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ORIGINAL ARTICLE

Mycophenolate mofetil combined with tacrolimus and minidose methotrexate after unrelated donor bone marrow transplantation with reduced-intensity conditioning

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Abstract We evaluated the efficacy of a post-grafting immunosuppressive regimen consisting of tacrolimus, methotrexate, and mycophenolate mofetil (MMF) in 21 adults (median age, 55 years) with poor-risk hematologic malignancy who underwent unrelated bone marrow transplantation after fludarabine-based reduced-intensity conditioning (RIC). In combination with intravenous tacrolimus and minidose methotrexate (5 mg/m^2 on days 1, 3, and 6), MMF was orally administered at 30 mg/kg daily in three divided doses between days 7 and 27. All patients achieved neutrophil recovery with donor-type chimerism at a median of 19 days (range, 13–35). Cumulative incidences of grades II–IV and III–IV acute graft-versus-host disease (GVHD) were 33% (95% CI, 15–53%) and 5% (95% CI, 0.3–20%), respectively. Five of 20 evaluable patients developed extensive chronic GVHD. Toxicities associated with the use of MMF were acceptable, although one patient experienced intractable GVHD immediately after the cessation of MMF. With a median follow-up of 24 months, overall survival at 3 years was 38% (95% CI, 14–63%). No late graft failure was observed. In conclusion, post-transplant MMF combined with tacrolimus and methotrexate was well tolerated and conferred stable donor cell engraftment, low risk of severe acute GVHD, and encouraging overall survival in unrelated donor marrow transplantation after RIC regimens.

Keywords Mycophenolate mofetil · Reduced-intensity conditioning · Unrelated donor · Bone marrow transplantation · Graft-versus-host disease

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1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) with reduced-intensity conditioning (RIC) regimens is increasingly employed as a treatment option for various hematologic disorders. RIC transplantations using cytokine-mobilized peripheral blood stem cells (PBSC) have been reported to yield comparable outcomes with conventional myeloablative HSCT at least in selected patients [1–4]. However, previous reports have consistently shown that RIC transplantations using bone marrow (BM) graft, especially from an unrelated donor, are associated with an increased risk of graft failure and treatment-related toxicity as compared with those using PBSC, although confirmatory data from randomized-controlled trials are currently unavailable [5–8]. To improve outcomes after unrelated BM transplantation conditioned with non-myeloablative or reduced-intensity regimens, it would be beneficial to introduce a newer post-transplant immunosuppressive protocol which can effectively prevent both graft rejection and severe graft-versus-host disease (GVHD).

Mycophenolate mofetil (MMF) is an esterified prodrug of mycophenolic acid (MPA), which has pleiotropic immunosuppressive actions [9, 10]. MPA preferentially inhibits de novo purine nucleotide synthesis in T-cells and B-cells via inhibition of inosine-5′-monophosphate

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dehydrogenase, interfering with their proliferation. MPA also suppresses dendritic cell maturation and can induce T-cell apoptosis. The use of MMF in combination with cyclosporine or tacrolimus was proven to be active in promoting hematopoietic stem cell engraftment after non-myeloablative HSCT using fludarabine and low-dose total-body irradiation (TBI) conditioning [11, 12], and was also shown to be as effective as the standard post-grafting immunosuppression with cyclosporine and methotrexate (MTX) in preventing severe acute GVHD after myeloablative HSCT from an HLA-matched related donor [13, 14].

A combination of tacrolimus and minidose MTX has been widely used as GVHD prophylaxis in RIC transplantation as well as in conventional HSCT from adult unrelated donors [15, 16]. Because MMF and tacrolimus are shown to have synergistic immunosuppressive actions in experimental and clinical organ transplantations [17, 18], we hypothesized that MMF in conjunction with tacrolimus and minidose MTX would be more efficacious than a combination of tacrolimus/MTX alone. In this single-center study, we retrospectively evaluated the efficacy of such triple combination as an alternative peritransplant immunosuppressive protocol in unrelated donor RIC transplantation using exclusively BM as a stem cell source.

2 Patients and methods

2.1 Patients

Among 61 consecutive adult patients who received BM transplantation from an unrelated donor between 2003 and 2006 at Kyoto University Hospital, those who fulfilled the following criteria were selected for the study: having a hematologic malignant disease; having an unrelated donor who was serologically matched at HLA-A, -B, and -DR antigens, allowing a single-allele mismatch identified by high-resolution DNA typing; receiving fludarabine-based RIC because of having a history of chemoradiotherapy precluding the use of myeloablative conditioning or having 55 through 69 years of age; receiving GVHD prophylaxis consisted of intravenous tacrolimus, minidose MTX and oral MMF. All patients had an adequate cardiac, pulmonary, hepatic, and renal function at the time of transplantation and did not have therapy-resistant central nervous system involvement or active infectious disease. A total of 21 patients fulfilled these criteria and considered evaluable for the study. With respect to disease status at transplant, patients who received transplant without prior cytotoxic chemotherapy or in first complete remission were considered to have an early disease, while those who underwent transplantation in all the other conditions were considered to have an advanced disease. All the patients with early disease were considered to have resistance to conventional chemotherapy or to have a high risk of relapse: those included two cases with untreated high-risk myelodysplastic syndrome, one with chronic active Epstein-Barr virus infection and one with adult T-cell leukemia/lymphoma in first remission. This study was approved by the Institutional Review Board and Ethic Committee of Kyoto University; written informed consent for transplantation was obtained from all participating patients.

2.2 Study end points

The primary end points of the study were donor cell engraftment and the occurrence of grade II–IV acute GVHD. Secondary end points included the neutrophil and platelet recovery, the occurrence of extensive chronic GVHD, progression or relapse of primary disease, and death from any cause.

Donor cell engraftment was defined as the detection of donor-type chimerism among unfractionated BM-nucleated cells with concomitant neutrophil recovery. Date of neutrophil recovery was defined as the first 3 consecutive days with the absolute neutrophil count (ANC) higher than 0.5×10^9/L. Date of platelet recovery was defined as the first 7 consecutive days with platelet count exceeding 20×10^9/L without transfusion. Acute GVHD was diagnosed and graded according to the conventional criteria [19]. Chronic GVHD was diagnosed and staged as limited or extensive on the basis of traditional criteria among patients who survived more than 90 days after transplantation [20]. Disease response and progression were defined by the standard criteria [21–25]. Toxicity observed between days 0 and 100 after transplantation was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events ver 3.0. Non-infectious pulmonary complications were diagnosed on the basis of clinical manifestations, radiologic findings, and the results of pulmonary function tests if available [26].

2.3 HLA typing and chimerism analysis

Compatibility at *HLA-A*, *-B*, and *-DRB1* loci between patients and donors was determined by standard serologic technique and high-resolution DNA typing as described elsewhere [27]. *HLA-C* compatibility was not included as a criterion for donor selection because routine *HLA-C* allele typing for the screening of unrelated donors was not available before April 2004. Donor cell chimerism levels among unfractionated BM-nucleated cells were evaluated on day 28 and thereafter at the appropriate time point by polymerase chain reaction-based analysis of polymorphic microsatellite regions for recipients of sex-matched graft or fluorescent in situ hybridization analysis of sex chromosomes for sex-mismatched pairs as described previously [28].

2.4 Transplantation procedure

Preparative regimens were assigned according to diagnosis and disease status at transplantation. Fourteen patients received fludarabine 25 mg/m^2/day for 5 consecutive days (days −6 to −2) in combination with oral busulfan 1 mg/kg every 6 h for 2 days (days −3 and −2) followed by 400 cGy of TBI in 2 fractions (on day −1 and/or day 0). One patient who had a history of TBI-based myeloablative allogeneic transplantation received the same dose schedule of fludarabine plus busulfan regimen without 400 cGy TBI. Four patients received fludarabine at the same daily dose from days −8 through −4 combined with melphalan 70 mg/m^2/day on days −3 and −2. The remaining two patients without a history of cytotoxic chemotherapy received 200 cGy TBI in a single fraction in addition to the fludarabine plus melphalan regimen.

All BM collections from unrelated donors were facilitated through the Japan Marrow Donor Program [27]. On day 0, BM graft was infused without T-cell depletion; ABO major-mismatched or bidirectionally mismatched graft was processed to isolate mononuclear cell suspension using COBE Spectra (Gambro BCT, Lakewood, CO, USA) or CS-3000 Plus (Baxter Corp., Deerfield, IL, USA) according to the manufacture's instruction, while ABO minor-mismatched graft was plasma depleted before infusion. Eleven patients who were suspected to develop bacterial infection during the first week after transplantation or who were considered to be at high risk for infectious complications because of prior history of allogeneic transplantation received infusional or subcutaneous granulocyte colony-stimulating factor 5 μg/kg/day from day 7 until ANC exceeded 0.5 × 10^9/L.

Continuous intravenous administration of tacrolimus in a dose of 0.02 mg/kg/day was started on day −3 in patients receiving busulfan-based conditioning or on day −1 in patients receiving melphalan-based conditioning with therapeutic monitoring which targeted blood levels of 10–15 ng/ml at least until day 28 after transplantation, converted to twice-daily oral administration at an appropriate time to maintain trough levels between 5 and 10 ng/ml until day 100, followed by stepwise tapering over 3–6 months if active GVHD was absent. MTX at a dose of 5 mg/m^2 was intravenously injected on days 1, 3, and 6; MMF 30 mg/kg/day was orally administered in three divided doses from days 7 to 27. After day 28, MMF was discontinued without tapering if acute GVHD was absent or gradually tapered if ongoing acute GVHD was present. Patients who developed grade II–IV acute GVHD were initially treated with methylprednisolone or prednisolone at a dose of 1–2 mg/kg/day. All patients received supportive care including blood product transfusion and prophylaxis against opportunistic infections according to our institutional protocols [29].

2.5 Statistical analysis

Probabilities of neutrophil recovery, platelet recovery, and grade II–IV or grade III–IV acute GVHD were calculated by cumulative incidence estimates, treating death without the respective event as a competing risk [30]. Overall survival from the date of transplantation until the date of death from any cause was estimated by the Kaplan–Meier method; progression-free survival was estimated from the date of transplantation until the date of disease progression, relapse, or death from any cause. Data on patients who were alive at the time of last follow-up were censored. All statistical analyses were performed using STATA version 10 software (Stata Corp., College Station, TX, USA) based on dataset available on 10 January 2008.

