failure due to obstruction of the bilateral ureters by abdominal mass on day 474.

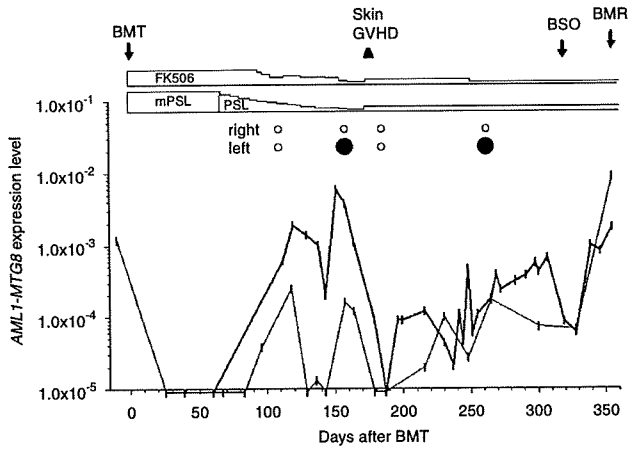

Figure 1 Clinical course and kinetics of the *AML1-MTG8* gene-transcript expression levels in bone marrow (BM) and peripheral blood (PB). Thin and thick lines indicate changes in *AML1-MTG8* expression levels in BM and PB, respectively. In the results of imaging studies of the ovary, open circles indicate the normal size ovary, whereas closed circles indicate the enlarged ovary. Real-time quantitative-PCR was performed with ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). *AML1-MTG8* expression levels in Kasumi-1 cell lines were defined as 1.0. BMR, bone marrow relapse; BMT, bone marrow transplantation; BSO, bilateral salpingo-oophorectomy; FK506, tacrolimus; GVHD, graft-versus-host disease; mPSL, methylprednisolone; PSL, prednisolone.

EMR of leukemia after transplant occurs in diverse sites such as the central nervous system and testis, which makes an early diagnosis difficult. One feasible approach to overcome this problem would be monitoring MRD that is involved in BM.[5,6] In the present case, continuous detection of *AML1-MTG8* chimeric transcripts not only in BM, but also in PB was quite helpful in detecting the presence of EMR. So far nested PCR detection of *AML1-MTG8* chimeric transcripts in BM and PB have not been the indicators of subsequent relapse in t(8;21) AML after BMT.[7] Meanwhile, recent studies using quantitative PCR methods reported that there is a threshold of *AML1-MTG8* expression levels at which subsequent relapse occurs.[1,8,9] Therefore, frequent monitoring of *AML1-MTG8* expression levels or systemic screening for extramedullary disease should be considered, especially as *AML1-MTG8* chimeric transcripts continued to be detected despite CR.

Interestingly, the *AML1-MTG8* levels in BM and PB showed reversal between day 112 and 179, suggesting that t(8;21)-positive leukemia cells originated from extramedullary disease were constantly present in PB, rather than in BM. Given that this unusual relationship returned to the original state at the time of BM relapse, higher *AML1-MTG8* expression levels in PB compared to BM suggest a sign of isolated EMR. However, further studies are required to determine whether screening of PB is superior to BM for early detection of isolated EMR by PCR-based monitoring *AML1-MTG8* expression levels.

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The B-cell calcium sensor predicts progression of chronic lymphocytic leukemia

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Identifying mechanisms responsible for the clinical heterogeneity of chronic lymphocytic leukemia (CLL) is important to develop better treatments for this disease. Variations in responsiveness to immunoreceptor signaling may be responsible for differences in proliferation of CLL cells *in vivo*.[1] Accordingly, we examined the status of the B-cell calcium sensor (Ca_oS) in primary CLL cells, as it responds to extracellular calcium (Ca_o^{2+}) fluctuations by modulating subsequent signal transduction through immunoreceptors.[2]

In contrast to normal B cells, nearly half (23/51) of the CLL samples examined (with approval from the Sunnybrook Health Sciences Center Research Ethics Board) did not release intracellular calcium (Ca_i^{2+}) in response to $CaCl_2$ (labeled Ca_o^{2+} non-responders) (Table 1; Figure 1a). This impaired Ca_oS activity was not due to decreased stores of Ca_i^{2+} in the endoplasmic reticulum, as the Ca^{2+} ATPase inhibitor, thapsigargin, was able to mobilize Ca_i^{2+} in these cells (not shown). While normal B-cells mobilized Ca_i^{2+} in response to as little as 250 μM $CaCl_2$, Ca_o^{2+} non-responder CLL cells remained insensitive to doses as high as 1.5 mM (above which, calcium was toxic) (not shown). These results suggested that the defective responses to Ca_o^{2+} were not due to reduced expression or

Table 1 Summary of clinical properties of CLL patients classified on the basis of release of Ca_i^{2+} stores by their tumor cells in response to Ca_o^{2+}

Variable	*All patients*	*Ca_o^{2+} responders*	*Ca_o^{2+} non-responders*	*P-value*
No. of patients	51	28	23	
Median age, years	61	60.5	63	NS
Sex, no. (%)				
Female	27 (52.9)	14 (50.0)	13 (56.5)	NS
Male	24 (47.1)	14 (50.0)	10 (43.5)	NS
Years after diagnosis, mean ± s.e.	6.1 ± 0.6	6.1 ± 0.9	6.1 ± 0.7	NS
WBC count, ⁄ 1000 cells/μl, mean ± s.e.	65.9 ± 11.2	62.7 ± 14.2	69.8 ± 18.3	NS
Rai stage III–IV, no. (%)	30 (58.8)	19 (67.9)	11 (47.8)	0.08
CD38%, mean ± s.e.	16.8 ± 3.4 (*n* = 46)	25.3 ± 5.4 (*n* = 26)	5.9 ± 0.9 (*n* = 20)	<0.02
β2-Microglobulin, mg/l, mean ± s.e.	2.3 ± 0.3 (*n* = 16)	2.6 ± 0.4 (*n* = 11)	1.8 ± 0.3 (*n* = 5)	0.05
Genomic aberrations, no. (%)				
Deletion 11	3 (8.1)	2 (9.1)	1 (6.6)	
Deletion 17	5 (13.5)	4 (18.8)	1 (6.6)	
Trisomy 12	3 (8.1)	2 (9.1)	1 (6.6)	
Deletion 13	21 (56.8)	15 (68.2)	6 (40.0)	
Normal	11 (29.7)	6 (27.3)	5 (33.3)	
Not available	14	6	8	
High-risk cytogenetics[a], no. (%)	10 (27.0)	7 (31.8)	3 (20.0)	NS
LDTs, months, mean ± s.e.	28.3 ± 5.6 (*n* = 44)	10.9 ± 3.3 (*n* = 22)	49.5 ± 9.4 (*n* = 22)	<0.001
Received treatment, no. (%)	25 (49.0)	17 (60.7)	8 (34.8)	0.03
No. of treatments/patient, mean ± s.e.	1.3 ± 0.3	1.9 ± 0.4	0.7 ± 0.3	0.02

Abbreviations: CLL, chronic lymphocytic leukemia; LDTs, lymphocyte doubling times; NS, not significant; WBC, white blood cell.
Assume $n = 51$, unless otherwise indicated.
[a]High-risk cytogenetics include patients with $17p^-$ deletions, $11q^-$ deletions, trisomy 12 or complex multiple abnormalities.

Hematopoietic stem cell transplantation for core binding factor acute myeloid leukemia: t(8;21) and inv(16) represent different clinical outcomes

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We analyzed 338 adult patients with acute myeloid leukemia (AML) with t(8;21) and inv(16) undergoing stem cell transplantation (SCT) who were registered in the Japan Society for Hematopoietic Cell Transplantation database. At 3 years, overall survival (OS) of patients with t(8;21) and inv(16) was 50% and 72%, respectively ($P = .002$). Although no difference was observed when restricted to allogeneic SCT in first complete remission (CR; 84% and 74%), OS of patients with t(8;21) and inv(16) undergoing allogeneic SCT in second or third CR (45% and 86% at 3 years; $P = .008$) was different. OS was not different between patients in first CR who received allogeneic SCT and those who received autologous SCT for both t(8;21) AML (84% vs 77%; $P = .49$) and inv(16) AML (74% vs 59%; $P = .86$). Patients with inv(16) not in CR did better after allogeneic SCT than those with t(8;21) (70% and 18%; $P = .03$). Patients with t(8;21) and inv(16) should be managed differently as to the application of SCT. SCT in first CR is not necessarily recommended for inv(16). For t(8;21) patients in first CR, a prospective trial is needed to clarify the significance of autologous SCT and allogeneic SCT over chemotherapy. (Blood. 2009;113:2096-2103)

Introduction

Core binding factor (CBF) acute myeloid leukemia (AML) including t(8;21)(q22;q22) and inv(16)(p13q22)/t(16;16)(p13;q22) [t(8;21) and inv(16)] is considered to be a favorable cytogenetic subgroup in clinical studies.[1-4] Patients with t(8;21) and inv(16) have shown a markedly improved outcome with repetitive use of high-dose cytarabine.[5-13] However, the major treatment failure is disease recurrence.[14-16] These patients frequently become stem cell transplantation (SCT) candidates.

Both t(8;21) and inv(16) AMLs are associated with disruption of genes encoding subunits of the CBF, a heterodimeric transcriptional factor involved in the regulation of hematopoiesis.[17,18] Although these 2 different cytogenetics also share common clinical characteristics, they are associated with different clinical features such as morphologic presentation and immunophenotypic marker expression.[19]

Several reports demonstrated inferior outcome of t(8;21) compared with inv(16), but the number of patients who underwent transplantation was limited.[14,15,20] A recent study from the Dana-Farber Cancer Institute reported that both patients with t(8;21) and inv(16) de novo AML who underwent allogeneic transplantation performed favorably compared with other karyotypes.[21] To identify the survival data and prognostic factors among the CBF leukemia population who received SCT, we conducted a retrospective analysis using a Japanese multi-institution database with a large number of patients.