3 Results

3.1 Patient and transplant characteristics

Table 1 shows the characteristics of the patients and transplantation procedures. A total of 17 patients (86%) had an advanced disease at transplantation, while the remaining 4 patients had an early disease. With respect to the compatibility at *HLA-A*, *-B*, and *-DRB1*, five patients (24%) received a single-allele mismatched graft, three had a mismatch at *HLA-A* and two at *HLA-DRB1*. Two of these five patients were found to have an additional allele mismatch at *HLA-C*. The median total number of nucleated cells included in the collected BM graft was 3.0 (range, 1.2–4.0) × 10^8 per kg of the recipient's body weight.

3.2 Engraftment

All patients achieved successful donor cell engraftment. The cumulative incidence of neutrophil recovery >0.5 × 10^9/L by day 35 was 100%, with a median time of 19 days (range, 13–35 days) (Fig. 1a). The cumulative probability of platelet recovery >20 × 10^9/L by day 42 was 81%, with a median time of 26 days (range, 13–91 days) (Fig. 1b). Two patients had experienced relapse on days 39 and 67 after transplantation without platelet recovery. No secondary graft failure was observed.

3.3 Acute and chronic GVHD

Acute GVHD was evaluable in all the patients. A total of seven patients developed grade II–IV acute GVHD: grade II in 6 and grade IV in 1. Cumulative incidence of developing grade II–IV acute GVHD at day 100 after transplantation was 33% (95% CI, 15–53%), and that of grade III–IV acute GVHD was 5% (95% CI, 0.3–20%)

Table 1 Patient and transplant characteristics

	$n = 21$
Median recipient age (range) (years)	52 (24–66)
Recipient sex, *n*	
Female/male	12/9
Diagnosis, *n*	
Acute myeloid leukemia	5
Myelodysplastic syndrome	4
Adult T-cell leukemia/lymphoma	4
Follicular lymphoma	3
Hodgkin lymphoma	1
Plasma cell myeloma	2
Chronic active EBV infection	1
Extranodal NK/T-cell lymphoma	1
Disease status at transplantation, *n*	
Early disease	
CR1	1
Untreated	3
Advanced disease	
CR > 1	4
PR	8
Progressive disease	5
Median donor age (range) (years)	34 (20–48)
HLA matching (at HLA-A, -B, -DRB1), *n*	
Match	16
Single-allele mismatch	5
ABO incompatibility, *n*	
Match	9
Minor	3
Major	5
Bidirectional	4
Conditioning, *n*	
Fludarabine + busulfan + 4 Gy TBI	14
Fludarabine + busulfan	1
Fludarabine + melphalan + 2 Gy TBI	2
Fludarabine + melphalan	4

EBV Epstein-Barr virus, *CR* complete remission, *PR* partial remission, *TBI* total-body irradiation

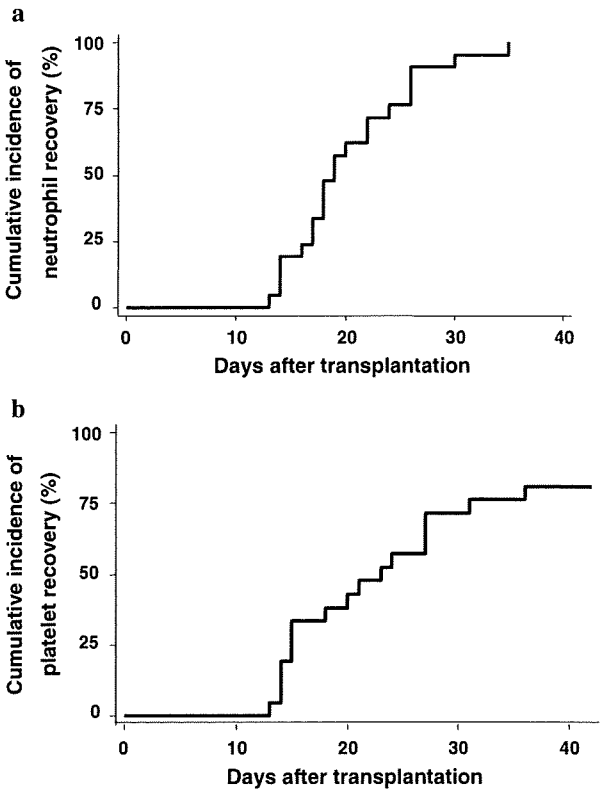

Fig. 1 Cumulative incidence of neutrophil recovery (**a**) and platelet recovery (**b**)

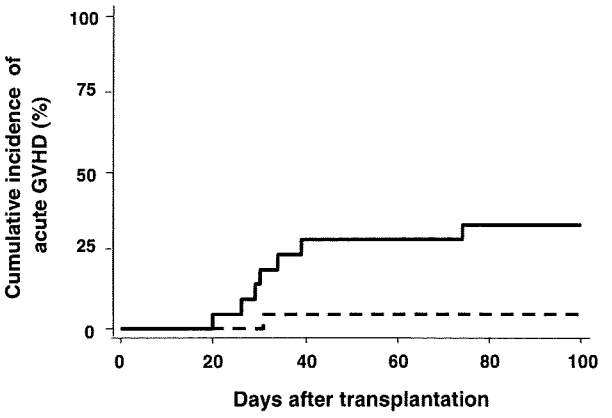

Fig. 2 Cumulative incidences of grade II–IV (*solid line*) and grade III–IV (*dashed line*) acute GVHD

(Figs. 2, 3). Chronic GVHD was observed in 11 of 20 (55%) evaluable patients who survived 100 days after transplantation: limited type in 6 and extensive type in 5.

3.4 Transplant-related toxicities and infectious complications

Transplant-related organ toxicities during the first 100 days after transplantation are shown in Table 2. Mild to moderate gastrointestinal symptoms considered to be associated with preparative regimens were frequently observed, although other adverse events were mostly moderate. One patient was required to discontinue MMF on day 9 because of grade III diarrhea.

Eighteen patients (86%) experienced 38 episodes of documented or suspected infectious complications (Table 3). Fourteen episodes of culture-negative neutropenic fever were reported. Five episodes of microbiologically documented bacterial infection were observed in four patients:

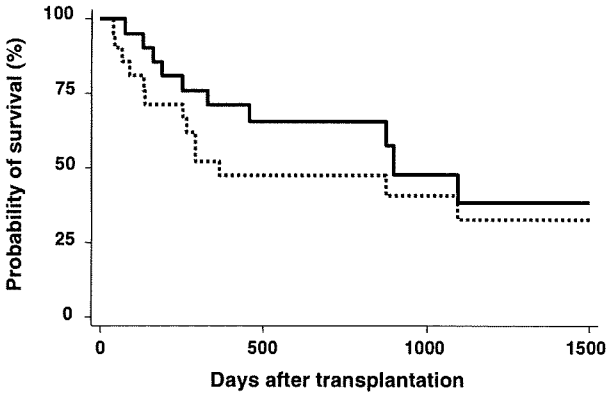

Fig. 3 Probabilities of overall survival (*solid line*) and progression-free survival (*dotted line*) after transplantation

Table 2 Transplant-related toxicities

	CTCAE grade I–II	CTCAE grade III
Vomiting	3 (14%)	6 (28%)
Stomatitis	5 (24%)	6 (28%)
Diarrhea	9 (43%)	3 (14%)
Liver dysfunction	5 (24%)	3 (14%)
Renal dysfunction	3 (14%)	0 (0%)
Headache	0 (0%)	2 (10%)
Pleural effusion	2 (10%)	0 (0%)
Myalgia	2 (10%)	0 (0%)

CTCAE common terminology criteria for adverse events

Table 3 Infectious complications

	No. of episodes
Culture-negative febrile neutropenia	14
Bacteremia	4
Clostridium difficile-associated diarrhea	1
Phlegmone	1
Neutropenic enterocolitis	1
CMV antigenemia	11
Cystitis	5
Aseptic meningitis	1
Total	38

CMV cytomegalovirus

bloodstream infection ($n = 4$) and *Clostridium difficile*-associated enteritis ($n = 1$). Two episodes of suspected bacterial infections were reported: phlegmone ($n = 1$) and neutropenic enterocolitis ($n = 1$). Eleven patients became positive for cytomegalovirus (CMV) antigenemia; one of them developed CMV-associated hepatitis and enteritis. Adenovirus was detected from the urine from one of five patients who developed cystitis. One patient experienced aseptic meningitis. No varicella-zoster virus infection was observed. There was no death directly attributable to infectious events until day 100.

Six patients developed non-infectious pulmonary complications between 3 and 14 months after transplantation: bronchiolitis obliterans ($n = 2$), bronchiolitis obliterans-organizing pneumonia ($n = 1$), diffuse alveolar hemorrhage ($n = 1$), and idiopathic interstitial pneumonia ($n = 2$). One patient developed secondary gastric cancer at 34 months after transplantation.

3.5 Survival and treatment-related mortality

Eleven patients were alive and 10 of them were disease-free at a median follow-up of 24 months (range, 3–31 months). Among four patients who had an early disease at transplant, one experienced relapse on day 292. Fifteen of 17 patients who had an advanced disease maintained or attained remission after transplantation, but seven of them eventually relapsed between days 67 and 363. Two of the relapsed patients received donor lymphocyte infusion after chemotherapy, and durable remission lasting more than 16 months was observed in one patient.

Ten patients were deceased between 76 and 1093 days after transplantation. Six patients succumbed to disease progression and four patients died of treatment-related complications including interstitial pneumonia ($n = 1$), bronchiolitis obliterans followed by diffuse alveolar damage ($n = 1$), intracranial hemorrhage during exacerbation of bronchiolitis obliterans ($n = 1$), and secondary gastric cancer ($n = 1$). The probabilities of overall survival and progression-free survival at 3 years after transplantation were 38% (95% CI, 14–63%) and 33% (95% CI, 12–55%), respectively.

4 Discussion

In this study, we evaluated the efficacy of a combination of tacrolimus, minidose MTX, and MMF as post-transplant immunosuppression in RIC transplantations using BM grafts from an HLA-A, -B, -DR antigen compatible unrelated donor. This triple regimen conferred stable donor cell engraftment, low risk of severe acute GVHD, and encouraging overall survival with acceptable toxicity profiles.

Recent introduction of RIC has provided the opportunity to enjoy long-term disease-free survival in patients with hematologic malignancies who were previously ineligible for allogeneic HSCT because of elder age or pre-existing comorbidity. It has been shown that RIC HSCT using alternative stem cell source is a feasible treatment option when an HLA-matched related donor is not available, albeit at the expense of substantial risk of more serious

transplant-related complications. Among the first 285 patients who underwent unrelated RIC HSCT through the National Marrow Donor Program, the respective incidence rates of primary graft failure, grade III–IV acute GVHD, and treatment-related mortality at 3 months after transplantation were 11, 22, and 19%, respectively [7].

It should also be noticed that RIC transplantations using BM as a stem cell source have been reported to be associated with a higher risk of graft failure when compared with those using cytokine-mobilized PBSC, especially in the unrelated donor setting [6, 7]. As compared with BM, PBSC grafts usually contain more than ten times higher number of T-cells and 2–4 times greater number of $CD34^+$ cells, which would have a beneficial impact on successful engraftment after RIC [31]. In a study which compared the engraftment kinetics after transplantation of PBSC and BM with an identical non-myeloablative conditioning, the number of patients who achieved full donor chimerism was significantly lower in the BM group [32]. These observations suggested that, to improve the outcomes after RIC transplantation using BM grafts from unrelated donors, it is important to develop more optimal post-transplant immunosuppressive protocol which can effectively prevent graft rejection as well as severe GVHD.

In preclinical canine models and clinical experiences of HSCT after truly non-myeloablative regimen using low-dose TBI with or without fludarabine as pre-transplant conditioning, post-transplant administration of MMF was shown to improve the rate of successful donor cell engraftment [11, 33]. Therefore, we hypothesized that the addition of MMF to the standard immunosuppression with tacrolimus plus minidose MTX might facilitate engraftment after unrelated BM allografting with RIC. In support of this hypothesis, all the patients in this study achieved durable donor cell engraftment without experiencing serious morbidity associated with delayed hematopoietic recovery or late graft failure. However, this promising result awaits further validation because the probability of engraftment can also be influenced by the type and intensity of RIC regimens or by the use of pre-transplant anti-thymocyte globulins or T-cell-depleting monoclonal antibodies. Onishi et al. reported the outcomes of unrelated BM transplantation after RIC with fludarabine, busulfan, and 4 Gy TBI among a cohort of 17 patients with various hematologic malignant diseases. Although all the patients in their report initially achieved successful engraftment with the use of conventional post-transplant immunosuppression composed of cyclosporine and MTX, 2 of them subsequently developed secondary graft failure [8]. This observation suggests that the intensification of conditioning with 4 Gy TBI does not always confer sustained engraftment, at least in the setting of unrelated marrow transplantation.

In contrast, the role of MMF in ameliorating acute GVHD has been controversial at least when administered solely with calcineurin inhibitors. Recently, Koh et al. [34] reported that the post-grafting MTX combined with cyclosporine and MMF significantly reduced the risk of grade III–IV acute GVHD as compared with a combination of cyclosporine and MMF. Consistent with their experience, the cumulative incidence of severe acute GVHD after our triple combination was 5%, encouragingly lower than those previously reported in the analysis of unrelated BMT through the Japan Marrow Donor Program, while the incidence of extensive chronic GVHD was apparently similar [27, 35]. However, an important concern regarding the intensification of post-transplant immunosuppressive regimen is an increased risk of infection or relapse. In this study, a substantial proportion of patients developed manageable infections and experienced disease progression within 1 year after transplantation. Although the incidence rates of these events might be adversely affected by the high proportion of patients who had an advanced disease at transplantation, further studies are needed to elucidate whether our triple immunosuppressive regimen may increase the risk of infectious complications or may compromise the graft-versus-tumor effect after RIC HSCT [36, 37].

An unresolved issue in the present study is a pharmacokinetic/pharmacodynamic profile of MMF when combined with tacrolimus and MTX. The increased mean total plasma MPA concentrations at steady state were reported to be associated with higher donor cell chimerism after unrelated non-myeloablative transplantation, while the lower MPA levels were shown to be a predictor of graft rejection [38]. We administered MMF in three divided doses rather than in twice-daily doses because the former is more likely to confer higher mean total MPA concentrations [38, 39]. Because it is speculated that the bioavailability of oral MMF is highly variable depending on the degree of gastrointestinal mucosal damage and donor-recipient pharmacogenomic backgrounds [40], it is important in the future studies to evaluate the association of MPA pharmacodynamics with the risk of post-transplant immunologic complications such as graft rejection, acute GVHD, and infections. Furthermore, appropriate dosing of MMF would be affected by the type of combined calcineurin inhibitor: cyclosporine is reported to decrease MPA exposure due to delay of the excretion of the MPA metabolites, while tacrolimus is less likely to cause drug interaction with MPA [41, 42].

In conclusion, our study demonstrated the feasibility and efficacy of using a triple combination of tacrolimus, minidose MTX and MMF as post-grafting immunosuppression after RIC BM transplantation from unrelated donors. Because this triple regimen conferred high

probability of sustained donor engraftment with an acceptable risk of transplant-related complications, further studies are warranted to confirm its efficacy in a larger population including patients who receive HLA-mismatched family donor grafts or unrelated cord blood units with dose-reduced conditioning.

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LETTER TO THE EDITOR

Impact of discontinuing fluoroquinolone prophylaxis on early mortality after allogeneic marrow or peripheral blood SCT with myeloablative conditioning

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Oral fluoroquinolones (FQs) or other antibiotics are commonly used as antibacterial prophylaxis after cytotoxic chemotherapy for malignant neoplasms, although significant practice variations have been reported among centers and countries.[1] Despite such practical variations, the efficacy of oral FQ as a prophylactic agent has not been fully evaluated in the SCT setting.[2,3] Further, the widespread emergence of multidrug-resistant microorganisms in hematology–oncology units has also increased the need for re-evaluating the role of antibacterial prophylaxis administered to patients undergoing cytotoxic chemotherapy or SCT.[4–9] In attempts to reduce the emergence of antibiotic-resistant bacterial organisms, since 2003, we have discontinued the administration of oral FQs as prophylactic agents for patients undergoing standard-dose chemotherapy other than SCT; further, since 2004, we have withdrawn the use of any antibacterial prophylaxis in auto- and allo-SCT recipients as well.[6] In allogeneic SCT after myeloablative conditioning regimens, the risk of bacterial infection is considerably high because high-dose chemotherapy and/or TBI may cause severe mucosal damage that facilitates bacterial translocation under profound post transplant immunosuppression. Therefore, we conducted a single-center retrospective study to evaluate the effect of such restriction of FQ prophylaxis on the incidence of bacterial infection and early mortality rate among patients receiving allo-SCT after myeloablative conditioning for hematologic malignancies.

The medical records were reviewed with respect to data on 145 consecutive adult patients with hematologic malignancies who underwent allogeneic myeloablative SCT with the use of T-cell-replete marrow or peripheral blood graft between January 2000 and December 2008 at our institution. Patients who had repeated episodes of bacterial infections, and those who had active infections before transplantation procedure were excluded; a total of 128 patients with the median age of 41.5 years (range, 17–61 years) were included in the analysis. Between January 2000 and August 2004, the patients received oral FQs (levofloxacin, tosufloxacin or ciprofloxacin) as antibacterial prophylactic agents (prophylaxis group). Patients who could not ingest oral drugs were temporarily administered anti-pseudomonal ß-lactams i.v. instead. From September 2004 to December 2008, the use of any prophylactic antibacterial agents, including oral FQs, was discontinued (non-prophylaxis group). In both groups, i.v. antibiotics with anti-pseudomonal activity were promptly administered in the episodes of febrile neutropenia or suspected bacterial infections.

There were no differences in patient characteristics between the prophylaxis and non-prophylaxis groups (Table 1), except with regard to the preferential use of G-CSF in the prophylaxis group, which conferred significantly earlier neutrophil engraftment as compared with the non-prophylaxis group (median day, 15 (range, 10–67) in the prophylaxis group and 19.5 (12–30), in the non-prophylaxis group, $P<0.001$). During the entire study period, a total of 12 episodes of bacterial infections were documented; these included 11 cases of bloodstream infections and a single case of pneumonia. All organisms detected in the prophylaxis group were resistant to FQs; most of these organisms were gram-positive cocci ($n=4$, 67%). On the other hand, four of the six organisms detected in the non-prophylaxis group were FQ-sensitive gram-negative bacilli. Organ failure and septic shock because of bacterial infection were not observed, except in one patient in the prophylaxis group who succumbed to infection with metallo-β-lactamase-producing multidrug-resistant *Pseudomonas aeruginosa*. In both groups, microbiologically documented infections developed during the early period after SCT (median day, 5.5 (range, 2–11) in the prophylaxis group and median day, 6.5 (1–13) in the non-prophylaxis group), and there was no statistically significant difference between the two groups in this regard ($P=0.750$). Although the cumulative incidence of microbiologically documented infections was slightly higher in the non-prophylaxis group (15%; 95% confidence interval (CI), 6–27%) than in the prophylaxis group (7%; 95% CI, 3%–13%), multivariate Cox analysis revealed this difference was not statistically significant (hazard ratio for the non-prophylaxis group vs prophylaxis group, 1.69; 95% CI, 0.40–7.08; $P=0.473$). The overall survival rates at 100 days after transplantation in the prophylaxis group and non-prophylaxis group were 89% (95% CI, 80–94%) and 90% (95% CI, 76–96%), respectively, with no significant difference between the groups in multivariate Cox analysis ($P=0.682$) (Figure 1).

The emergence of multidrug-resistant microorganisms is becoming a serious problem in clinical settings in which SCT and cytotoxic chemotherapy are performed. Frère *et al.*[5] studied the drug susceptibility of bacterial organisms isolated from 492 patients who underwent auto- or allo-SCT between 1982 and 1999. They reported that the susceptibility to ciprofloxacin among gram-positive and

Table 1 Characteristics of patients

Characteristics	*Prophylaxis group* (n = 87)	*Non-prophylaxis group* (n = 41)	P-*value*
Age, years			
Median (range)	39 (17–61)	43 (19–58)	0.505
Sex			
Male	48 (55%)	21 (51%)	0.675
Female	39 (45%)	20 (49%)	
Diagnosis[a]			
Myeloid neoplasms	53 (61%)	26 (63%)	0.786
Lymphoid neoplasms	34 (39%)	15 (37%)	
Disease status at transplant[b]			
Standard risk	43 (49%)	24 (59%)	0.336
High risk	44 (51%)	17 (42%)	
Type of donor			
HLA-identical sibling donor	29 (33%)	16 (39%)	0.162
Other related donor	15 (17%)	2 (5%)	
Unrelated donor	43 (49%)	23 (56%)	
Stem cell source			
BM	72 (83%)	39 (95%)	0.054
Peripheral blood	15 (17%)	2 (5%)	
Conditioning regimen			
TBI-based regimen	76 (87%)	34 (83%)	0.134
Busulfan-based regimen	7 (8%)	7 (17%)	
Other non-TBI regimen	4 (5%)	0 (0%)	
Courses of chemotherapy before transplant			
<5	42 (48%)	21 (51%)	0.756
⩾5	45 (52%)	20 (49%)	
Interval days between the last chemotherapy and transplant			
<60	34 (39%)	17 (42%)	0.797
⩾60	53 (61%)	24 (59%)	
Hospitalized months before transplant			
<3	18 (21%)	10 (24%)	0.417
⩾3, <6	33 (38%)	19 (46%)	
⩾6	36 (41%)	12 (29%)	
Use of G-CSF			
Yes	76 (87%)	20 (49%)	<0.001
No	11 (13%)	21 (51%)	