Methods

Study population

A total of 2802 adult patients who underwent autologous or allogeneic SCT from 1996 and 2004 for AML were registered in the Japan Society for Hematopoietic Cell Transplantation (JSHCT) database. Patients who underwent SCT from unrelated donors were registered in the different registry in the study period, but not all of the patients undergoing unrelated SCT were registered in the JSHCT database. Demographic, diagnostic, clinical, cytogenetics, induction, and outcome information were collected for each patient, and were sent to a central registration center. Cytogenetic studies were performed in each center, but a central review of cytogenetic analysis was not performed.

Patients with de novo AML aged 16 to 70 years who received hematopoietic SCT as the first transplant were included in the study. No patients with prior history of autologous or allogeneic SCT were included in the study. Of the remaining 2164 patients, 178 patients with t(15;17) or PML/RARα were excluded from the analysis below (Table 1). Finally, of the 1986 patients included in the analysis, 255 were reported to have t(8;21) abnormality, and 83 to have inv(16). A total of 194 patients had no available cytogenetic data. The remaining 1454 patients with normal karyotype and other cytogenetic abnormalities were further coded and analyzed according to published Southwest Oncology Group (SWOG) criteria.[3] The intermediate risk category included patients characterized by +8, −Y, +6, del(12p), or normal karyotype. The unfavorable risk category was defined by the presence of one or more of −5/del(5q), −7/del(7q), abn 3q, 11q, 20q, or

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Table 1. Cytogenetic risk groups of patients with AML who received autologous SCT and allogeneic SCT

Cytogenetic risk groups	No. patients		
	Auto-SCT	Allo-SCT	Total
t(8;21)	61	194	255
inv(16)	17	66	83
t(15;17)*	65	113	178
Intermediate	140	749	889
Unfavorable	35	325	360
Unknown			
Unknown cytogenetic risk	27	178	205
No available cytogenetic data	44	150	194
Total	389	1775	2164

Auto-SCT indicates autologous stem cell transplantation; Allo-SCT, allogeneic stem cell transplantation.

*Patients with t(15;17) were excluded from the analysis.

21q, del(9q), t(6;9), t(9;22), abn 17p, and complex karyotypes defined as 3 or more abnormalities. Patients with other cytogenetic aberrations were considered an unknown risk group, and were analyzed together with 194 patients with no cytogenetic data.

This study was approved by the Committee for Nationwide Survey Data Management of the JSHCT. Informed consent was obtained in accordance with the Declaration of Helsinki.

Transplantation

A total of 1662 patients underwent allogeneic SCT, and 324 underwent autologous SCT. Patients were treated with various conditioning regimens, but most of those who underwent autologous transplantation received non–total body irradiation (TBI) regimens (97%), including busulfan (BU), cytarabine (CA), and etoposide. The most frequently used conditioning regimens before allogeneic SCT were cyclophosphamide (Cy) plus TBI (n = 327 patients), and BU plus Cy (n = 267). Conditioning regimens before allogeneic SCT also included more intensified regimens such as CA plus Cy plus TBI (n = 262) and BU plus Cy plus TBI (n = 146), or reduced-intensity conditioning regimens with fludarabine (n = 241) or cladribine (n = 19).

Stem cell sources for allogeneic SCT were bone marrow in 871 patients, peripheral blood stem cell in 570 patients, bone marrow plus peripheral blood stem cell in 23 patients, and cord blood in 190 patients. A total of 1242 patients underwent allogeneic SCT from a related donor, and 404 patients underwent SCT from an unrelated donor.

Of the 1637 patients who had available data, 74% received transplants from human leukocyte antigen (HLA)–matched donors. Among patients who received unrelated bone marrow transplants, 156 patients were HLA genotypically matched and 51 were HLA mismatched. HLA data for 39 mismatched unrelated bone marrow transplantation patients were available. A total of 32 patients were one locus mismatched, and 7 patients were 2 loci mismatched. Among patients receiving unrelated cord blood transplants, 19 patients were serologically HLA matched and 170 patients were mismatched. HLA incompatibility was 5 of 6 HLA matched in 57 patients, 4 of 6 HLA matched in 99 patients, 3 of 6 HLA matched in 7 patients, and 1 of 6 HLA matched in 1 patient.

Graft-versus-host disease (GVHD) prophylaxis mostly consisted of methotrexate and a calcineurin inhibitor, either cyclosporin A or tacrolimus. Several other prophylaxes include mycophenolate mofetil, antithymocyte globulin, and $CD34^+$ selection. The incidence of acute GVHD was evaluated in 1488 patients who survived more than 28 days, and chronic GVHD was evaluated in 1302 patients who survived more than 100 days after allogeneic SCT. GVHD was evaluated in each center.

Statistical analysis

Correlation between the 2 groups was examined with the chi-square test, Fisher exact test, and the Mann-Whitney *U* test. Disease-free survival (DFS) was calculated from the date of transplantation until the date of relapse or the date of death in CR. Patient survival data were analyzed with the method of Kaplan and Meier and compared by the log-rank test.

Univariate and multivariate analyses for OS were performed with the aid of the Cox proportional hazard regression model, and variables were selected with the stepwise method. The following variables were evaluated: age, sex, and disease status at transplantation; CR versus not in CR; the number of induction courses to achieve CR; one course versus more than one course and failure; type of transplantation (allogeneic SCT vs autologous SCT); conditioning regimen (reduced intensity vs myeloablative); TBI regimen or not; and the existence of additional karyotype abnormalities or not. For those who received allogeneic SCT, in addition to these variables, the following were also evaluated: type of GVHD prophylaxis; short-course methotrexate plus cyclosporin A or short methotrexate plus FK506; acute GVHD, grade II to IV or grade III to IV; chronic GVHD; HLA mismatch; donor; and donor source. The doses of methotrexate were not surveyed. Each factor was considered to be prognostic if the *P* value was less than .05. Data were analyzed with the Stata 9.2 statistical software (College Station, TX).

Results

Initial characteristics of patients

The median age of all patients with AML in total was 41 years old (range, 16-70 years old). Median follow-up period of living patients was 37.3 months (range, 0.4-108 months). Patients were categorized into 5 cytogenetic subgroups: with t(8;21), with inv(16), intermediate risk cytogenetics, unfavorable cytogenetics, and an unknown risk group. Table 1 shows the number of patients in each cytogenetic subgroup and patients with t(15;17), who were excluded from the analysis.

Characteristics of the patients with CBF who underwent allogeneic SCT or autologous SCT are shown in Table 2. No significant difference was observed between characteristic of 2 groups of patients with CBF who received autologous SCT, except for the initial white blood cell count.

Of the 259 patients with CBF who received allogeneic SCT, significantly more patients with t(8;21) had failed to achieve CR with a single course of induction chemotherapy at diagnosis ($P = .002$), and were not in CR at the time of transplantation ($P < .001$). Among patients in CR at transplantation, the ratio of those in first, second, or third CR was not different between t(8;21) and inv(16) subgroups. Significantly more patients with inv(16) received transplants from an unrelated donor ($P = .004$). Table 3 and Table S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) summarize the transplantation data of those undergoing allogeneic SCT. More patients with inv(16) received unrelated transplants compared with t(8;21) patients ($P = .004$).

Overall survival

The OS of 1986 patients with AML at 3 years was 48%, and those with t(8;21), inv(16), intermediate, unfavorable, and unknown cytogenetic risks showed OS of 50%, 72%, 52%, 35%, and 45%, respectively ($P < .001$). Figure 1 shows survival curves of patients with AML patients who underwent allogeneic SCT in first CR (Figure 1A), in second or third CR (Figure 1B), or not in CR (Figure 1C), categorized by the cytogenetic abnormalities. Survival data are listed in Table 4. The OS of patients with t(8;21), inv(16), and intermediate, unfavorable, and unknown risk undergoing allogeneic SCT in first CR was 84%, 74%, 69%, 53%, and 52%, respectively ($P < .001$), and that of patients undergoing allogeneic-SCT

Table 2. Characteristics of patients with CBF AML

	Auto-SCT			Allo-SCT		
	t(8;21) (n = 61), no.	inv(16) (n = 17), no.	P	t(8;21) (n = 194), no.	inv(16) (n = 66), no.	P
Median age, y (range)	44 (17-68)	37 (19-61)	.59	39 (16-70)	34 (16-64)	.054
Median WBC, g/L (range)	8.8 (0.2-94)	33 (2.1-199)	.02	11 (.6-366)	53 (1.8-284)	< .001
Sex						
Male	41	12	.79	117	40	.93
Female	20	5		74	26	
No. of induction chemotherapy at diagnosis of AML						
1 course	48	15	.72	125	55	.002
> 1 or failure*	11	2		56	7	
Additional cytogenetic abnormalities						
None	53	15	> .999	153	54	.61
Positive	8	2		41	12	
Disease status at SCT						
CR	55	16	> .999	108	52	< .001
Not in CR	6	1		85	11	
CR1	43	13	.98	49	21	.29
CR2	7	1		45	26	
CR3	0	1		5	4	
Conditioning regimen						
TBI	0	1	.22	118	47	.078
Not TBI	61	16		71	16	

Correlation between the two groups was examined.
WBC indicates white blood cell count; g/L, 10^9/L; CR1, first complete remission; and CR2 or 3, second or third CR.
*More than 1 or failure includes patients who did not achieve complete remission after first course of induction chemotherapy, and those who were resistant to induction chemotherapy.

in second or third CR was 45%, 86%, 57%, 44%, and 64%, respectively ($P = .09$). OS of patients undergoing allogeneic SCT not in CR was 18%, 70%, 25%, 15%, and 18%, respectively ($P = .003$).

Table 3. Summary of allogeneic SCT

	t(8;21) (n = 194), no.	inv(16), (n = 66), no.	P
Conditioning regimen			
RIST	31	9	.66
Myeloablative	161	56	
GVHD prophylaxis*			
sMTX+CyA	136	48	.78
sMTX+FK	20	8	
HLA			
Match	146	47	.5
Mismatch	45	18	
Donor			
Related	161	44	.004
Unrelated	32	22	
Stem cell source			
BM	101	40	.27
PB	72	17	
CB	18	7	
aGVHD grade			
0-I	117	37	.54
II-IV	60	22	
cGVHD type			
None	64	28	.28
Lmt/Ext	67	20	

Correlation between the two groups was examined. Some of the missing data was not available, and total numbers do not add up to the number of the patients in each group.
RIST indicates reduced intensity stem cell transplantation; sMTX, short-course methotrexate; CyA, cyclosporin A; FK, tacrolimus; BM, bone marrow; PB, peripheral blood; CB, cord blood; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; Lmt, limited; and Ext, extensive.
*Dose of methotrexate was not surveyed in the study. Detail of other GVHD prophylaxis regimens are in Table S1.

When patients undergoing allogeneic SCT in first CR were analyzed, 3-year OS was not significantly different between patients with t(8;21) and inv(16) (84% and 74%, respectively; $P = .28$), between inv(16) and intermediate risk groups (74% and 69%, respectively; $P = .84$), or between t(8;21) and intermediate risk groups (84% and 69%, respectively; $P = .06$). However, when patients undergoing allogeneic SCT in second or third CR were analyzed, the 3-year OS of patients with inv(16) was significantly better than patients with t(8;21) (86% and 45%, respectively; $P = .008$), and better than intermediate risk patients (86% and 57%, respectively; $P = .03$). Difference was not significant between patients in the intermediate risk group and t(8;21) undergoing allogeneic SCT in second or third CR ($P = .36$). The OS of inv(16) patients undergoing allogeneic SCT not in CR was 70% at 3 years, which was also significantly better than that of t(8;21) (18%; $P = .03$) and the intermediate risk group (25%; $P = .045$).

In addition, the OS of t(8;21) undergoing allogeneic SCT in first CR was significantly better than that of the unfavorable risk group (84% and 53%, respectively; $P < .001$), but the difference between the 2 groups was not significant among patients undergoing allogeneic SCT in second or third CR. In contrast, OS was not different between inv(16) and unfavorable groups undergoing allogeneic SCT in first CR, but it was significantly different when they underwent allogeneic SCT in second or third CR (86% and 44%, for inv(16) and unfavorable groups, respectively; $P = .01$) or allogeneic SCT in non-CR (70% and 15%, respectively; $P = .006$).

Survival curves of patients who underwent autologous SCT in first CR, second or third CR, and not in CR are shown in Figure 2A, 2B, and 2C, respectively. The overall survival of patients with t(8;21), inv(16), and intermediate, unfavorable, and unknown cytogenetic risks in first CR was 77%, 59%, 74%, 38%, and 71%, respectively ($P = .049$), while that of patients undergoing autologous SCT in second or third CR was 43%, 50%, 59%, 44%, and 42%, respectively ($P = .8$). The OS of patients undergoing autologous SCT not in CR with t(8;21), inv(16), intermediate, and

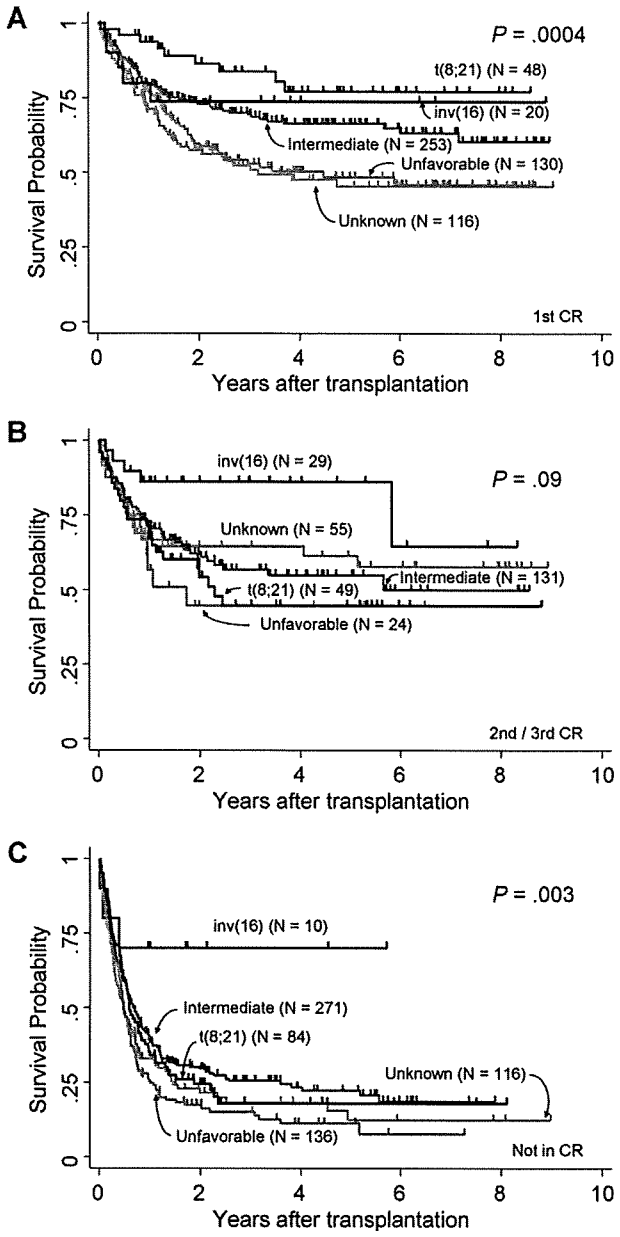

Figure 1. OS difference of patients undergoing allogeneic SCT between cytogenetic subgroups. (A) Survival curves of patients undergoing allogeneic SCT in first CR. (B) Survival curve of patients undergoing allogeneic SCT in second or third CR. (C) Survival curves of patients undergoing allogeneic SCT not in CR. Each are categorized by cytogenetic risk groups, respectively.

unknown risks was 17%, 100%, 25%, and 13%, respectively, and the survival curve of patients in the unfavorable risk group did not reach 3 years ($P = .35$).

Figure 3A and B focus on t(8;21) and inv(16) patients, stratified according to the type of (allogeneic or autologous) and disease status at the time of transplantation (first CR, second or third CR, and not in CR). The 3-year overall survival of t(8;21) patients in first CR was not different between allogeneic and autologous transplantation (84% and 77%, respectively), as well as that of patients in second or third CR (45% and 43%, respectively) and patients not in CR (18% and 17%, respectively). Similarly, the 3-year OS of inv(16) patients was not different between allogeneic and autologous transplantation when they underwent transplantation in first CR (74% and 59%). A significant difference was observed among the 3 disease status groups of t(8;21) patients ($P < .001$; Figure 3A), but not inv(16) patients ($P = .75$; Figure 3B).

The OS of allogeneic SCT, excluding cord blood transplantation, was not different from the analysis presented here, including bone marrow, peripheral blood, and cord blood transplantation (Table S2; Figures S1,S2).

DFS after SCT was also different among cytogenetic risk groups ($P < .001$). DFS of patients with inv(16) (69% at 3 years) was better compared with t(8;21) (49%), intermediate (46%), unfavorable (31%), and unknown (41%) risk groups. Among patients undergoing allogeneic SCT in first CR, DFS was also different among cytogenetic subgroups ($P < .001$). When t(8;21), inv(16), and intermediate cytogenetic subgroups undergoing allogeneic SCT in first CR were compared, the difference was not statistically significant between t(8;21) and inv(16) (78% and 73% at 3 years; $P = .58$), between t(8;21) and intermediate risk group (78% and 63%; $P = .1$), nor between inv(16) and intermediate risk group (73% and 63%; $P = .65$). DFS of patients with t(8;21) undergoing allogeneic SCT in first CR was better than that of the unfavorable risk group (78% and 47%, respectively; $P < .001$), but the difference was not significant between inv(16) and unfavorable risk groups (73% and 47%, respectively; $P = .16$).

DFS was not significantly different when 5 cytogenetic subgroups among patients undergoing allogeneic SCT in second or third CR were compared ($P = .32$). The DFS of patients undergoing allogeneic SCT in second or third CR was not significantly different between t(8;21) and inv(16) (43% and 71% at 3 years; $P = .053$), t(8;21) and the intermediate group (43% and 47%; $P = .76$), or inv(16) and the intermediate group (71% and 47%; $P = .06$). The difference was also not significant between t(8;21) and unfavorable risk groups (43% and 42%; $P = .7$), nor between inv(16) and unfavorable risk groups (71% and 42%; $P = .06$). The DFS of patients undergoing allogeneic SCT who were not in CR was significantly different among the 5 cytogenetic subgroups ($P = .005$), and that of inv(16) (75% at 3 years) was significantly better than t(8;21) (18%; $P = .02$), the intermediate risk group (22%; $P = .03$) and the unfavorable risk group (10%; $P = .003$).

Relapse and TRM

The relapse rate (RR) after SCT also differed among cytogenetic subgroups ($P < .001$). The RR of patients with inv(16) (18% at 3 years) was lower than t(8;21) (38%), intermediate (38%), and unfavorable (56%) risk groups. The RR of t(8;21) and inv(16) after allogeneic SCT was not statistically different in either first CR (16% and 6%; $P = .45$) or second or third CR (34% and 16%, respectively; $P = .09$).

Transplantation-related mortality (TRM) of all patients with AML was 22% at 3 years. The TRM of t(8;21) (18%), inv(16) (11%), and intermediate (21%), unfavorable (24%), and unknown risk groups (27%) was significantly different among cytogenetic risk groups ($P = .02$).