Abbreviations: Busulfan-based regimen = conditioning regimen including 16 mg/kg of busulfan; Other non-TBI regimen = myeloablative conditioning regimen including ranimustine and melphalan without TBI nor busulfan; TBI-based regimen = conditioning regimen including more than 8 Gy of TBI.
Data are no. of patients (%) except for age.
[a]Myeloid neoplasms include AML, MDS, CML, and other myeloproliferative neoplasms, while lymphoid neoplasms include ALL, adult T-cell leukemia/lymphoma, and other mature B-cell or T-cell neoplasms.
[b]Patients were considered to have standard-risk disease if they received transplant without previous chemotherapy or in CR, while those who received transplant in all the other status were considered to have high-risk disease.

gram-negative bacterial isolates was more than 70% in 1990, while it was <30% in 1997–1998; this observation suggested that FQ prophylaxis might no longer promise its

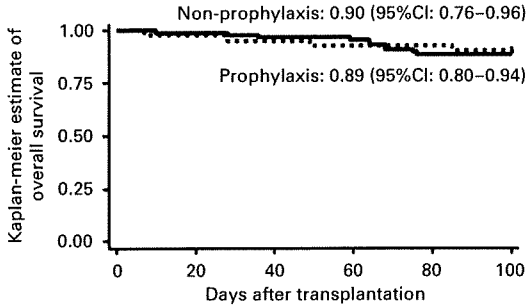

Figure 1 Kaplan–Meier estimate of overall survival within 100 days after SCT. Solid black line, the prophylaxis group ($n = 87$); dotted black line, the non-prophylaxis group ($n = 41$); CI, confidence interval.

clinical benefit at least in SCT settings. Given the increasing prevalence of FQ-resistant microorganisms, trials involving neutropenic patients with hematologic malignancies have been conducted to evaluate the effects of withdrawal of FQs as prophylactic agents. It is noteworthy that these studies showed that the susceptibility of enterobacterial isolates to FQ was significantly restored after FQ prophylaxis was discontinued.[6,9] In addition, routine administration of FQs can induce cross-resistance to other antibiotics, such as β-lactams, through various mechanisms.[10] Therefore, the routine use of antibacterial prophylaxis in patients undergoing SCT should be carefully re-evaluated to reduce the prevalence of multidrug-resistant microorganisms.

Our present findings suggested that withdrawal of FQ as antibacterial prophylaxis is feasible without significant increase in the early mortality rate in allogeneic T-cell-replete BM or peripheral blood SCT after myeloablative conditioning, provided appropriate antibiotic treatment is promptly initiated in the event of febrile neutropenia. However, the retrospective study design, the heterogeneous underlying diseases in the small number of patients involved, and the variability in transplantation procedures used may have caused a bias in the results. Therefore, larger well-controlled prospective studies are needed to evaluate the role of antibacterial prophylaxis in BM or peripheral blood SCT after myeloablative conditioning. In addition, the significance of antibacterial prophylaxis in other SCT settings such as cord blood transplantation or SCT after reduced-intensity conditioning is also worth evaluating in the future study.

Conflict of interest

The authors declare no conflict of interest.

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Experimental Hematology

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Recovery from established graft-vs-host disease achieved by bone marrow transplantation from a third-party allogeneic donor

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Objective. **We investigated whether established graft-vs-host disease (GVHD) could be successfully treated by a second allogeneic bone marrow transplantation (BMT) through elimination of first donor–derived lymphocytes responsible for GVHD.**

Materials and Methods. **In a murine GVHD model of BDF1 (H-$2^{b/d}$)→B6C3F1(H-$2^{b/k}$), GVHD mice underwent a second BMT using a graft (1×10^7 bone marrow and 3×10^7 spleen cells) from a major histocompatibility complex (MHC) antigen haploidentically mismatched (to host and also to first donor) mouse strain, B6B10F1(H-$2^{b/s}$), following low-dose total body irradiation (TBI) 2 to 3 weeks after the first BMT.**

Results. **Results demonstrated that severe GVHD could be successfully and stably treated by a second allogeneic BMT. For successful treatment of GVHD, rapid achievement of full second–donor T-cell chimerism was required. Furthermore, we showed that mice with GVHD could easily accept MHC haploidentically mismatched second-donor hematopoietic cells even after minimal conditioning (2–4 Gy TBI) because they were in a profoundly immunosuppressed state, and that the mice were relatively resistant to new development of GVHD by second-donor grafts. Furthermore, the timing of the second BMT, the intensity of conditioning treatment (GVHD mice are very sensitive), and donor selection were also found to be important for obtaining successful outcomes. Increased regulatory T cells and reduction of interferon-γ levels may be involved in tolerance induction.**

Conclusions. **We demonstrated that established GVHD in a murine GVHD model could be successfully treated by a second BMT from a third-party allogeneic donor.** **© 2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Graft-vs-host-disease (GVHD) is a major obstacle to successful allogeneic bone marrow transplantation (BMT), and greatly limits the application and efficacy of allogeneic BMT. GVHD is initiated by donor T cells that recognize differences in major or minor histocompatibility antigens between the recipient and donor. Alloactivated donor T cells attack recipient tissues, especially the skin, liver, and gastrointestinal tract, directly or indirectly, through cellular- and cytokine-mediated effectors, ultimately resulting in the death of recipients [1].

Autoimmune diseases are caused by self-reactive lymphocytes, which attack various organs, including the muscles, joints, or skin. In murine models, allogeneic BMT was reported to be effective for the prevention [2–4] and treatment [4–7] of autoimmune diseases. In this situation, donor lymphocytes are considered to have the capacity to eliminate all residual self-reactive host lymphocytes through a process known as graft-vs-autoimmunity effects, with analogy to graft-vs-leukemia in leukemia [8]. GVHD resembles autoimmune diseases in that host-reactive lymphocytes induce life-threatening disorders involving various organs, although there is a difference in lymphocyte origin, namely, allogeneic or autologous; therefore, allogeneic (second) BMT may improve established, life-threatening GVHD by eliminating the harmful lymphocytes responsible for GVHD.

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For the successful treatment of established GVHD by allogeneic BMT, the engraftment of second-donor grafts, and the absence of new development of GVHD by second-donor lymphocytes are required; however, mice with ongoing GVHD may have several special features. For example, they might not be able to endure a high dose of total body irradiation (TBI) because of profound organ damage due to GVHD. To overcome this problem, we adopted low-dose TBI as the conditioning treatment for the second BMT. For autoimmune diseases, major histocompatibility complex (MHC)–mismatched murine BMT using a nonmyeloablative regimen has been reported to induce mixed chimerism between the donor and recipient, and to succeed in reversing autoimmunity [9,10]. However, unlike autoimmune diseases, the organ damage caused by GVHD generally progresses rapidly because of the vigorous alloreactive response. Because the GVH reaction must be stopped before organ damage becomes irreversible, the mixed chimerism strategy between first and second donors may be insufficient; therefore, to rapidly eradicate the first-donor lymphocytes responsible for GVHD under nonmyeloablative conditioning, we used a T-cell–replete graft, namely, unmanipulated bone marrow (BM) and spleen cells from the second donor.

In the present study, to investigate whether severe GVHD could be successfully treated by a second BMT, GVHD in MHC haploidentically mismatched BMT of BDF1(H-$2^{b/d}$)→B6C3F1(H-$2^{b/k}$) was treated by a second BMT under various conditions, in which donor selection, TBI dose, and BMT timing varied. The results demonstrated that severe GVHD could be successfully treated by performing a second BMT using a T-cell–replete graft from another MHC haploidentically mismatched donor. Furthermore, we clarified several conditions for successfully treating severe GVHD by allogeneic BMT.

Materials and methods

Mice

Female B6C3F1(B6 × C3H/He; H-$2^{b/k}$), BDF1(B6 × DBA/2; H-$2^{b/d}$), B6B10F1(B6 × B10.s; H-$2^{b/s}$), DBA/1J(H$2^{q/q}$), DBA/2(H-$2^{d/d}$), and C3DF1 (DBA/2 × C3H/He; H-$2^{d/k}$) mice were purchased from Japan SLC (Shizuoka, Japan). Mice used for experiments were between 6 and 11 weeks of age, were housed in sterile microisolator cages in a specific pathogen-free facility, and received autoclaved food and water ad libitum.

BMT

BM cells were harvested from the tibia and femur of donor mice by flushing with RPMI-1640 medium. Spleen cells were isolated from donor mice using the nylon-wool purification method as a source of lymphocytes. T-cell purification rate after the nylon-wool purification was 28% to 31%. Because the purification rate was very constant, T-cell normalization was not performed in the following experiments. All BMTs were performed by transfusion of a fixed number of donor cells (1×10^7 BM cells and 3×10^7 spleen cells) after TBI on the previous day. TBI (137Cs source) was given to recipients in a single dose: 8.5 Gy to induce GVHD, and 0 to 5 Gy to treat GVHD. 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 GVHD clinical score was assessed weekly using a scoring system incorporating five clinical parameters: body weight, posture (hunching), mobility, fur texture, and skin integrity, as described by Cook et al. [11]. All animal protocols were approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

Flow cytometric analysis

Fluorescein isothiocyanate-, phycoerythrin-, peridinin chlorophyll protein-, and allophycocyanin-conjugated monoclonal antibodies against mouse antigens (H-2^d, H-2^b, H-2^k, H-2^s, CD3) were purchased from BD Pharmingen (San Diego, CA, USA). Cells were first incubated with monoclonal antibody 2.4G2 (specific for Fcγ receptor) for 10 minutes at 4°C to block nonspecific staining. They were then incubated with the relevant fluorescein isothiocyanate-, phycoerythrin-, peridinin chlorophyll protein-, or allophycocyanin-conjugated monoclonal antibodies for 30 minutes at 4°C, and then washed with RPMI-1640 medium containing 1% fetal calf serum and 1 mM ethylenediamine tetraacetic acid. Three- or four-color cytometric analysis was performed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) using Cell Quest software.

T-cell chimerism was analyzed in peripheral blood obtained by eye bleeding. Chimerism analysis of $CD3^+$ cells was performed by three-color flow cytometry. Based on expression of MHC determinants, second-donor cells were discriminated from recipient or first-donor cells.