Evaluation of prognostic variables in CBF

Univariate analyses of t(8;21) showed that age ($P = .004$), not in CR at transplantation ($P < .001$), allogeneic SCT ($P = .01$), and TBI regimen ($P = .006$) were significant prognostic factors indicating poor OS (Table 5). Multivariate analysis for OS revealed older age ($P = .01$) and not in CR at transplantation ($P < .001$) as the independent prognostic variables. Univariate analyses of t(8;21) patients who received allogeneic SCT in CR showed that age ($P = .02$), TBI regimen ($P = .01$), and second and third CR at

Table 4. Outcome of the AML patient population by cytogenetic risk groups

	t(8;21)		inv(16)		Intermediate		Unfavorable		Unknown		
	%	N	%	N	%	N	%	N	%	N	*P*
OS											
Allogeneic SCT											
CR1	84	48	74	20	69	253	53	130	52	116	< .001
CR2/CR3	45	49	86	29	57	131	44	24	64	55	.09
Non-CR	18	84	70	10	25	271	15	136	18	116	.003
Autologous SCT											
CR1	77	42	59	13	74	89	38	15	71	39	.05
CR2/CR3	43	7	50	2	59	15	44	6	42	18	.8
Non-CR	17	6	100	1	25	16	0	10	13	8	.35
DFS											
Allogeneic SCT											
CR1	78	48	73	19	63	249	47	129	48	113	< .001
CR2/CR3	43	48	71	27	47	129	42	22	57	54	.32
Non-CR	18	81	75	8	22	255	10	128	16	107	.005
Autologous SCT											
CR1	73	41	62	13	64	81	33	15	61	36	.09
CR2/CR3	43	7	50	2	36	14	50	6	39	18	.89
Non-CR	17	6	100	1	25	16	0	10	17	6	.45

transplantation ($P < .001$) were also significantly prognostic for poor OS. These variables remained significant after multivariate analysis. Univariate analyses for inv(16) patients showed only age ($P = .009$) to be a significant prognostic factor (Table 5). The univariate analysis of inv(16) patients who underwent allogeneic SCT in CR showed only additional karyotype abnormalities to be an unfavorable prognostic variable ($P = .009$).

Additional cytogenetic abnormalities to CBF

A total of 49 patients with t(8;21) and 14 with inv(16) had additional cytogenetic abnormalities. Data for additional cytogenetic abnormalities were obtained in 42 patients with t(8;21) and 13 patients with inv(16) (Table 6). Additional abnormalities were selected that have been reported to be prognostic by others, including loss of sex chromosome (X or Y), trisomy 8, trisomy 4, del(7q), and del(9q) for the t(8;21) group, and trisomy 22, trisomy 8, trisomy 21, del(7q), and del(9q) for the inv(16) group.[14,15,20,22,23] There were no patients with trisomy 21 in the data of patients with CBF. Patients with t(8;21) and patients with inv(16) were analyzed separately. Among t(8;21) patients undergoing allogeneic SCT, survival was not different between patients with and without additional karyotype abnormalities. When patients with inv(16) were analyzed, the survival was not different between patients with (n = 13) and without (n = 67) additional abnormalities (61% and 74%, respectively; $P = .07$). The survival of patients undergoing allogeneic SCT without additional abnormality (n = 52) was significantly better than that with additional abnormality (n = 11), (85% and 53%, respectively; $P = .004$). When analysis was restricted to patients in CR with inv(16) undergoing allogeneic SCT, a similar difference was observed (86% without additional abnormality [n = 42], and 60% with additional abnormality [n = 8], respectively; $P = .03$). Difference in OS was observed among non-CR patients with (n = 9) and without (n = 1) additional abnormality, but this difference may not be relevant with too few patients in the analysis. We further analyzed subgroups of additional abnormalities of the patients with inv(16). Although the number of patients were limited, significant difference was found among 3 groups of patients; trisomy 8 or trisomy 22 as a sole abnormality (n = 4), without additional abnormality (n = 69), and other additional abnormality to inv(16) (n = 10). The OS at 3 years were 100%, 74%, and 42%, respectively ($P = .002$). The OS of patients undergoing allogeneic SCT was also different among these 3 groups (100%, n = 3; 85%, n = 52; and 33%, respectively; $P < .001$).

Discussion

We analyzed the outcome of a large group of patients with adult CBF AML in Japan who were treated with SCT. The current study focused on the different outcome of the 2 different cytogenetic subgroups of patients with CBF AML undergoing SCT. Our study demonstrated a comparable outcome between patients with t(8;21) and inv(16) undergoing SCT in first CR, but the prognosis between these 2 cytogenetic subgroups was different beyond first CR.

In the literature, there have been several reports showing inferior survival of patients with t(8;21) compared with inv(16) patients undergoing induction chemotherapy and SCT.[14,15,20] Other studies categorized both patients with t(8;21) and inv(16) undergoing allogeneic SCT together as good-risk CBF AML,[1,21] with a relatively comparable prognosis. In our study, OS of patients with t(8;21) undergoing allogeneic SCT in first CR was not statistically different from intermediate cytogenetic subgroup (84% and 79% at 3 years, respectively; $P = .058$). Moreover, the survival of inv(16) (74% at 3 years) and intermediate cytogenetic subgroups showed no statistically significant difference.

In contrast, we have here demonstrated that the prognosis of patients with t(8;21) undergoing allogeneic SCT with second or third CR disease was significantly poor compared with those with inv(16). This finding is consistent with those of other studies reporting differences between the 2 types of CBF AML.[14,15] In the present study, non-CR disease with t(8;21) was also significantly poor compared with patients with inv(16). The Acute Leukemia French Association reported that allogeneic donor availability among patients with CBF AML who were in second CR was a prognostic factor for better survival.[16] We believe that different treatment strategies should be applied for patients with t(8;21) and those with inv(16) other than first CR.

Patients with t(8;21) undergoing allogeneic SCT and autologous SCT had a similar survival rate when they underwent transplantation in first CR, and in further CR. No survival difference between allogeneic SCT and autologous SCT was also

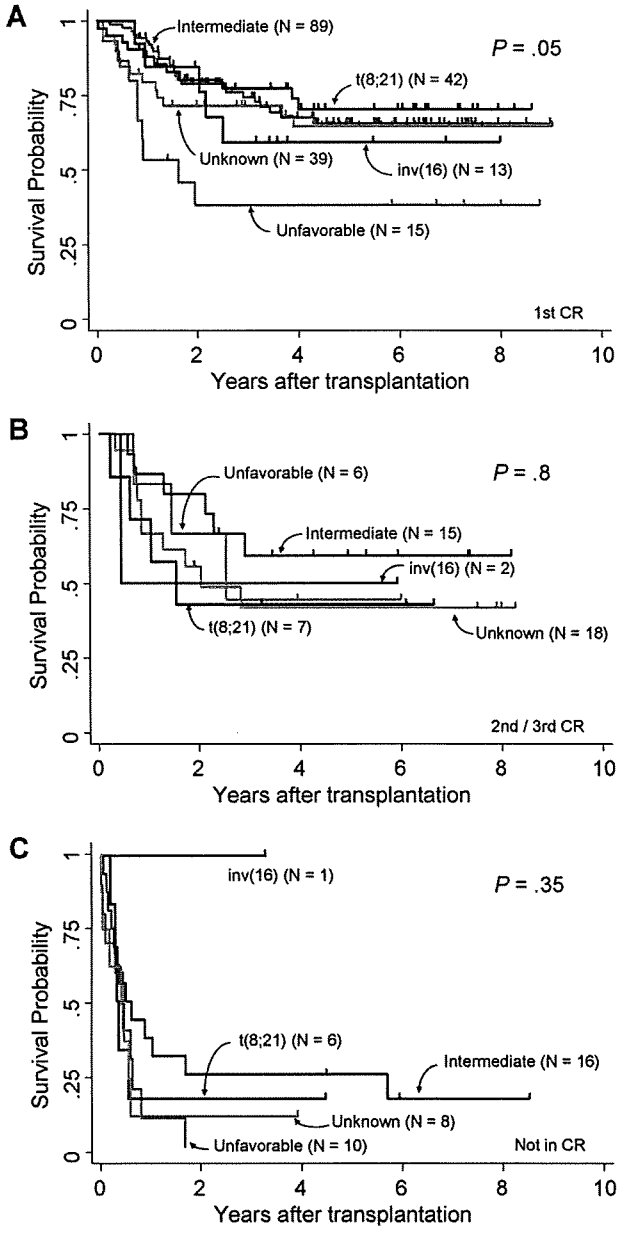

Figure 2. OS difference of patients undergoing autologous SCT between cytogenetic subgroups. (A) Survival curves of patients undergoing autologous SCT in first CR. (B) Survival curves of patients undergoing autologous SCT in second or third CR. (C) Survival curves of patients undergoing autologous SCT not in CR. Each are categorized by cytogenetic risk groups, respectively.

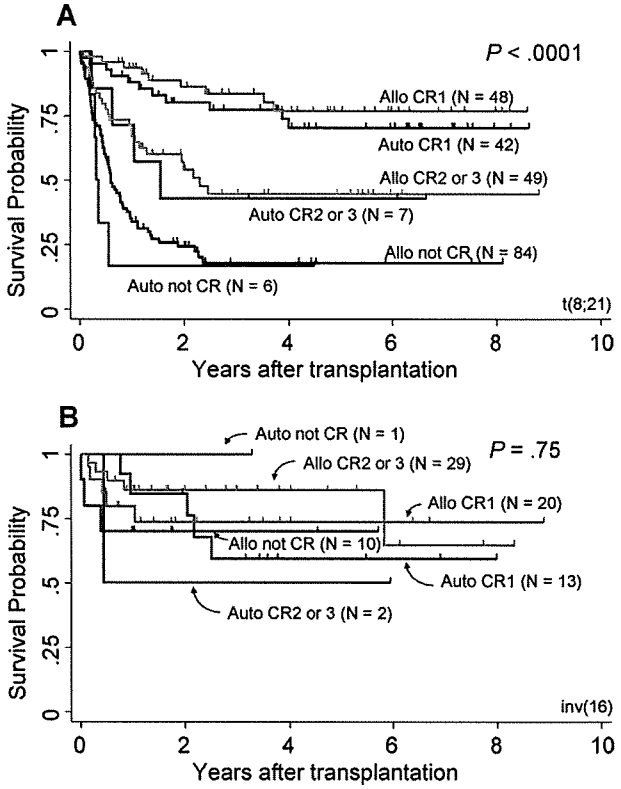

Figure 3. OS of patients with CBF. Survival curves of patients with t(8;21) (A) and with inv(16) (B). Both are stratified according to the type of transplantation (allogeneic or autologous) and disease status at the time of transplantation (first CR, second or third CR, and not in CR).

observed among inv(16) patients receiving SCT in first CR (74% and 59%, respectively). The University of California, San Francisco (UCSF) group described the good results of patients with advanced AML undergoing autologous SCT in second or third remission, including patients with CBF.[24] As in our study, the European Group for Blood and Marrow Transplantation (EBMT) reported that the survival rate of t(8;21) patients who received allogeneic bone marrow transplantation was not significantly different from that of patients who received autologous SCT.[1] Results by others showed that allogeneic SCT in first CR did not benefit good-risk cytogenetic subgroups.[3,25,26] Schlenk et al also demonstrated that t(8;21) patients receiving allogeneic SCT or chemotherapy showed no difference in outcome.[23] These results suggest that autologous SCT can be considered as postremission therapy for patients with CBF AML, but it remains unclear whether SCT is more beneficial for patients with CBF than high-dose cytarabine. Survival of patients with inv(16) was favorable beyond first CR. Patients with inv(16) in second or third CR, or even non-CR patients, are good candidates for allogeneic SCT. There are long-term survivors after allogeneic SCT in non-CR disease, so t(8;21) patients with no other choice of treatment, such as those in further CR or non-CR, can proceed to allogeneic SCT. In order to confirm the appropriate treatment for t(8;21) patients in first CR, a prospective trial is needed to compare the results of autologous SCT for t(8;21) in first CR with standard chemotherapy. t(8,21) patients with suitable related or well-matched donors should be recommended to participate in a risk-adopted prospective trial when they receive allogeneic SCT in first CR.

There were differences between the 2 types of CBF AML with respect to prognostic valuables. Age was a significant and independent prognostic variable in both t(8;21) and inv(16) patients, a finding in agreement with reports from some,[14,27] but not all,

Table 5. Prognostic factors affecting overall survival of patients with t(8;21)

Variables	Unfavorable factors	Hazard ratio	95% CI	P
t(8;21)				
Age		1.02	1.01-1.04	.004
Disease status at SCT	Not in CR	4.4	3.1-6.5	< .001
Transplantation	Allo-SCT	1.9	1.2-3.0	.01
Conditioning regimen	TBI	1.7	1.2-2.5	.005
inv(16)				
Age		1.1	1.0-1.1	.009

CI indicates confidence interval.

Table 6. Additional cytogenetic abnormalities among patients with CBF

Additional cytogenetic abnormalities	t(8;21), no.	inv(16), no.
None	206	69
With additional abnormalities	49	14*
−Y	10	0
−X	5	0
Trisomy 22	0	3†
Trisomy 8	0	2†
Trisomy 4	2*	0
Complex	7	4
del(7q)	1†	2
del(9q)	6	0
Other abnormalities	27	9‡
Unknown	7	1

*Patients with additional change to inv(16) and trisomy 4 with t(8;21) tended to show poor survival tendency, with $P < .1$.

†All patients with trisomy 22, trisomy 8 with inv(16), and del(7q) with t(8;21) were alive and censored at survival analysis.

‡Other abnormalities with inv(16) was poorly prognostic, with $P < .001$.

investigators.[28] Transplantation in CR was a significant and independent prognostic factor for patients with t(8;21), but not for those with inv(16). The Cancer and Leukemia Group B (CALGB) also reported differences between t(8;21) and inv(16) in prognostic factors, in terms of race, sex, and secondary cytogenetic abnormalities.[14] Among patients with CBF AML, t(8;21) and inv(16) patients undergoing SCT should be considered 2 separate clinical entities in future clinical studies.

Several specific additional karyotype abnormalities have been reported to be prognostic in patients with CBF AML. Among t(8;21) patients, no specific additional karyotype abnormality was prognostic for overall survival. The poor prognosis of t(8;21) patients with trisomy 4 has been reported by others,[22] but the survival difference was not statistically significant ($P = .085$) in our case series. Since there were limited numbers of patients with additional abnormalities, the real significance of each additional abnormality should be investigated in large numbers of patients.

The reason for the different survival results between patients with t(8;21) and inv(16) undergoing allogeneic SCT in our study remains unclear. The impact of additional mutational events such as c-Kit, FLT3, RAS, and gene-expression profiles was reported to be associated with the clinical outcome of patients with CBF AML.[29-34] The effects of these additional mutational events and gene-expression profiles on the clinical outcome of autologous and allogeneic SCT have not yet been studied. Which proportion of the patients with CBF AML benefited from earlier SCT remains to be identified in future clinical studies. Recent studies by others also suggested that prognosis of CBF AML could differ among different ethnic groups or races.[14,35-37] The background molecular basis among the Japanese population must also be taken into account in future studies.

In conclusion, the survival outcome of patients with CBF AML was similar when they received allogeneic or autologous SCT in first CR. However, the outcomes were significantly different between t(8;21) and inv(16) when they received allogeneic SCT beyond first CR. Therefore, these 2 kinds of CBF AML should be managed differently when applying SCT.

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Authorship

Contribution: Y. Kuwatsuka, K.M., and R.S. contributed to data collection, designed and performed the study, analyzed the data, and wrote the manuscript; M.K., A.M., H.O., R.T., S.T., K.K., K.Y., Y.A., T.Y., and H.S. contributed to data collection and analysis and writing of the paper; and Y. Kodera contributed to data collection and writing of the paper, conceived the study, and provided intellectual input.

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ORIGINAL ARTICLE

Separation of antileukemic effects from graft-versus-host disease in MHC-haploidentical murine bone marrow transplantation: participation of host immune cells

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Abstract Allogeneic hematopoietic stem cell transplantation (HSCT) is associated with both graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) effects. In clinical studies of HLA-mismatched HSCT, strong GVL effects have been reported. In the present study, we addressed the mechanism of the GVL and GVH response using MHC-haploidentical murine bone marrow transplantation (BMT) models. Recipient BDF1 (H-$2^{b/d}$) mice received T cell-depleted bone marrow and spleen cells from B6C3F1 (H-$2^{b/k}$) or C57BL/6 (H-2^{b}) mice with or without P815 mastocytoma cells (H-2^{d}) after receiving lethal total body irradiation. B6C3F1 → BDF1 (hetero-to-hetero type) recipients showed more powerful antileukemic effects with less severe GVHD than C57BL/6 → BDF1 (parent-to-F1 type) recipients. Compared with C57BL/6 → BDF1 recipients, significantly higher in vitro cytotoxic activity against P815 cells was observed in B6C3F1 → BDF1 recipients. Significantly lower CXCR3 expression on donor T cells and higher interferon (IFN)-γ expression were considered to be associated with strong antileukemic effects with less severe GVHD in B6C3F1 → BDF1 recipients. Furthermore, host immune cells, especially natural killer cells and $CD8^{+}$ T cells, were found to contribute remarkably to high IFN-γ production in B6C3F1 → BDF1 recipients. Thus, in MHC-haploidentical HSCT, host immune cells may change the balance between GVH and GVL response through IFN-γ production.

Keywords MHC-mismatched hematopoietic stem cell transplantation · GVHD · GVL · Interferon-γ · Natural killer cell

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1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) has been a potentially curative therapy for patients with a variety of diseases, especially for hematologic malignancies [1, 2]; however, more than 70% of patients who could benefit from allogeneic bone marrow transplantation (BMT) do not have a matched sibling donor. On the other hand, there is a greater than 90% chance of promptly identifying a human leukocyte antigen (HLA)-haploidentical donor within the family. A major obstacle of HLA-mismatched HSCT is the high incidence of graft-versus-host disease (GVHD) [3, 4]; therefore, separating beneficial GVL effects from deleterious GVHD is a goal for HLA-mismatched HSCT.

In this context, we have reported, in a series of clinical studies on unmanipulated HLA-haploidentical HSCT, that strong graft-versus-leukemia (GVL) effects are maintained in many patients even after complete suppression of GVHD by the use of reduced-intensity conditioning treatment, or the use of steroids and/or anti-T-lymphocyte globulin as

GVHD prophylaxis [5–8]. However, the cellular and molecular mechanisms of the separation of GVL reaction from GVHD observed in HLA-haploidentical HSCT remain unclear. In murine BMT studies, parent-to-F1 (homo-to-hetero) BMTs, in which donor type engraftment can be achieved without total body irradiation (TBI), have usually been used as major histocompatibility complex (MHC)-haploidentical BMT models. Furthermore, these models have contributed to the progress of GVHD study [9] because the influence of radiation on tissue damage can be avoided; however, whether the parent-to-F1 murine models correctly reflect clinical HLA-haploidentical HSCTs that are mostly performed in transplant settings of HLA hetero-to-hetero combinations remains unclear.

To enable comparison between homo-to-hetero and hetero-to-hetero transplants, we therefore established two major MHC-haploidentical murine BMT models, in which recipient BDF1 (H-$2^{b/d}$) mice received T cell-depleted (TCD) bone marrow (BM) and spleen cells from B6C3F1 (H-$2^{b/k}$) or C57BL/6 (H-2^{b}) mice with or without P815 mastocytoma cells after receiving lethal TBI. In the present study, we found that B6C3F1 → BDF1 (MHC hetero-to-hetero-type) BMT showed more powerful antileukemic effects with less severe GVHD than C57BL/6 → BDF1 (MHC homo-to-hetero-type) BMT. Furthermore, we found that, compared with C57BL/6 → BDF1 recipients, B6C3F1 → BDF1 recipients showed lower CXCR3 expression on donor T cells in recipient spleens and higher interferon (IFN)-γ production. This high IFN-γ milieu with low expression of the inflammatory chemokine receptor was considered to be associated with the induction of strong antileukemic effects with less severe GVHD, since recent studies demonstrated that IFN-γ augmented lymphohematopoietic GVH reactions [10–12], namely, GVL reaction, and that IFN-γ mediated the protective effect against GVHD [13, 14]. Furthermore, donor immune cells as well as host immune cells, especially host natural killer (NK) cells and $CD8^{+}$ T cells, were found to home to spleens after transplantation, and to produce IFN-γ highly in B6C3F1 → BDF1 recipients.