Regulatory T ($CD4^+foxp3^+$) cells in the spleen were quantitated by flow cytometry as reported previously [12]. Th1 and Th2 cells in spleens were quantitated based on the intracellular expression of cytokines, as reported previously [13]. Cells were treated with phorbol myristate acetate and ionomycin, and were examined for their intracellular interferon (IFN)-γ (Th1) or interleukin-4 (Th2) expression status by flow cytometry.

Mixed lymphocyte culture

Spleen cells were isolated and suspended in RPMI-1640 medium supplemented with 10% fetal calf serum. One million responder cells were incubated with 1×10^6 irradiated (30 Gy) spleen cells (stimulator) in triplicate using 96-well plates at 37°C in 5% CO_2 in air for 3 days. Cells were pulsed on the third day with 1 μCi of [^{3}H]-thymidine, and harvested on the fourth day, and thymidine incorporation was measured using a liquid scintillation counter.

Measurement of cytokine gene expression levels

Tumor necrosis factor-α (TNF-α) and IFN-γ gene expression levels in spleen cells were measured using quantitative reverse transcription polymerase chain reaction as reported previously.[14] TNF-α and IFN-γ polymerase chain reaction products were normalized in relation to the β-actin internal control.

Histology

Formalin-preserved liver, skin, and small and large intestines were embedded in paraffin, cut into 5-μm–thick sections, and stained with hematoxylin and eosin for histologic examination. Slides were coded without reference to prior treatment and evaluated

in a blinded fashion by one individual (Y. Hoshida). A semiquantitative scoring system for GVHD was used as described previously,[15,16] with some modifications: small intestine: villous blunting, crypt regeneration, loss of enterocyte brush border, luminal sloughing of cellular debris, crypt cell apoptosis, outright crypt destruction and lamina propria lymphocytic infiltrate; large intestine: crypt regeneration, colonocyte vacuolization, surface colonocyte attenuation, crypt cell apoptosis, outright crypt destruction, lamina propria lymphocytic infiltrate, and mucosal ulceration; liver: portal tract expansion, mononuclear cell infiltration of the portal tracts, nuclear pleomorphism of the bile ducts, vascular endothelialitis, and hepatocellular changes, including mitotic figures, confluent necrosis, acidophilic bodies, foamy changes, neutrophil accumulation, and macrophage aggregates; skin: intraepithelial apoptosis, exocytosis, liquefaction, dermal lymphocyte infiltration. The scoring system denoted 0 as normal, 0.5 as focal and rare, 1.0 as focal and mild, 2.0 as diffuse and mild, 3.0 as diffuse and moderate, and 4.0 as diffuse and severe. Scores were added to provide a total score for each specimen: the maximum score for small and large intestines was 28 each, for liver 40, and for skin 16.

Statistical analysis

Statistical analysis of GVHD clinical scores and histopathological scores was performed with the nonparametric unpaired Mann-Whitney *U*-test. Data from mixed-lymphocyte culture (MLC) analysis were compared with Tukey's HSD test. Estimates of survival probability were calculated by the Kaplan-Meier method. Survival data were compared based on the results of log-rank tests. Cytokine gene expression levels, regulatory T cells, and Th1/Th2 balance between first and second BMT recipients were compared by Student's *t*-test. Results were considered significant when p was <0.05.

Results

Treatment of GVHD by second BMT from a syngeneic donor

In a murine GVHD model (BDF1(H-$2^{b/d}$)→B6C3F1(H-$2^{b/k}$)), we first tested whether established GVHD can be alleviated by a second BMT from a mouse syngeneic to the recipient. In this GVHD model, in which recipients received donor BM (1×10^7) and spleen (3×10^7) cells after receiving a lethal TBI dose (8.5 Gy), recipients began to show clinical features of GVHD 1 week after BMT. Mice began to die from GVHD progression 3 weeks after BMT (Fig. 1A), and only 20% of recipients survived until day 70.

The timing for the second BMT was fixed at day 14 after the first BMT. After receiving TBI of 3 Gy, 4 Gy, or 5 Gy, mice with GVHD underwent a second BMT using BM (1×10^7) and spleen (3×10^7) cells from B6C3F1 mice (syngeneic to the recipient). The survival of mice that underwent the second BMT using TBI of 3 Gy was not significantly improved compared with control groups that did not undergo a second BMT (Fig. 1A). On the other hand, survival of mice undergoing the second BMT using TBI of 4 Gy was significantly improved compared with control mice; however, all mice that underwent a second BMT using TBI of 5 Gy died by 9 days after the second BMT due to TBI toxicity. The clinical score for GVHD of mice that underwent the second BMT is shown in Fig. 1B. The extent of GVHD in mice that underwent the second BMT using TBI of 3 Gy or 4 Gy was not significantly different from that of the

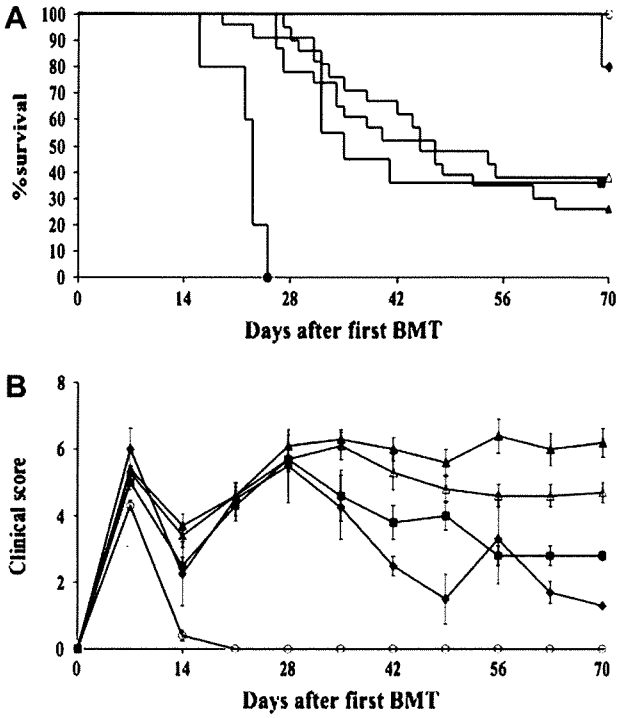

Figure 1. Treatment of graft-vs-host disease (GVHD) by second bone marrow transplantation (BMT) using grafts from mice syngeneic to host. B6C3F1 (H-$2^{b/k}$) received bone marrow (BM) (1×10^7) and spleen (3×10^7) cells from an major histocompatibility complex (MHC) haploidentically mismatched donor, BDF1 (H-$2^{b/d}$) after lethal total body irradiation (TBI) (8.5 Gy). On day 14, recipients underwent a second BMT using BM (1×10^7) and spleen (3×10^7) cells from mice syngeneic to the host (B6C3F1) after receiving low-dose TBI (3, 4, or 5 Gy). Open circles, B6C3F1→(B6C3F1→B6C3F1) (n = 9); closed triangles, BDF1→B6C3F1 (n = 15); open triangles, BDF1→B6C3F1, in which mice received TBI 3 Gy on day 13 without undergoing a second BMT (n = 21); closed squares, B6C3F1→(BDF1→ B6C3F1) using TBI of 3 Gy (n = 15); closed diamonds, B6C3F1→(BDF1→B6C3F1) using TBI of 4 Gy (n = 8); closed circles, B6C3F1→(BDF1→B6C3F1) using TBI of 5 Gy (n = 10). (**A**) Survival. Data were calculated by the Kaplan-Meier method. The survival of mice that underwent the second BMT using TBI of 3 Gy was not significantly improved compared with GVHD control groups that did not undergo a second BMT with ($p = 0.591$) or without ($p = 0.224$) receiving TBI of 3 Gy. In contrast, the survival of mice undergoing a second BMT using TBI of 4 Gy was significantly better than that of control groups with ($p = 0.048$) or without ($p = 0.004$) receiving TBI. (**B**) GVHD clinical score. Animals were scored for clinical GVHD, as described in Materials and Methods. GVHD scores are shown as the mean ± standard error of the mean. The extent of GVHD in mice that underwent a second BMT using TBI of 3 Gy or 4 Gy was not significantly different from that of the control group by 2 weeks after the second BMT, but thereafter, mice undergoing the second BMT showed GVHD improvement. In particular, remarkable improvement of GVHD was observed in mice undergoing a second BMT using TBI of 4 Gy.

control groups at 2 weeks after the second BMT, but thereafter, mice that underwent the second BMT showed improved GVHD. In particular, remarkable improvement of GVHD was observed in mice undergoing the second BMT using TBI of 4 Gy.

Next, T-cell chimerism in the peripheral blood (PB) of mice that underwent a second BMT was analyzed. In this GVHD model, $CD3^+$ cells in the PB of recipients became composed entirely of BDF1-derived cells by day 14 (of the first BMT) (data not shown). Mice that underwent a second BMT using TBI of 3 Gy showed mixed chimerism between BDF1 and B6C3F1 on day 7 (of the second BMT), and thereafter converted to almost complete chimerism of B6C3F1 on day 14 (experiment 1 in Table 1). On the other hand, mice that underwent the second BMT using 4 Gy achieved almost complete chimerism of B6C3F1 by 7 days after the second BMT (experiment 2 in Table 1). This result suggests that rapid achievement of complete chimerism of B6C3F1 is required to improve the survival of GVHD mice, and that the range of the TBI dose useful for conditioning before the second BMT is narrow, as judged by the improved survival in this system.

Treatment of GVHD by second BMT from a third-party allogeneic donor

Next, in this GVHD model (BDF1 ($H\text{-}2^{b/d}$) → B6C3F1 ($H\text{-}2^{b/k}$)), we examined whether GVHD could be treated by a second BMT using a graft from a third mouse strain, B6B10F1 ($H\text{-}2^{b/s}$). In this BMT sequence, the three mouse strains (recipient, first donor, and second donor) shared one MHC haplotype, while the other MHC haplotype was different. Mice with GVHD (BDF1 → B6C3F1) received a graft (1×10^7 BM cells and 3×10^7 spleen) from B6B10F1 on day 14 (of the first BMT) without TBI or following various doses of TBI (2, 3, 4, or 5 Gy). Mice that underwent a second BMT without TBI did not show a significant improvement of the GVHD score by day 28 after the second BMT compared with the control groups that did not undergo a second BMT (Fig. 2B), and the survival rate of these mice was comparable to that of the control groups (Fig. 2A). In contrast, all mice that underwent a second BMT after receiving TBI of 2, 3, or 4 Gy showed an improvement of GVHD symptoms by 7 days after the second BMT, and survived until at least day 70. The survival of these mice was significantly better than that of control mice (Fig. 2A). Some of the mice were observed for a long period (> 150 days), and all survived with or without mild GVHD. All mice receiving TBI of 5 Gy died of severe organ damage (data not shown).

The T-cell chimeric status in the PB was analyzed. Mice undergoing a second BMT without TBI showed mixed chimerism with a low percentage (< 30%) of persistent second-donor components (experiment 4 in Table 1). In contrast, mice undergoing a second BMT using TBI 2 to 4 Gy showed almost complete T-cell chimerism of the second-donor origin by 7 days after the second BMT (experiment 3 in Table 1). These findings indicate that, in the GVHD model of BDF1 → B6C3F1, the second BMT from B6B10F1 ameliorated GVHD and significantly improved the survival of mice by effectively eliminating the first-donor–derived cell components responsible for GVHD, and that the range of the TBI dose (for the second BMT) useful for improving survival was clearly wider in the second BMT using a third-party allogeneic donor than when using a donor syngeneic to recipients. We named the effect by which allografts counteract GVHD "graft-vs-GVHD." To clarify the relationship between the T-cell chimeric status of the first- and second-donors posttransplantation and the graft-vs-GVHD effect, GVHD mice that had received BDF1 → B6C3F1 BMT underwent a second BMT under different conditions, as shown in Table 1 (experiments 5, 6, and 7). The results revealed that the improvement of GVHD occurred only when recipients achieved almost complete T-cell chimerism of the second-donor type, indicating that mixed chimerism was insufficient to improve GVHD symptoms.

Table 1. T-cell chimerism between first and second donors after second bone marrow transplantation

Experiment	BMT sequence	TBI dose for second BMT (Gy)	Second donor components (%)[a] Day 7	Second donor components (%)[a] Day 14	Improvement in GVHD score
1	B6C3F1 → (BDF1 → B6C3F1)	3	47.1 ± 20	95.7 ± 3.4	+
2		4	99.4 ± 0.8	99.1 ± 1.1	+
3	B6B10F1 → (BDF1 → B6C3F1)	3	99.3 ± 0.9	99.0 ± 1.1	+
4		0	25.9 ± 10	18.2 ± 18.5	−
5	C3DF1 → (BDF1 → B6C3F1)	3	2.6 ± 2.5	1.8 ± 3.6	−
6	DBA/2 → (BDF1 → B6C3F1)	0	40.9 ± 7.1	25.1 ± 3.1	−
7		3	78.9 ± 8.6	96.0 ± 2.5	+

TBI = total body irradiation.

BDF1($H\text{-}2^{b/d}$), B6C3F1($H\text{-}2^{b/k}$), B6B10F1($H\text{-}2^{b/s}$), C3DF1($H\text{-}2^{d/k}$), and DBA/2($H\text{-}2^{d/d}$) were used in these experiments.

Flow cytometric analysis of T-cell lineage of peripheral blood (PB) samples was performed on days 7 and 14 of the second bone marrow transplantation (BMT).

[a]PB samples from seven recipients that underwent a second BMT were analyzed. Data are shown as the mean ± standard deviation.

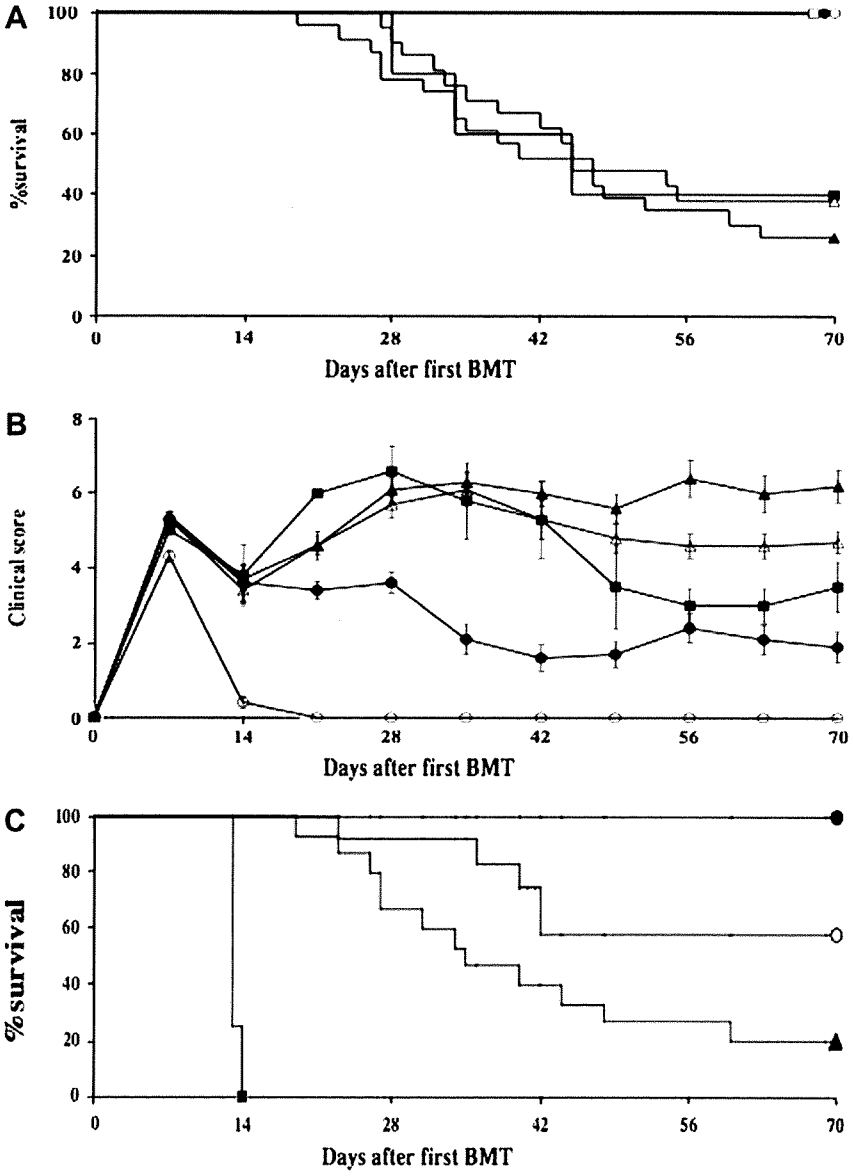

Figure 2. Treatment of graft-vs-host disease (GVHD) by second bone marrow transplantation (BMT) using grafts from mice that were major histocompatibility complex (MHC) haploidentically mismatched to the host and first donor. (**A**) Survival of mice receiving a second BMT. Mice that developed GVHD after BMT of BDF1(H-$2^{b/d}$)→B6C3F1 (H-$2^{b/k}$) underwent a second BMT using grafts (1×10^7 BM cells and 3×10^7 spleen cells) from B6B10F1 (H-$2^{b/s}$) without total body irradiation (TBI) or after receiving 2, 3, or 4 Gy of TBI on day 14 after the first BMT. As a control, the survival of mice that did not undergo a second BMT with (open triangles, n = 20) or without (closed triangles, n = 20) receiving TBI of 3 Gy on day 13 of the first BMT was assessed. The survival of mice that underwent a second BMT is shown by the following symbols (closed squares, no TBI [n = 8]; open squares, 2 Gy TBI [n = 8]; closed circles, 3 Gy TBI [n = 8]; open circles, 4 Gy TBI [n = 8]). Mice with GVHD that underwent a second BMT after receiving TBI of 2 Gy, TBI of 3 Gy, or TBI of 4 Gy showed significantly improved survival compared with GVHD control groups with or without TBI ($p < 0.01$, log-rank test). The survival rate of mice that underwent a second BMT without TBI was not significantly different from that of the GVHD control groups with ($p = 0.963$) or without ($p = 0.388$) TBI. (**B**) GVHD clinical score of mice that did or did not undergo a second BMT from B6B10F1 in the GVHD model of BDF1→B6C3F1. Mice not undergoing a second BMT with (open triangles) or without (closed triangles) TBI of 3 Gy; closed squares, second BMT mice without TBI; closed circles, second BMT mice following TBI of 3 Gy; open circles, mice undergoing repeated syngeneic BMT (B6C3F1→[B6C3F1 →B6C3F1]) (n = 9). Scores are shown as the mean ± standard error of mean. Mice that underwent a second BMT with TBI of 3 Gy showed significantly improved GVHD symptoms 7 days and later after the second BMT compared with control mice that did not undergo a second BMT. (**C**) Timing of the second BMT. Mice with GVHD after receiving BDF1→B6C3F1 BMT underwent a second BMT using grafts (1×10^7 BM cells and 3×10^7 spleen cells) from B6B10F1 after receiving a fixed TBI dose (3 Gy) on day 7, 14, or 21 after the first BMT. Closed triangles, no second BMT (n = 15); closed squares, second BMT on day 7 (n = 8); closed circles, second BMT on day 14 (n = 15); open circles, second BMT on day 21 (n = 15). The survival of mice that underwent a second BMT on day 14 ($p = 0.0004$) and on day 21 ($p = 0.031$) was significantly better than that of control mice that did not undergo a second BMT.

We next investigated the influence of the timing of the second BMT on the graft-vs-GVHD effect. To this end, GVHD mice that had received BDF1 → B6C3F1 BMT underwent a second BMT using a graft (1×10^7 BM cells and 3×10^7 spleen) from B6B10F1 after receiving a fixed TBI dose (3 Gy) at various times after the first BMT (Fig. 2C). All mice that underwent a second BMT on day 7 died within 1 week after the second BMT because of severe organ toxicity. On the other hand, all mice that underwent a second BMT on day 14 and 58.3% on day 21 survived at least until day 70 (after the first BMT), with the survivors showing improved GVHD symptoms. The survival of these mice was significantly better than that of control mice that did not undergo a second BMT. Mice undergoing a second BMT on day 21 tended to have a lower survival rate than those undergoing BMT on day 14 ($p = 0.0629$).

Next, to verify that the graft-vs-GVHD effect was observed regardless of donor–recipient pairings, we exchanged the first-donor strain with the second-donor strain. In the GVHD model with BMT of B6B10F1 (H-$2^{b/s}$) → B6C3F1 (H-$2^{b/k}$), in which recipients received 1×10^7 BM cells and 3×10^7 spleen cells from the donor after a lethal TBI dose (8.5 Gy), all the mice died of GVHD progression by day 35. Fifty percent of mice that underwent a second BMT on day 14 (after the first BMT) using a graft (1×10^7 BM and 3×10^7 spleen cells) from BDF1 (H-$2^{b/d}$) after receiving 3 Gy TBI survived until day 70, with the survivors showing improvement of GVHD symptoms. Although the extent of GVHD occurring in B6B10F1 → B6C3F1 BMT was found to be slightly greater than that in BDF1 → B6C3F1, a second BMT from BDF1 ameliorated the extent of GVHD, and significantly improved the survival rate compared with that of the control mice that did not undergo a second BMT ($p = 0.002$, log-rank test, Fig. 3A).