2 Materials and methods

2.1 Mice

Female C57BL/6 (B6, H-2^{b}), B6C3F1 (B6 × C3H/HeJ; H-$2^{b/k}$) or BDF1 (B6 × DBA2; H-$2^{b/d}$) mice were purchased from Japan CLEA (Osaka, Japan), or Shizuoka Laboratory Animal Center (Shizuoka, Japan). Mice used for experiments were 8–12 weeks of age, were housed in sterile microisolator cages in a specific pathogen-free mouse facility, and received autoclaved food and water ad libitum.

2.2 BMT

BM cells were harvested from the tibia and femur of donor mice by flushing with RPMI-1640 medium. T cell depletion of BM cells was performed by treatment with anti-Thy1.2 monoclonal antibody (mAb) (clone 30-H-12; PharMingen, San Diego, CA, USA) plus rabbit complement (Cedarlane, Hornby, ON, Canada). Spleen cells were isolated from donor mice using the nylon-wool-purification method as a source of lymphocytes. All BMTs were performed by the transfusion of a fixed number of donor cells after TBI the previous day. TBI was given in a single dose at a dose rate of 50 cGy/min. Cells from donors were resuspended in 0.5 ml RPMI-1640 medium and transplanted by tail-vein infusion into recipients.

Survival was monitored daily, and the presence of GVHD was judged by clinical symptoms, including body weight, posture (hunching), mobility, fur texture, and skin integrity [15]. All animal protocols were approved by the Ethics Review Committee for Animal Experimentation of Hyogo College of Medicine.

2.3 Challenge of tumor cells

In experiments to estimate the strength of antileukemic effects, recipient mice received P815 mastocytoma cells derived from DBA/2 (H-2^{d}). The tumor cells were injected intravenously through the tail vein on the day of transplantation.

2.4 Histopathological analysis

Tissues were fixed in 10% buffered formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin and were examined by light microscopy. Immunohistochemical analysis was performed as previously described [16], with some modifications. In brief, frozen sections were fixed in 4% paraformaldehyde. After being blocked with phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS) for 15 min at room temperature, the origin of infiltrating T cells was determined by staining with mouse anti-H-$2K^{d}$ mAb (SF1-1.1; host-specific) and rat anti-CD4 mAb (GK1.5) or rat anti-CD8 mAb (H35-17.2) at 4°C overnight and visualized using Alexa-Fluor 488-labeled anti-rat and Alexa-Fluor 546-labeled anti-mouse antibody. 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain the nucleus. Sections for fluorescent staining were analyzed with a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany) [16].

2.5 In vivo spectral fluorescence imaging analysis

For in vivo imaging analysis, P815 cells were engineered to express mCherry fluorescent protein by a lentiviral vector

transduction system, as previously described [17, 18]. Recipient mice received modified P815 cells with BM and spleen cells via the tail vein on day 0. Prior to imaging, mice were anesthetized with sodium pentbarbital (Nembutal), and hair was removed with a hair removal cream, Epilat (Kracie, Tokyo, Japan), and rinsed with water. Spectral fluorescence imaging analysis was performed using the Maestro in vivo fluorescence imaging system (CRi; Woburn, MA, USA), as previously described [19]. Whole body images (0.05- to 0.5-s exposure) were taken and analyzed over sequential days.

2.6 Flow cytometric analysis

Anti-Fc receptor (2.4G2) monoclonal antibody (mAb), fluorescein isothiocyanate (FITC)-conjugated anti-mouse H-2K^d (clone SF1–1.1) mAb, phycoerythrin (PE)- indotricarbocyanine (Cy7)-conjugated anti-mouse CD3 (clone 145-2C11) mAb, anti-mouse CD4 (clone GK1.5) mAb, allophycocyanin (APC)-conjugated anti-mouse CD8 (clone 53–6.7) mAb, and PE-conjugated anti-mouse NK1.1 (clone PK136) mAb were purchased from PharMingen (San Diego, CA, USA). PE-conjugated anti-mouse CXCR3 (clone 220803) mAb and rat anti-mouse IgG_{2A} isotype control were purchased from R&D Systems (Minneapolis, MN, USA). Cell suspensions were prepared in PBS-containing 1% FCS and 0.1% sodium azide. Cells were incubated with an anti-Fc receptor mAb for 10 min at 4°C to block nonspecific staining and then incubated with FITC-, PE-Cy7-, APC-, and PE-conjugated mAb for 30 min. The stained cells were washed twice, resuspended, and analyzed using FACSCalibur (Becton–Dickinson, Mountain View, CA, USA) using CellQuest software (Becton–Dickinson).

Intracellular IFN-γ staining was performed using the BD Cytofix/Cytoperm^{TM} Fixation/Permeabilization kit (BD Bioscience, San Jose, CA, USA). In brief, cells were retrieved from the recipient spleen, and resuspended at 10^6/ml and cultured with phorbol myristic acid at 50 ng/ml plus ionomycin at 500 ng/ml for 5 h, including monensin during the last 2 h of culture. Cells were harvested, washed, and resuspended in PBS-containing 1% FCS and 0.1% sodium azide. Cell-surface antigens were then stained as described above, and cells were resuspended in 100 µl per well of a microwell plate of fixation/permeabilization solution, and incubated for 20 min at 4°C. After washing, cells were stained with APC-conjugated anti-IFN-γ (clone XMG1.2; PharMingen) or isotype control: rat IgG1-APC (Clone R3-34).

2.7 Mixed lymphocyte culture (MLC) and ^{51}Cr release assay

BDF1 mice were transplanted using TCD-BM (5×10^6) and spleen cells (2×10^7) after receiving TBI 9 Gy. Spleen cells of the recipient mice on day 14 were used as responders for MLC. Cells (3×10^5/200 µl/well) were cultured with irradiated (20 Gy) BDF1 spleen cells (3×10^5/200 µl/well) in 24-well flat-bottomed plates (Falcon Labware, Lincoln Park, NJ, USA). After 72 h culture, IFN-γ concentrations of the culture supernatants were measured by Bio-Plex (Bio-Rad Laboratories, Hercules, CA, USA). For cytotoxic T lymphocyte (CTL) assay, BDF1 mice were transplanted using TCD-BM (5×10^6) and spleen cells (2×10^7) with P815 cells (1×10^4) after receiving TBI 9 Gy. Spleen cells of the recipient mice on day 14 were recovered, and directly measured for CTL activity against P815 cells by ^{51}Cr release assay, as described elsewhere [20]. Effecter cells were tested in triplicate at four effector:target (E:T) ratios, and the percent lysis was calculated according to the following formula: [(sample cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)] × 100%. Results are shown as the mean percent lysis of the E:T cell ratio for each treatment group.

2.8 Statistical analysis

Values were compared by two-tailed Student's t test. Survival data were plotted by the Kaplan–Meier method and were analyzed by the log-rank test. A P value of less than 0.05 was considered significant.

3 Results

3.1 B6C3F1 → BDF1 recipients showed less severe GVHD than C57BL/6 → BDF1 recipients

To investigate the pathophysiology of GVH or GVL reactions in MHC-haploidentical BMT, we established 2 MHC-haploidentical murine BMT models: BDF1 (H-2$^{b/d}$) mice were transplanted from B6C3F1 (H-2$^{b/k}$) or C57BL/6 (H-2^b) mice. B6C3F1 → BDF1 is an MHC hetero-to-hetero (donor/recipient combination) BMT model, where one MHC haplotype is identical between the donor and recipient but the other is different. C57BL/6 → BDF1 is an MHC homo-to-hetero (parent-to-F1) BMT model, where MHC is haplotypically mismatched in the graft-versus-host (GVH) direction but not in the host-versus-graft (HVG) direction.

Recipient BDF1 mice received donor TCD-BM (5×10^6) and spleen (2×10^7) cells after a lethal TBI dose (9 Gy) the previous day. There was no significant difference in total cell numbers, T cell doses, and the CD4:CD8 ratio of spleen cells transfused between the 2 BMT models (data not shown). Two weeks after BMT, the majority of C57BL/6 → BDF1 recipients began to present

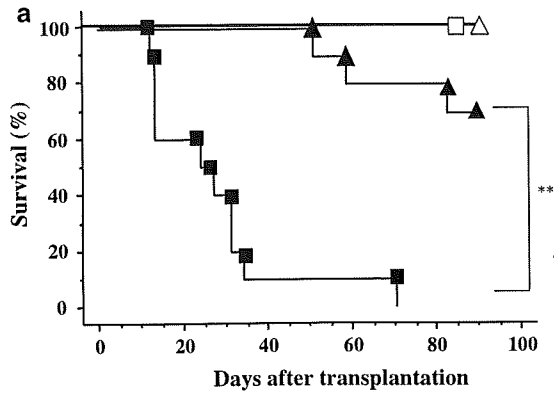
GVHD signs, such as body weight loss and a hunching posture, and 90% of mice had died of GVHD by day 40 (Fig. 1a). In contrast, B6C3F1 → BDF1 recipients showed fewer GVHD signs, and only 30% of mice had died of GVHD by day 80, with significantly improved survival observed in B6C3F1 → BDF1 recipients compared with C57BL/6 → BDF1 recipients. Histopathological examination of C57BL/6 → BDF1 recipients on day 14 revealed prominent lymphocyte infiltration in the periportal area of the liver, and various pathological changes in the large intestine compatible to GVHD (Fig. 1b, left panel). In the immunohistochemical study, these lymphocytes infiltrating the liver or large intestine were found to be donor-derived CD4 or CD8 T cells (Fig. 1c, upper panel). In contrast, liver or large intestine samples from B6C3F1 → BDF1 recipients showed few pathological changes (Fig. 1b, right panel) with almost no infiltration of donor T cells (Fig. 1c, lower panel). These results indicate that B6C3F1 → BDF1 recipients developed less severe GVHD than C57BL/6 → BDF1 recipients, leading to improved survival in B6C3F1 → BDF1 recipients.