Pathological demonstration of graft-vs-GVHD effects

The graft-vs-GVHD effect was confirmed histologically. Representative histologic images for the graft-vs-GVHD model of B6B10F1 → (BDF1 → B6C3F1) are shown in Fig. 4A to 4E. The GVHD lesions observed on day 14 in the first BMT (BDF1 → B6C3F1) were almost completely healed on day 50 (36 days after the second BMT) (Fig. 4B and C). In contrast, GVHD-related pathological changes continued in surviving mice in control groups on day 50 (Fig. 4D and E). We evaluated the pathology of GVHD-target organs using a semiquantitative index in which a number of histological parameters were scored. As shown in Fig. 4F, the second BMT group had significantly lower pathological GVHD scores in the skin and liver than control groups. For the large intestine, the second BMT group had a significantly lower score than the control group not receiving a second BMT ($p = 0.007$), and tended to have a lower score than the control group receiving only TBI on day 13 ($p = 0.098$). There was no significant difference in the small intestine among the three groups.

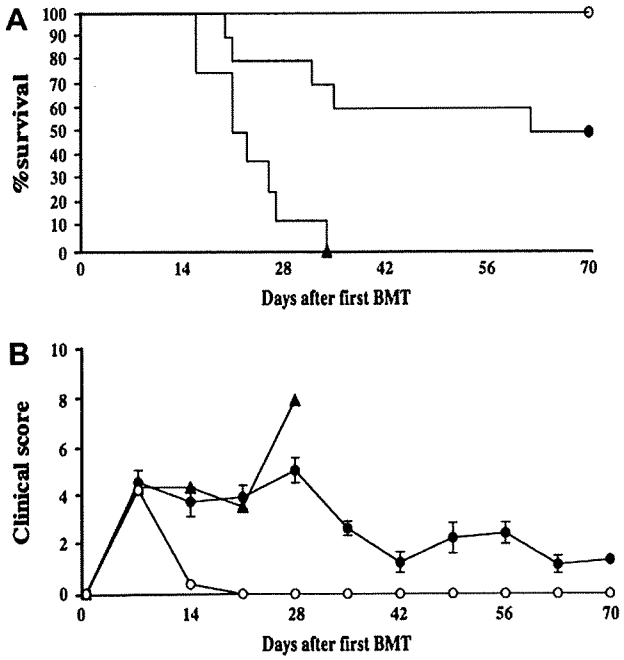

Figure 3. Treatment of graft-vs-host disease (GVHD) occurring in bone marrow transplantation (BMT) of B6B10F1 → B6C3F1 by second BMT from BDF1. B6C3F1(H-$2^{b/k}$) received BM (1×10^7) and spleen (3×10^7) cells from B6B10F1(H-$2^{b/s}$) after lethal total body irradiation (TBI) (8.5 Gy). All recipients (n = 8, closed triangles) died of GVHD by day 35. In this GVHD model, mice with GVHD underwent a second BMT using a graft (1×10^7 BM cells and 3×10^7 spleen cells) from BDF1 (H-$2^{b/d}$) after receiving 3 Gy TBI. GVHD clinical score is shown as the mean ± standard error of mean. Closed circles, BDF1 → (B6B10F1 → B6C3F1) (n = 10); open circles, B6C3F1 → (B6C3F1 → B6C3F1) (n = 10). (**A**) Survival. The survival of mice undergoing a second BMT was significantly better than that of control mice that did not undergo a second BMT ($p = 0.002$). (**B**) GVHD clinical score.

Analysis of the allogeneic response of recipient spleen cells by in vitro MLC

To clarify the immunological state of GVHD mice, the allogeneic response of recipient spleen cells just before the second BMT (on day 14 of the first BMT) was analyzed by in vitro MLC. As shown in Fig. 5A, cells from the recipient spleen, in which T cells of recipient origin (B6C3F1) had been completely replaced by those of first-donor origin (BDF1) by day 14 (data not shown), responded much more weakly to all stimulators, including B6C3F1 (host), B6B10F1 (second donor), and DBA/1J (third party), than control spleen cells obtained from BDF1, although their alloreactive response to the host was still detected.

Furthermore, in the model of graft-vs-GVHD of B6B10F1 → (BDF1 → B6C3F1), to verify the low immunological responsiveness to the host after the second BMT, spleen cells were harvested from recipients 2 months after the second BMT, and were analyzed for their response to

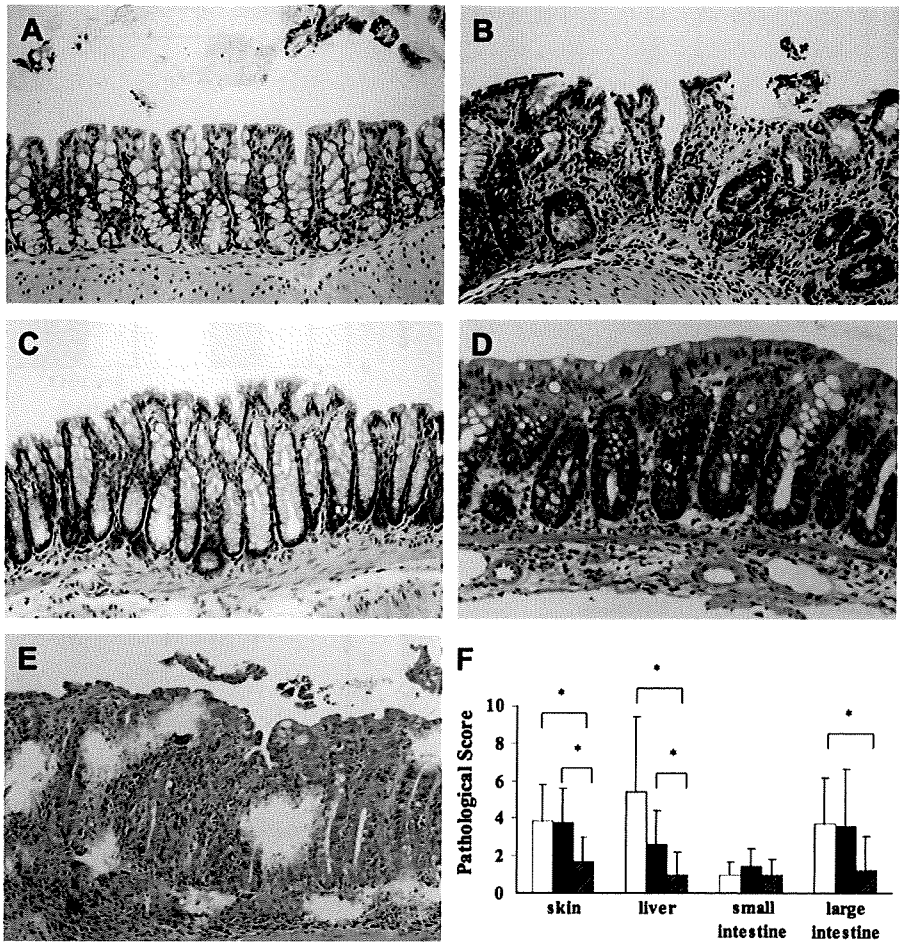

Figure 4. Pathological analysis of graft-vs-host disease (GVHD). In the graft-vs-GVHD model of B6B10F1 → (BDF1 → B6C3F1), histological analysis was performed. Representative histologic images of the large intestine are shown. (**A**) On day 14 of syngeneic (B6C3F1 → B6C3F1) bone marrow transplantation (BMT), the image was almost normal. (**B**) On day 14 of allogeneic (BDF1 → B6C3F1) BMT, luminal sloughing, a decrease in the number of glands, apoptosis of the glandular epithelium, and infiltration of small lymphoid cells in the stroma and glandular structures were observed. Arrows indicate apoptosis of the glandular epithelium. (**C**) On day 50 (36 days after the second BMT from B6B10F1), the abnormalities shown in Figure 4B were almost healed. (**D**) GVHD control mouse that did not undergo the second BMT (day 50). (**E**) GVHD control mouse that received total body irradiation (TBI) of 3 Gy only on day 13 but did not undergo the second BMT (day 50). (**F**) GVHD pathological score in GVHD target organs on day 50. The histopathological scoring system used is detailed in Materials and Methods. All data are the mean ± standard deviation. Open bars, GVHD control group (BDF1 → B6C3F1) that did not undergo the second BMT (n = 10); filled bars, GVHD control group (BDF1 → B6C3F1) receiving TBI 3 Gy only on day 13 that did not receive the second BMT (n = 8); hatched bars, second BMT group (B6B10F1 → (BDF1 → B6C3F1) (n = 12). Asterisks indicated statistically significant differences.

BDF1 (first donor) and B6C3F1 (recipient) by in vitro MLC analysis. As shown in Fig. 5B, spleen cells from survivors showed a significantly decreased response to B6C3F1 (host) compared with that to BDF1 (first donor), while the allogeneic response to a third-party antigen (DBA/1J) was relatively well-maintained. These findings are consistent with the conclusion that a second BMT eliminated first-donor lymphocytes without newly inducing GVHD against the host.

Analysis of cytokine levels, regulatory T cells and Th1/Th2 balance in mice undergoing BMT

In order to address the mechanisms of tolerance induction after second BMT, we performed relevant experiments using the graft-vs-GVHD system of B6B10F1 → (BDF1 → B6C3F1). TNF-α and IFN-γ gene expression levels in the spleen were compared between first- and second-BMT recipients. As shown in Fig. 6A, IFN-γ levels were significantly repressed in second-BMT recipients compared with first-BMT recipients. There was no significant difference in TNF-α levels between the two groups. Furthermore, to clarify the participation of regulatory T cells in tolerance induction, regulatory T cells were quantitated by analyzing $CD3^+CD4^+foxp3^+$ cells in spleen cells by flow cytometry. As shown in Fig. 6B, a significantly increased percentage of $CD3^+CD4^+foxp3^+$ cells on days 7 and 14 of BMT was observed in second BMT recipients compared with first BMT recipients. On the other hand, there was no significant

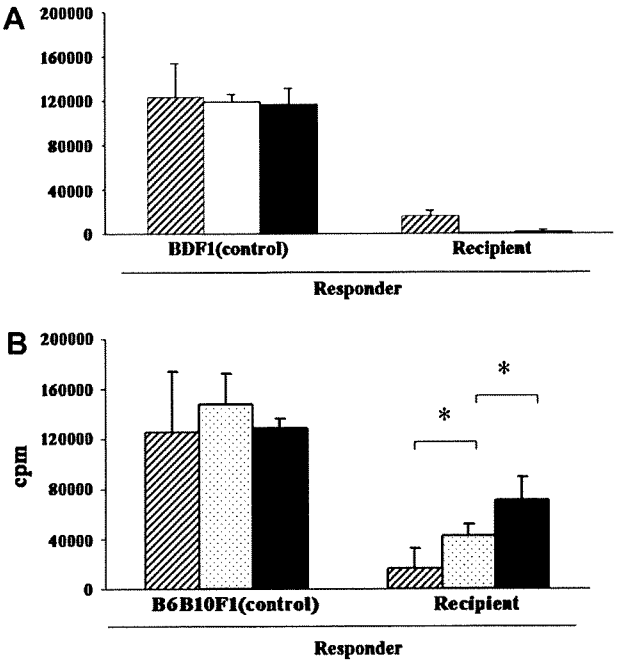

Figure 5. Allogeneic response of spleen cells recovered from recipients by in vitro mixed lymphocyte culture (MLC). In the graft-vs-GVHD model of B6B10F1 → (BDF1 → B6C3F1), spleen cells were harvested from hosts immediately before the second bone marrow transplantation (BMT) (on day 14 of the first BMT) (**A**), and 2 months after the second BMT (**B**), and their allogeneic response was analyzed by in vitro mixed-lymphocyte culture (MLC) (see Materials and Methods). Three independent experiments were performed. [^{3}H] Thymidine incorporation in the reaction containing responder cells was subtracted. Values are shown as the mean ± standard deviation. Hatched bars, B6C3F1 (host); stippled bars, BDF1 (first donor); open bars, B6B10F1 (second donor); filled bars, DBA/1J (third party). Asterisks indicate significant differences.

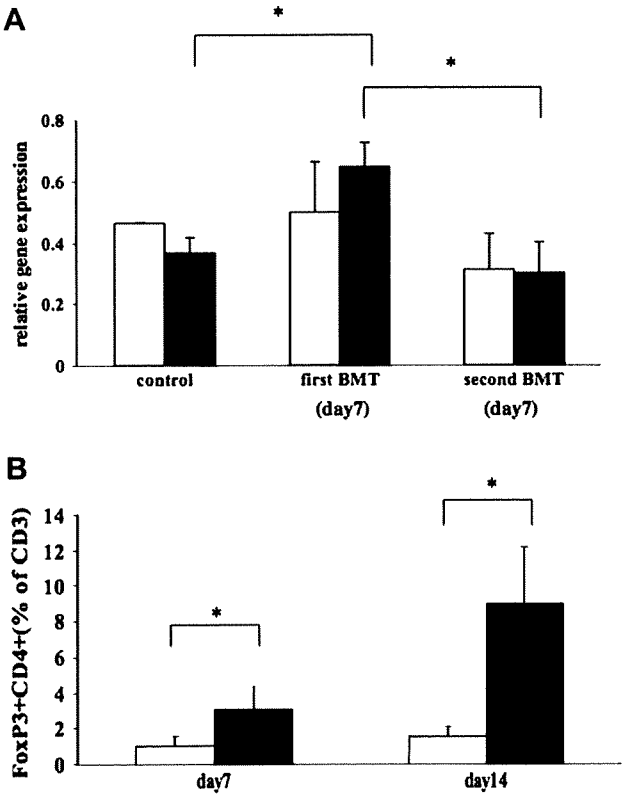

Figure 6. Comparison of cytokine gene expression levels or regulatory T-cell counts in the spleen between first and second bone-marrow transplantation recipients. Using the graft-vs-GVHD system of B6B10F1 → (BDF1 → B6C3F1), analyses of cytokine gene expression and regulatory T cell were performed. Values are shown as the mean ± standard deviation. (**A**) Cytokine gene expression levels in spleen cells that were measured by using quantitative reverse transcription polymerase chain reaction. Open bars, tumor necrosis factor-α (TNF-α); closed bars, interferon-γ (IFN-γ). Control, B6C3F1 mice without treatment; cytokine levels were examined 7 days after the first or second BMT. Data present the relative expression of cytokines polymerase chain reaction products to β-actin internal control. (**B**) Regulatory T-cell counts. Regulatory T cells in spleens were measured by flow cytometry on days 7 and 14 of the first or second BMT. Values are expressed as a ratio of $CD4^+Foxp3^+$ cells to CD3 cells. Open bars, first BMT recipients; closed bars, second BMT recipients. Asterisks indicate significant differences.

difference in Th1/Th2 balance between the two groups (data not shown).

Discussion

In the present study, we addressed the problem of whether a second allogeneic BMT from a third-party donor could eliminate the first-donor–derived harmful lymphocytes responsible for severe GVHD, and whether it could improve survival of recipients by ameliorating GVHD, with analogy to "graft-vs-autoimmunity," namely, the treatment of autoimmune diseases by allogeneic BMT [8]. Results obtained in a mouse BMT model, in which the recipient, first donor, and second donor were each MHC haploidentically mismatched, demonstrated that established GVHD could be successfully treated by a second BMT.

As the graft used to treat GVHD, two grafts, syngeneic and allogeneic (MHC-mismatched) to the host, were first considered. However, syngeneic grafts were found to have problems: the range of TBI dose useful for conditioning before the second BMT was narrow (Fig. 1A) and, in the clinical setting, the majority of patients who underwent autologous stem cell transplantation for life-threatening GVHD were reported to have a relapse of leukemia, although a marked improvement of GVHD symptoms might be observed [17–19]; therefore, we focused on GVHD treatment using allogeneic grafts.

GVHD mice were found to have two unique features that were advantageous for the application of this "graft-vs-GVHD" strategy. First, mice with ongoing severe GVHD were in a profoundly immunodeficient state (Fig. 5A) as a result of GVHD-related activation-induced cell death [20,21]; therefore, GVHD mice easily accepted second donor grafts with minimal conditioning (2–4 Gy TBI), which is an advantage in mice with GVHD, because they are very sensitive to the conditioning treatment. Second, mice with

GVHD were relatively resistant to the new development of GVHD by second-donor grafts even under conditions in which complete T-cell chimerism of second-donor origin was rapidly achieved (Table 1). Consequently, the TBI window in mice with GVHD was clearly widened, as shown in Fig. 2, which should be a key factor for obtaining a stable positive "graft-vs-GVHD" effect. Dendritic cells in the recipient spleen were found to have already been replaced by those of first-donor origin at the time of the second BMT (data not shown). Antigen presentation by first-donor–derived dendritic cells is considered to activate second-donor T cells mainly toward elimination of first-donor components responsible for GVHD rather than host tissues.

In fact, MLC analysis performed 2 months after the second BMT showed establishment of tolerance between the host and second donor, in which the allogeneic response against a third party was, however, maintained (Fig. 5B). Several mechanisms may have been involved in preventing the development of GVHD by second-donor grafts. In addition to GVHD-reducing effects by first-donor lymphocytes that survived after conditioning treatment [22], the allogeneic response of second-donor lymphocytes should have been reduced by a deletion mechanism through activation-induced cell death [20,23]. Conditioning treatment–related cytokine storm is known to enhance the magnitude of the GVH reaction [24]. In the present study, a reduced TBI dose was given to recipients in the second BMT (2–4 Gy) compared with in first BMT (8.5 Gy). In fact, regarding inflammatory cytokine levels after BMT, significantly reduced IFN-γ levels were observed in the second BMT compared with the first BMT, although there was no significant difference in TNF-α levels (Fig. 6A). Furthermore, the magnitude of the GVH response may have been further reduced by second-donor–derived regulatory T cells, which can prevent the occurrence of clinical GVHD when alloreactive T cells do not have a high burden [25,26]. In fact, a significantly increased percentage of $CD3^+CD4^+foxp3^+$ cells [27,28] was observed in second BMT recipients, compared with first BMT recipients (Fig. 6B). Thus, decreased cytokine production and increased regulatory T cells may have contributed to the induction of immunological tolerance in this graft-vs-GVHD. On the other hand, there was no significant difference in Th1/Th2 balance between first and second BMT recipients.

For the successful treatment of severe GVHD by second BMT, rapid achievement of full T-cell chimerism by second-donor components was found to be required, in contrast to treatment of autoimmune diseases, in which mixed chimerism may be beneficial as well as safer [8]. This difference is due to the rapidity and vigor of the GVH reaction, and the resultant need to eliminate the causative lymphocytes before they induce irreversible organ changes. In parental to F1 hybrid transplants, rejection by natural killer (NK) cells but not T cells (hybrid resistance) was reported by Bennett et al [29]. Although we could not address this problem, we consider that the rejection occurring in experiments 5 and 6 (Table 1) may have been induced by first-donor–derived T cells as well as NK cells. Particularly in experiment 6, which was a parental to F1 hybrid transplant (H-2^d to H-$2^{b/d}$) in the second BMT, first donor–derived NK cells may have partly contributed to the rejection.

Furthermore, at least three elements, namely, BMT timing, the intensity of conditioning treatment, and donor selection were found to be important to achieve successful outcomes. Regarding BMT timing, mice needed to undergo the second BMT after they recovered from organ damage induced by conditioning treatment for the first BMT and before they suffered irreversible organ damage due to GVHD. In our model of BDF1 (H-$2^{b/d}$) → B6C3F1 (H-$2^{b/k}$), the optimum timing was 2 to 3 weeks after the first BMT (Fig. 2C). Regarding the intensity of conditioning treatment, mice with GVHD were very sensitive because of severe GVHD-induced organ damage. In our model, they could not tolerate even 5 Gy TBI. Regarding second-donor selection, as indicated by the fact that the alloreactive response of first-donor–derived cells in recipient spleens was detected only in the host cells on day 14 (Fig. 5A), when the second-donor strain had MHC determinants that could be major targets for the GVH reaction in the first BMT, recipients were found to be relatively resistant to engraftment of the second donor, as shown in BMT experiments from syngeneic or allogeneic strains (Fig. 1 and Table 1).

We have presented here the novel concept of "graft-vs-GVHD" and demonstrated that, using appropriately reduced TBI and T-cell–replete grafts from MHC haploidentically mismatched donors, a second allogeneic BMT succeeded in eliminating harmful lymphocytes responsible for GVHD without the new development of GVHD. Mice with GVHD were relatively resistant to the new development of GVHD by second-donor grafts and, therefore, GVHD may be a good target disease for allogeneic BMT. Based on this graft-vs-GVHD concept, we are now testing a protocol for human leukocyte antigen–haploidentical nonmyeloablative stem cell transplantation for the treatment of life-threatening GVHD in a clinical study, and have already accumulated several successful cases (manuscript in preparation).

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