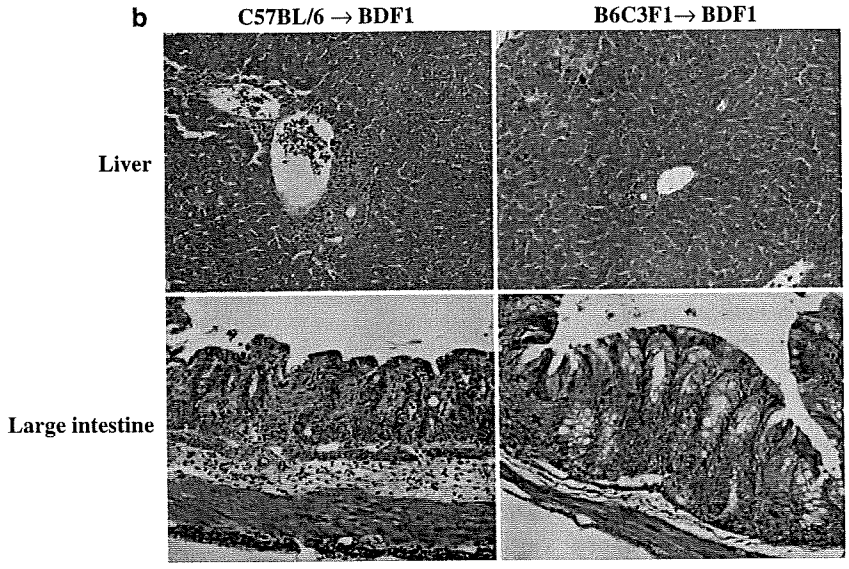
3.2 B6C3F1 → BDF1 recipients induced more powerful antileukemic effects than C57BL/6 → BDF1 recipients

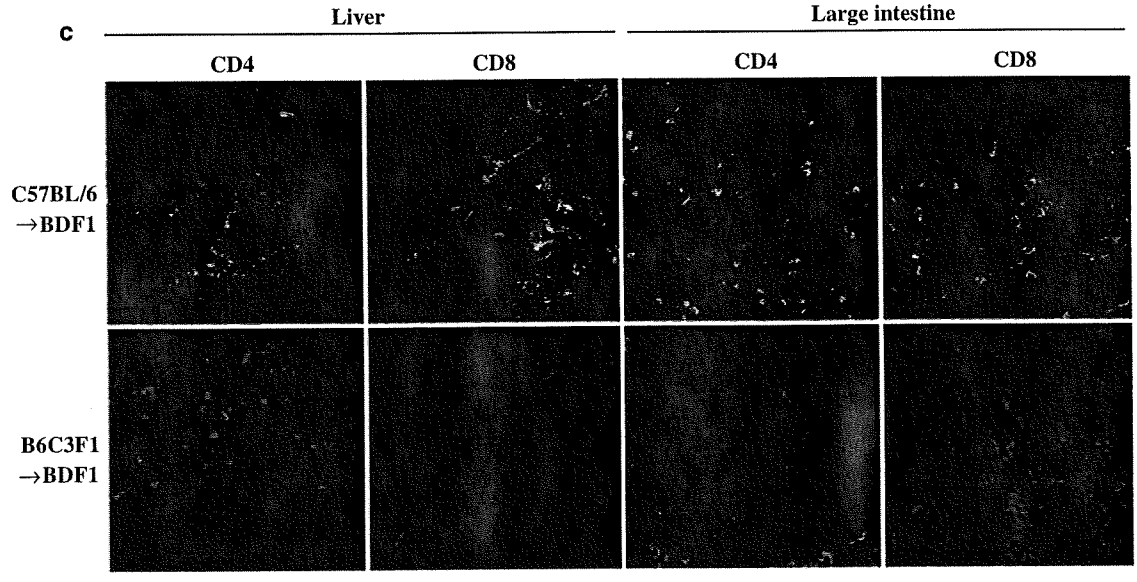
To compare antileukemic effects in the 2 MHC-haploidentical BMTs, recipient BDF1 mice received P815 mastocytoma cells (H-2^d, 1×10^4) with donor TCD-BM cells (5×10^6) with or without donor spleen cells (2×10^7) after receiving TBI 9 Gy the previous day. In mice receiving TCD-BM cells alone, P815 cells proliferated mainly in the liver, spleen, and BM of the recipient, and tended to form macroscopic nodules in the liver or spleen. Some animals developed lower limb paralysis, and histological analysis revealed infiltration of P815 cells around the spinal cord. Thus, death of recipient mice accompanied by these signs or symptoms was considered leukemic death. When recipient mice presenting with clinical signs of GVHD died without any signs of leukemia progression, they were considered as death by GVHD. All mice receiving TCD-BM cells alone with P815 cells had died of leukemia progression by day 20 (Fig. 2a). Compared with mice receiving TCD-BM cells alone, mice receiving spleen cells showed a significantly improved survival in the 2 groups (Fig. 2a); however, none of them died of tumor progression (some mice died of GVHD). We could demonstrated antileukemic effects of donor spleen cells, but could not compare antileukemic effects in the 2 BMT models under these conditions.

Fig. 1 Survival and histological change in B6C3F1 → BDF1 and C57BL/6 → BDF1 recipients. **a**. Survival of B6C3F1 → BDF1 and C57BL/6 → BDF1 BMT recipients. All recipients receiving TCD-BM cells alone from C57BL/6 or B6C3F1 mice survived. For mice receiving TCD-BM and spleen cells, B6C3F1 → BDF1 mice showed significantly improved survival than C57BL/6 → BDF1 mice. All mice that died showed severe clinical signs of GVHD. *Open rectangles* C57BL/6 → BDF1 mice receiving TCD-BM cells only ($n = 4$); *open triangles* B6C3F1 → BDF1 mice receiving TCD-BM cells alone ($n = 4$), *closed rectangles* C57BL/6 → BDF1 mice receiving TCD-BM and spleen cells ($n = 10$), *closed triangles* B6C3F1 → BDF1 mice receiving TCD-BM and spleen cells ($n = 10$). $^{**}P$ value < 0.01. The results are representative of 2 separate experiments. **b** Histological analysis of the liver and large intestine from recipient mice receiving TCD-BM and spleen cells. Prominent lymphocyte infiltration in the periportal area of the liver and severe intestinal histopathological changes, including surface erosion, decreased numbers of goblet cells, and cellular infiltration in the lamina propria, were observed in samples from C57BL/6 → BDF1 recipients on day 14. In contrast, few pathological changes were observed in samples from B6C3F1 → BDF1 recipients. Representative data are shown (×200). **c** Immunohistochemical analysis of GVHD-target organs on day 12. Data represent multicolored immunofluorescent staining: anti-CD4 (*green*) or anti-CD8 (*green*), anti-H2Kd (host-specific; *red*) and DAPI staining (*blue*) of the nucleus. Donor and host T cells were visualized as *green* and *yellow*, respectively. Massive lymphocytes infiltrating the liver or large intestine in C57BL/6 → BDF1 recipients were found to be donor-derived CD4 or CD8 T cells. In contrast, fewer infiltrates of donor T cells into these organs were observed in B6C3F1 → BDF1 recipients (×300)

Therefore, we decreased the number of spleen cells transfused to 5×10^5 cells. At this spleen cell dose, no mice died of GVHD. All mice receiving TCD-BM cells alone had died of tumor progression by day 14. Recipients receiving spleen cells survived significantly longer than mice receiving TCD-BM cells alone. All of C57BL/6 → BDF1 recipients receiving spleen cells had died of tumor progression by day 28, while only 20% of B6C3F1 → BDF1 recipients receiving spleen cells died of tumor progression during the observation period (Fig. 2b). For mice receiving spleen cells, compared with C57BL/6 → BDF1 recipients, B6C3F1 → BDF1 recipients showed a significant lower tumor mortality rate (Fig. 2b). To visualize the kinetics of tumor progression, P815 cells that were engineered to express mCherry fluorescent protein by a lentiviral transduction system were applied to the experiment in Fig. 2b. As shown in Fig. 2c, in mice receiving TCD-BM alone, fluorescence tumor signals appeared in the abdominal region (e.g. liver and spleen) and the femoral and sternal bones on day 10. In C57BL/6 → BDF1 recipients receiving TCD-BM and spleen cells, fluorescence tumor signals appeared in the femoral and sternal bones on day 10, and extended to the abdominal region by day 12. These fluorescence signals continued to strengthen, with signals continuing to spread







out to other organs, including the humeral bones and mesenteric lymph nodes, until death. Once mice became positive for fluorescence tumor signals, they always succumbed to tumor. We killed some mice, and confirmed mCherry-positive P815 cells in the liver, spleen, and abdominal lymph nodes, as well as the bone marrow in the femoral, sternal,

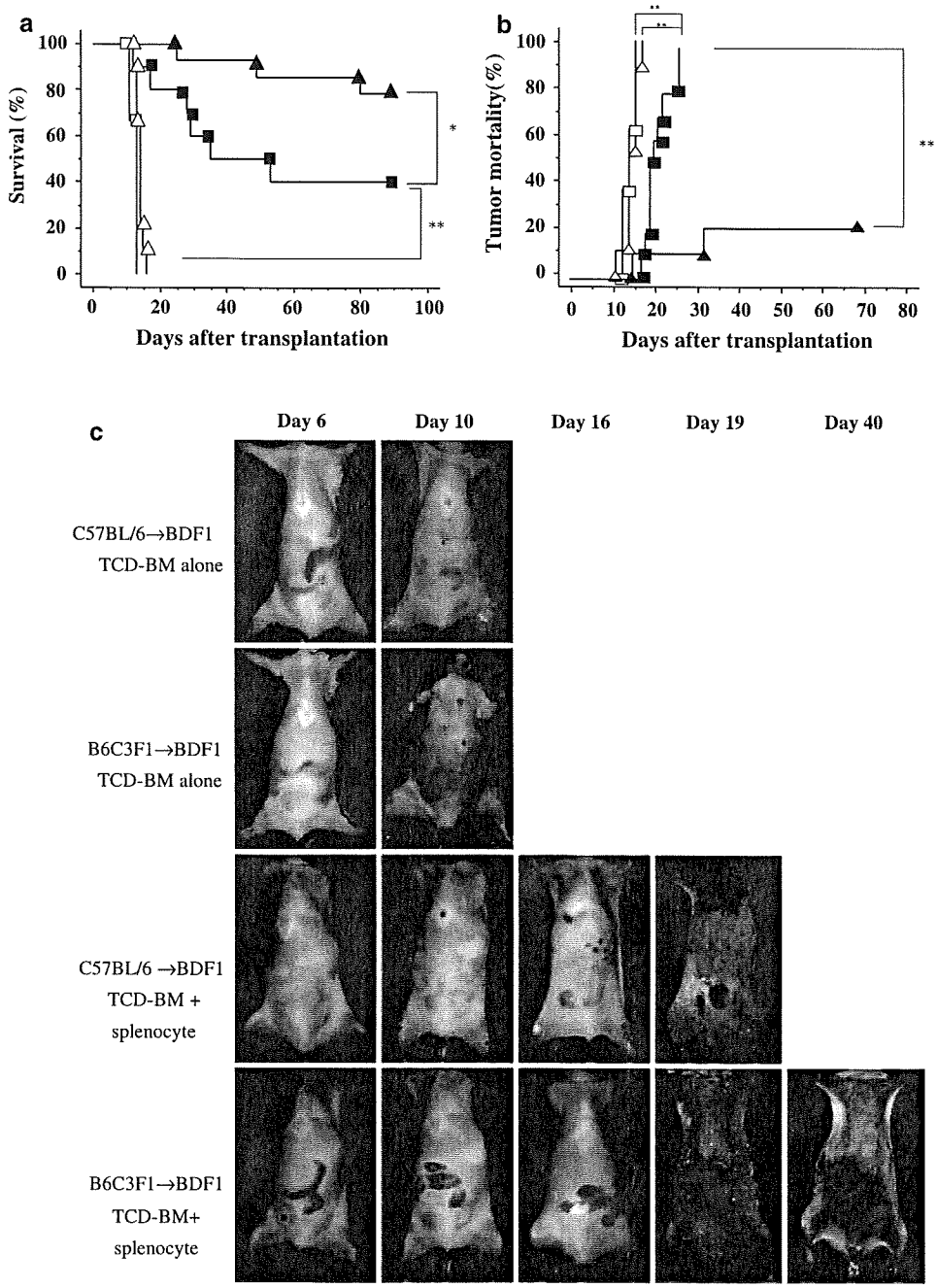


Fig. 2 B6C3F1 → BDF1 recipients developed stronger antileukemic effects than C57BL/6 → BDF1 recipients. **a** BDF1 mice received P815 mastocytoma cells (H-2^d, 1 × 10^4) with donor TCD-BM cells (5 × 10^6) with or without donor spleen cells (2 × 10^7) after receiving TBI 9 Gy the previous day. *Open rectangles* C57BL/6 → BDF1 mice receiving TCD-BM cells and P815 cells ($n = 6$), *open triangles* B6C3F1 → BDF1 receiving TCD-BM cells and P815 cells ($n = 9$), *closed rectangles* C57BL/6 → BDF1 mice receiving TCD-BM, spleen cells and P815 cells ($n = 10$), *closed triangles* B6C3F1 → BDF1 receiving TCD-BM, spleen cells and P815 cells ($n = 14$). *P value < 0.05. Representative data from 3 separate experiments are shown. **b** The same experiments as in **a** were performed except for the reduced number of spleen cells transplanted to 5 × 10^5. Each *symbol* indicates the same mice as shown in **a**. **P value < 0.01. Representative data from 3 separate experiments are shown. **c** In vivo imaging analysis confirmed the difference in the kinetics of tumor progression between the 2 BMT groups. Transplantation was performed in the same condition as shown in **b**. P815 cells were engineered to express mCherry fluorescent protein by a lentiviral gene transduction system (see "Materials and methods"). Fluorescent imaging in mice was checked every other day from days 6 to 21, and thereafter once a week until day 40. Tumor mass of P815 cells and gastrointestinal contents are visualized as *red* and *blue*, respectively. Representative images from 2 independent experiments are shown

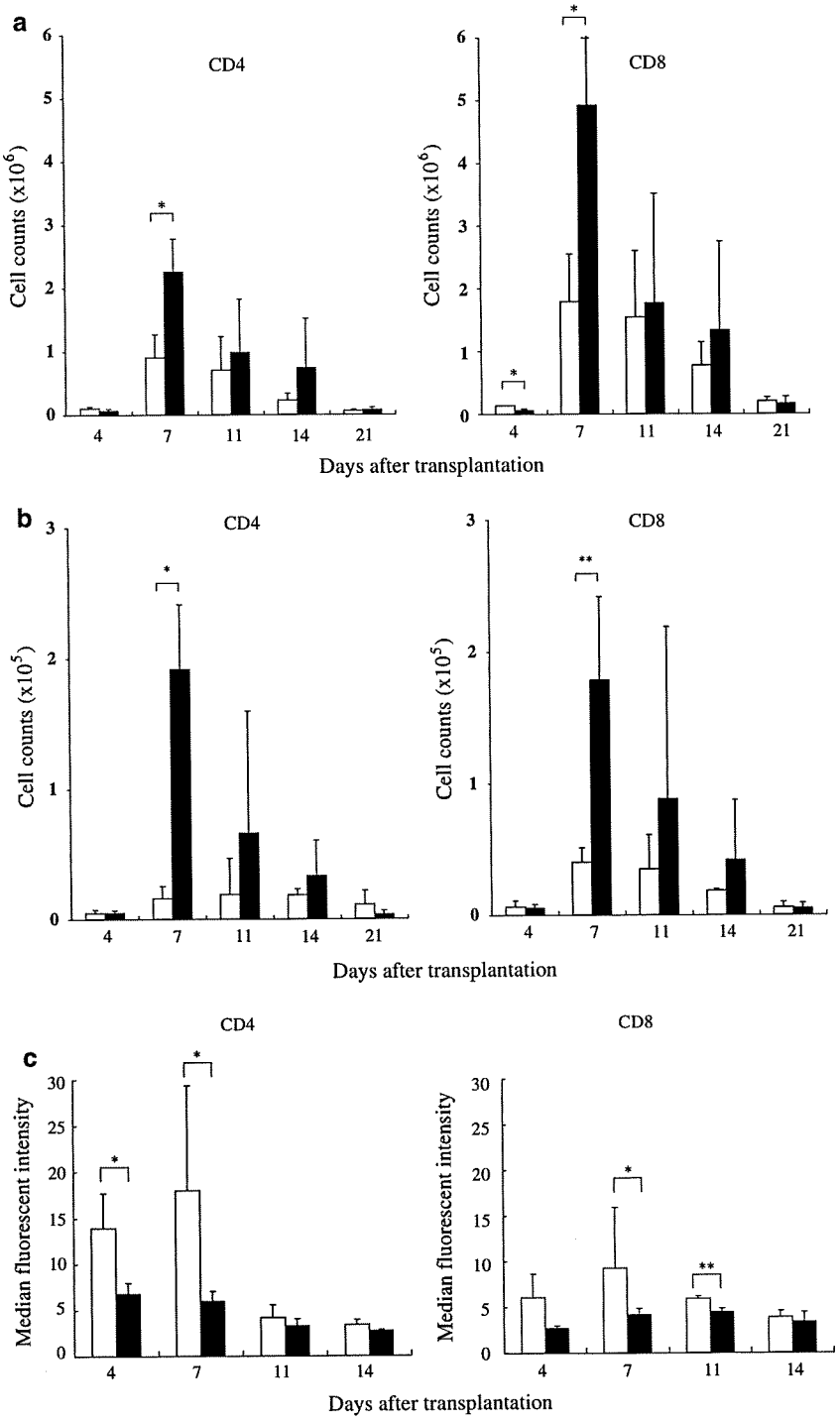


Fig. 3 The kinetics and characterization of T cells proliferating in recipient spleen. **a** Kinetic analysis of donor T cells engrafted to recipient spleens. B6C3F1 → BDF1 and C57BL/6 → BDF1 BMTs were performed as shown in Fig. 1. The number of donor $CD4^+$ or $CD8^+$ T cells was calculated based on multi-colored flow cytometry data, as described in "Materials and methods". *Open bars* C57BL/6 → BDF1 recipients, *closed bars* B6C3F1 → BDF1 recipients. Values were calculated based on experiments using at least 4 mice. Data are expressed as the mean ± standard deviation (SD). $*P < 0.05$. Representative data from 2 separate experiments are shown. **b** Kinetic analysis of host T cells recruited to recipient spleens. Each *symbol* indicates the same mice as shown in **a**. $*P < 0.05$, $**P < 0.01$. Representative data from 2 separate experiments are shown. **c** Median fluorescent intensity of CXCR3 expression on donor $CD4^+$ and $CD8^+$ T cells was compared in the 2 groups ($n = 3$). *Open bars* C57BL/6 → BDF1 recipients, *closed bars* B6C3F1 → BDF1 recipients. Results are representative of 2 experiments. $*P < 0.05$, $**P < 0.01$

and humeral bones by fluorescence microscope and flow cytometry (data not shown). On the other hand, no detectable signals were observed in most B6C3F1 → BDF1 recipients receiving TCD-BM and spleen cells during the observation period. These results demonstrated that B6C3F1 → BDF1 recipients developed more powerful antileukemic effects despite presenting with less severe GVHD compared with C57BL/6 → BDF1 recipients.

3.3 Lower expression of CXCR3 on donor T cells engrafted to B6C3F1 → BDF1 spleens was associated with less severe GVHD

To address the difference in the extent of GVHD between B6C3F1 → BDF1 and C57BL/6 → BDF1 recipients (Fig. 1), we examined the kinetics of and characterized donor T cells engrafted to recipient spleens by flow